Biomimetic nanotechnology relates to the most basic aspects of living systems, and the transfer of their properties to human applications. Biological materials, structures, and processes are predominantly based on functionalities at the nanoscale. These nanoscale functionalities are often peppered with added components embedded in beautiful hierarchical layers, moving from the nano- to the micro-, through the meso-, and finally to the macroscale [1]. This is of relevance in materials science, medicine, physics, sensor technologies, smart materials science, and various further fields. Biomimetics of nanoscale features of living systems is highly challenging, interesting, and rewarding. Yet, because of the inherent multifunctionality of most biological functions, sometimes it is complicated to isolate specific features that are interesting for potential novel applications in technology. Here, both smart approaches and a focus on properly identifying the underlying principles of Nature are necessary for us to be able to transfer lessons from living systems to technology, science, engineering, and the arts.

The Special Issue “Biomimetic Nanotechnology Vol. 2” [2] of the MDPI Open Access Journal Biomimetics succeeds the Biomimetics Special Issue “Biomimetic Nanotechnology” [3]. The current issue was edited because various important developments have taken place since its predecessor was published, especially in medical biomimetic nanotechnology. Moreover, biomimetics as a field is growing, and has become more consolidated. This issue comprises four original research articles and five review articles. The contributions come from researchers and thinkers in all realms of biomimetic nanotechnology, and features theoretical, experimental, and review contributions from biologists, biomimeticians, chemists, clinicians, engineers, experts in interface and colloid science, food and nutrition specialists, manufacturing specialists, material scientists, microelectronics experts, nanotechnologists, physicists and systems scientists, who are engaged and interested in this fast-growing field.

The specialist fields covered are deep and wide, and comprise original and review articles on biomimetic nanotechnology from dementia research to dentistry (two contributions regard the oral cavity: one focuses on acidic challenges to restorative materials and one on the prevention of detrimental biofilm formation), energy (the generation of hydrogen by water-splitting), nanomembranes (with their vast number of applications), therapies against cancer and multiresistant bacteria, tissue engineering (two contributions: one focuses on the kidney, the other on vascularization) and vaccine design.

The articles highlight the importance of hierarchical materials, nanomaterials, nanoparticles, nanosystems and nanostructures, nanoscale functionalities, programmable materials, safe nanotechnology and tunable materials with nanoscale functionalities in biomimetic nanotechnology.

Especially in the time of the COVID-19 pandemic, there is high interest in vaccines and vaccine design. The review article “Cationic nanostructures for vaccines design”, by the Brazilian author team Carmona-Ribeiro and Pérez-Betancourt [4], approaches this important field from the angle of structuring antigen/adjuvant combinations into four categories. Antigens from the pathogen guarantee an immune response, and adjuvants help create a stronger immune response. The intranasal route is an advantageous mucosal route that allows rapid administration for large populations in the case of pandemics; the
development of effective intranasal vaccines is of great interest due to their potential to induce both mucosal and systemic immunity. Cationic nanostructures can further protect the carried antigen for vaccine administration by the oral route, and increase the permanence time of the antigen/carrier assembly at the mucosae, enhancing systemic immunity. The mRNA technology for vaccines has been recognized as a transformative technology to control infectious diseases and to fight cancer. Lipid nanoparticles are a vital component of mRNA vaccines; they protect and transport the mRNA to the place it is needed, and they are currently used in the Pfizer/BioNTech and Moderna COVID-19 vaccines. The huge variety of cationic nanostructured materials available from nanomaterials science may trigger further valuable research on vaccine design with novel cationic nanostructures.

Nanomembranes are the principal building blocks of, essentially, all living organisms. Their artificial counterparts are essential in fields such as separation science, chemical engineering, toxicology, forensics, sensing technology, the life sciences, medicine, the food and drinks industry, process engineering, and green and renewable energy. The Serbian author team Zoran and Olga Jakšić [5] define synthetic nanomembranes as structures with a thickness below 100 nanometers, and a large thickness-to-lateral-size aspect ratio, resulting in, at times, surprising and counter-intuitive phenomena such as extreme toughness combined with extraordinary foldability and stretchability. Their review article “Biomimetic nanomembranes: An overview” critically deals with purely synthetic biomimetic nanomembranes, their fabrication and functionalization. Nanomembranes define boundaries and shapes, and yet can enable complex exchange when properly functionalized. The classes and types of synthetic nanomembranes comprise inorganic nanomembranes, hybrid organic/inorganic nanomembranes, organic nanomembranes and synthetic biological nanomembranes. They can be fabricated top-down, bottom-up, and via exfoliation approaches. Functionalization in biological nanomembranes is mainly via built-in protein structures; in synthetic nanomembranes the toolbox is vastly larger, allowing for the incorporation of various non-biological properties such as plasmonic, magnetic, electrical and optical ones. An example for the functionalization of synthetic biomimetics nanomembranes are water channels inspired by aquaporins that ensure the rapid passing of water combined with the outstanding rejection of undesired ions. The outlook further presents visionary approaches such as biomimetic metamaterials and quantum functionalities.

Decentralized systems, such as those found in social insects and more than 70 different species of bacteria, can coordinate joint behavior and perform complex tasks by using a process called quorum sensing. In this decision-making process, the decentralized systems correlate stimulus and response to population density. Inhibiting the quorum sensing of certain harmful bacteria in the oral cavity by certain molecules can prevent the formation of detrimental biofilm, providing a treatment for dental plaque. Such antibacterial molecules can be encapsulated in nanoparticles, which are then transported via targeted delivery to the teeth and mucins. The structure-giving component of the mucus of organisms such as mucosal surfaces in the oral cavity are also present in saliva. This can provide the patient’s immune system with essential time to fight an infection, and thereby represents an alternative system to antibiotics, against which microbial resistance can arise. The nanostructures can additionally be coated with substances such as chitosan, that react electrostatically with bacterial membranes, providing additional antibacterial activity. In their research article “Characterisation of the interaction among oil-in-water nanocapsules and mucin” [6], a multinational author team from the University of Leeds (UK) investigate a potential new treatment against biofilms that consists of oil-in-water nanocapsules that are coated with chitosan, and loaded with a quorum-sensing inhibitor. The group analyses the interaction with mucin molecules (in a concentration that is comparable to the one in saliva) and the stability of the nanocapsules via dynamic light scattering and asymmetrical flow field-flow fractionation. Such studies form an important knowledge base for the rational design of drug delivery systems in oral environments and shall—in the future—also include
investigations regarding the stability and drug-release potential of nanocapsules in more realistic situations.

Intrinsic (such as vomiting) or extrinsic (such as acidic drinks) acidic factors in the oral cavity contribute to the fading of the hydroxyapatite in teeth, leading to caries and potential tooth loss. The demineralizing action of acids on the teeth may lead to loss of the outer enamel. The second article in the Biomimetics Special Issue of Biomimetic Nanotechnology Vol. 2 that deals with the oral cavity is the research article “Exposure of biomimetic composite materials to acidic challenges: Influence of flexural resistance and elastic modulus” [7] written by an Italian team, dealing with the effect of acidic conditions on biomimetic composite materials that are used for restorative purposes. The group exposed five different, conventionally used, restorative, biomimetic composite materials to three different treatments (either storage in distilled water, or storage in a carbonated soft drink for some time, and then in distilled water for some time, or storage in the carbonated soft drink for the whole period). Afterwards, the flexural strength and the elastic modulus of the composites was measured with a universal testing machine. Teeth are not the only studied item influenced by acids: restorative materials (that should, in principle, guarantee good mechanical properties) were subjected to acid-induced corrosion, resulting in changes in their initial characteristics, such as a reduction in microhardness, flexural strength and elastic modulus. Future studies shall be conducted in vivo (allowing the buffering capacity of saliva to be taken into account), on the acidic conditions that naturally appear in the oral cavity.

Biological substitutes for medical applications are an important aspect of tissue engineering. For the development of artificial organs, the combination of natural blood vessels and artificial tissues is of high relevance. In their research article “Fabrication of nanoporous polylactic acid microtubes by core-sheath electrospinning for capillary vascularization” [8], the authors report on the development of base ingredients for future complex synthetic tissues, that allow for the creation of vascular networks in engineering constructs. They use the core-sheath electrospinning process (via which a fiber is formed by simultaneous flow of core and sheath solutions from separate capillaries) to fabricate nanoporous microtubes that mimic the structure of fenestrated capillary vessels. Fenestrated capillary vessels have small pores and are located mainly in places of the body that require considerable exchange between blood and tissues. The microtubes are electrospun and then post-processed. The surfaces and cross-sections of the microtubes are subsequently characterized with scanning electron microscopy. The outer diameter of the artificial vascular channels reported in the article is between one and eight micrometers—this comes close to the natural situation of small human capillaries, which have a diameter of five to ten micrometers. The average size of the surface nanopores ranges from 100 to 800 nm. Furthermore, attachment of the microtubes to human dermal microvascular endothelial cells is preliminarily tested with fluorescence microscopy, indicating the compatibility and potential use as scaffolds for capillary vessel engineering. Future studies will include measurement of the wall thickness, and various solvent mixture combinations.

The use of hydrogen is a possible alternative to the use of fossil fuels. Inspired by the photosystem-II that splits water into hydrogen and oxygen, the authors of the research article “Biomimetic catalysts based on Au@ZnO-graphene composites for the generation of hydrogen by water splitting” [9] report on their studies regarding high-surface area catalysts contributing to artificial photosynthesis. The photosystem-II is a part in the photosynthetic system in plants, algae and cyanobacteria, which stores the energy of light in the form of a redox potential by generating a proton gradient across the thylakoid membranes, i.e., the membranes of the chloroplasts, in which the photosynthetic light reactions and the associated electron transport, as well as proton transport and ATP formation, take place. Hydrogen is abundant, has a high energy yield, can be easily stored, and has environmental compatibility. ZnO is chemically stable, easy to produce and abundant, and provides—in nanosize form—a large surface area, as does the graphene that is used as a co-catalyst, and the gold nanoparticles with diameters of less than 10 nanometers. Their smart combination
results in the favored transfer of photogenerated electrons and improved photocatalytic efficiency. The catalysts are characterized with various methods (such as transmission electron microscopy, photoelectron spectroscopy, Raman and X-ray diffraction and UV-vis spectroscopy), and the photocatalytic activity regarding hydrogen production is measured by gas chromatography.

The review article “Biomimetic nanocarrier targeting drug(s) to upstream-receptor mechanisms in dementia: Focusing on linking pathogenic cascades” [10] is written by a single author, the CEO and founder of a US-based company dealing with the continued development of nanoemulsion technology for the “actively targeted” therapy of neuroinflammation, neurodegeneration, and Alzheimer’s disease. Apolipoprotein from the blood can absorb to the surface of certain colloidal lipid nanocarriers that are injected into the blood stream. Apolipoprotein mediates the crossing of the blood–brain barrier. Thereby, these artificial biomimetic nanocarrier particles can transport the pharmaceuticals they carry across the blood–brain barrier, potentially providing a preventive and therapeutic strategy against cognitive decline, dementia and Alzheimer’s disease. Inflammation and oxidative stress, as they occur in microvascular dysfunction, can precede cognitive decline. The author reviews papers that state that endothelial modulation and repair is feasible by the pharmacological targeting of certain endocytic receptors. Oxidative stress and inflammation impair the functionality of high-density lipoproteins (HDL, the so-called “good cholesterol”) in Alzheimer’s disease. Moreover, the gut–brain axis is of high relevance: inflammation in the gut may induce—especially when combined with increased permeability of the blood–brain barrier—proinflammatory cytokine concentration in the brain. Inhibiting these inflammatory cascades may attenuate Alzheimer’s disease. HDL might include some proteins that help inhibit these inflammatory cascades, and could also be an early or even proactive treatment with lipid nanoemulsion vehicles loaded with targeted delivery agents and further drug molecules, allowing for a localized drug treatment of brain tissue in vivo.

The kidney is a paired organ of the urinary system that prepares the urine and regulates water balance, as well as electrolyte balance in vertebrates. It is the most sought-after organ for transplantation. Therefore, a plenitude of attempts is undertaken to replace its function. In their review article “Enhancing kidney vasculature in tissue engineering—current trends and approaches: A review” [11] the author pair from a US American university mainly deal with the current primary obstacle in the development of clinically relevant kidney tissue engineering: the precise formation of blood vessels (i.e., vascularization) for the large-scale growth of whole engineered kidneys for transplantation. Another important aspect in their review is a related comparison between top-down and bottom-up approaches, with regard to vascularization. The general goal of tissue engineering is to develop tissues to assist, maintain and enhance tissue function. Tissue engineering is very successful for avascular structures such as skin or cartilage; however, the engineering of larger organs with intricate structures and vascularization, especially small blood vessels with a diameter less than six millimeters, remains a challenge. The four major kidney tissue engineering methodologies reviewed are: whole kidney tissue engineering (top-down approach), kidney organoids (bottom-up approach), vascular corrosion casts and organs-on-a-chip (microphysiological systems). The authors summarize that although kidney tissue engineering is most challenging and much further research is needed, some highly effective attempts are either already established or hold great promise.

The review article “Learning from Nature: Bioinspired Chlorin-based photosensitizers immobilized on Carbon materials for combined photodynamic and photothermal therapy” [12] by authors from Brazil, gives an extensive review of articles published in this field over the last ten years, comprising the mechanisms and applications of photodynamic and photothermal therapy, carbon materials applied in photodynamic and photothermal therapy and a detailed update on chlorine-based photosensitizers immobilized on carbon materials for photodynamic and photothermal therapy. Photosensitizers induce reactions that may destroy cancerous cells via oxidative pathways and inactivate bacteria, with
only a low molar concentration of photosensitizer and low light doses. Certain carbon nanomaterials synergistically support this action. This is also attributed to their large surface area. For example, graphene is a one-atom thin layer of carbon that combines a large surface area, extreme mechanical strength, chemical purity and the possibility of easy functionalization for bioapplications combined with various other supreme properties [13]. Chlorin-based photosensitizers are bioinspired by biomolecules such as chlorophylls. Carbon nanomaterials such as graphene, carbon nanotubes and fullerenes are considered as promising anticancer agents; however, their potential cytotoxicity against healthy cells still poses challenges towards their successful clinical application. Such new technological approaches open new pathways for treating cancer and multi-resistant bacteria.

The guest editor thanks all the contributors, reviewers and the staff of MDPI for their valuable work related to this special issue of the journal Biomimetics, and looks forward to great future developments in the exciting field of biomimetic nanotechnology.

Due to the dynamic developments in the field of biomimetic nanotechnology, and the new interesting contributions we receive, we are glad to inform you that we will continue the successful series of Biomimetics Special Issues in the field of biomimetic nanotechnology with a Volume 3 [14]. The focus of this new special will be the contributions of biomimetics and nanotechnology in the age of a global pandemic.

Conflicts of Interest: The author declares no conflict of interest.

References
10. D’Arrigo, J.S. Biomimetic nanocarrier targeting drug(s) to upstream-receptor mechanisms in dementia: Focusing on linking pathogenic cascades. Biomimetics 2020, 5, 11. [CrossRef] [PubMed]
Fabrication of Nanopores Polylactic Acid Microtubes by Core-Sheath Electrospinning for Capillary Vascularization

Yingge Zhou 1, Dilshan Sooriyaarachchi 2 and George Z. Tan 2,*

Abstract: There has been substantial progress in tissue engineering of biological substitutes for medical applications. One of the major challenges in development of complex tissues is the difficulty of creating vascular networks for engineered constructs. The diameter of current artificial vascular channels is usually at millimeter or submillimeter level, while human capillaries are about 5 to 10 µm in diameter. In this paper, a novel core-sheath electrospinning process was adopted to fabricate nanoporous microtubes to mimic the structure of fenestrated capillary vessels. A mixture of polylactic acid (PLA) and polyethylene glycol (PEO) was used as the sheath solution and PEO was used as the core solution. The microtubes were observed under a scanning electron microscope and the images were analyzed by ImageJ. The diameter of the microtubes ranged from 1–8 microns. The diameter of the nanopores ranged from 100 to 800 nm. The statistical analysis showed that the microtube diameter was significantly influenced by the PEO ratio in the sheath solution, pump rate, and the viscosity gradient between the sheath and the core solution. The electrospun microtubes with nanoscale pores highly resemble human fenestrated capillaries. Therefore, the nanoporous microtubes have great potential to support vascularization in engineered tissues.

Keywords: core-sheath electrospinning; nanoporous microtubes; capillary vessels

1. Introduction

Vascularization has been challenging for several decades in the tissue engineering field. The introduction of blood vessels into artificial tissues is one of the most critical steps toward viable organ transplant substitutes. In the last years, remarkable progress was made in the development of bioprinted microchannel networks and decellularized matrices for both artery and vein vascularization [1,2]. However, the finest of the fabricated microchannels are within a scale of several hundred microns in diameter, which is not aligned with the human capillary vessels’ diameter range (5–10 µm) [3,4]. There are three different lining structures for capillary vessels: continuous, fenestrated, and sinusoidal [5,6]. The basement membrane layer and endothelial layer are closed in continuous capillaries, while the endothelial layer is porous in fenestrated and sinusoidal capillaries. The porous structure is believed to improve the efficiency of transportation of biological factors between inside and outside of the capillaries [7]. Similarly, the incorporation of nanoporous microchannels into biomimetic scaffolds can significantly improve the viability of cultured cells inside scaffolds. Therefore, there is a research gap to create biological substitutes for capillary vessels in relevant scale and nanoporous structures.

At centimeter to millimeter scale, electrospun nanofiber mat can be rolled into a tubular structure as a scaffold for engineering vessels [8,9]. However, the diameters of human capillaries are at micron level. To better address the challenge of capillary vascularization in biomimetic scaffolds, numerous efforts have been made. For example, Wong et al. used microcontact imprinting to generate 2D patterns of adhesive proteins.
on non-cell-adhesive substrates and adhered human umbilical vein endothelial cells (HUVECs) on it. The capillary-patterned cells can migrate and sprout into hydrogels that cover the cells, thereby forming a patterned vasculature formation in hydrogel [10]. Moya et al. combined tissue engineering and microfluidic technology to fabricate a 3D stroma that contains a perfused and interconnected human capillary network [11]. Other advanced manufacturing techniques that were deployed for capillary fabrication include laser-assisted bioprinting [12], electrospinning [13–15], and 3D printing [16]. For example, coaxial spinnerets were adopted to fabricate nano-to-micrometer tubes with average tube diameters within 1 µm [17,18]. Polycaprolactone (PCL) is one of the most commonly used materials. The diameter can be modulated by electrospinning process parameters [19]. Studies showed that blending or co-electrospun natural and synthetic polymers could improve the mechanical strength of the fibers and promote vascular formation [20–22]. For example, the biocompatibility of the electrospun tubes could be improved by adding collagens into the polymer solutions [18,23,24]. Meanwhile, recent work in bioprinting focused on extruding multi-type materials in a coaxial extrusion system [25,26]. However, the limitation of extrusion nozzle size hinders the formation of microtubes.

Recent research projects have shown the possibility of fabricating 3D aligned nanofibers scaffolds and 2D aligned nanoporous microtubular scaffolds [27–29]. The results indicate that the fiber geometry and microtube size can be changed by changing the process parameters and ambient environment settings. Other studies focused on customizing the composition and degree of cross-linking by adding different hydrogels into bioinks [25,30,31]. Overall, there are several drawbacks associated with these fabrication techniques, such as low fabrication efficiency and nonrelevant capillary diameter. Therefore, efficiently fabricated microtubular structures with a diameter of less than 10 µm and surface nanopores remains challenging.

To address the challenge, a novel core-sheath electrospinning strategy has been developed to fabricate polylactic acid (PLA) microtubes with surface nanopores to mimic the fenestrated capillaries. With phase separation and water-soluble polymer core, the porous surface and tubular structure can be obtained [27]. The objectives of this research were to investigate the effects of process parameters, such as flow rate, and material properties, such as solution viscosity on tube diameter and pore size. Our hypothesis was that tubular structure is determined by viscosity ratio of core to the sheath solutions and that nanopores will form on the microtube surfaces due to the rapid evaporation of the solvent. The polymer composition in the sheath solution can also influence the nanopores formation. To test the hypotheses, various combinations of polymer solution flow rate, viscosity levels, as well as different polymer ratios in sheath polymer solution were tested. The results show that the solution viscosity levels, flow rates, and polymer composition in sheath solution all had significant influence on micropore size, and the solution viscosity levels and polymer composition in sheath solution had significant influence on nanopores size. The microtube closure rate was higher when the sheath solution flow rate was high or when there was more polyethylene glycol (PEO) in the sheath solution.

2. Materials and Methods
2.1. Preparation of Polymer Solutions
Polyactic acid (PLA, Mw = 194,000, Ingeo Biopolymer 4032D) pellets were purchased from Jamplast Inc. (Ellisville, MO, USA). Polyethylene glycol (PEO, molecular weight = 300,000) powder and dichloromethane (DCM, ≥99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water (DI water) was obtained from PURELAB Classic Water Purification System (ELGA Lab water, High Wycombe, UK).

The 15% and 16% (w/v) PLA solutions were prepared by dissolving PLA in DCM through magnetic stirring for 4 h at room temperature. Similarly, 3.8% and 4.5% (w/v) PEO solutions were prepared by dissolving PEO in DCM through magnetic stirring for 4 h at room temperature. The solution ratios were selected based on solubility, viscosity levels, and ease of electrospinning jet formation. Sheath solutions with different PLA
and PEO ratios were prepared by adding PEO solution into PLA solution with respective volume ratios. The viscosity of the polymer solutions was measured by a digital rotational viscometer (Brookfield AMETEK, Middleboro, MA, USA).

2.2. Electrospinning of Microtubes

The electrospinning process was performed on the TL-Pro-BM robotic electrospinning platform (Tongli Tech, Shenzhen, China) with a 50 kV high voltage power source. A concentric core-sheath spinneret was adopted for this study. The PEO solution and the PLA/PEO solution were extruded from a two-channel syringe pump with independently controlled pump rates. The PLA/PEO solution was delivered to the sheath of the spinneret, and the PEO solution was delivered to the core of the spinneret. The solution and parameter settings are summarized in Table 1. The nozzle size for core and sheath solution were gauge 25 and gauge 18, respectively. The tip-to-ground distance was 150 mm. A positive voltage of 10 kV was applied to the spinneret to induce the electrospinning. The humidity of the chamber was set at 40% by a humidifier. The electrospinning time was set to be 3–5 min.

**Table 1.** Materials for the core-sheath electrospinning.

<table>
<thead>
<tr>
<th>Group Number</th>
<th>Sheath</th>
<th>Core</th>
<th>Viscosity (Sheath) (mPa·s)</th>
<th>Viscosity (Core) (mPa·s)</th>
<th>Flow Rate (Sheath) (mL/h)</th>
<th>Flow Rate (Core) (mL/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15% PLA</td>
<td>3.8% PEO</td>
<td>350</td>
<td>350</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>15% PLA/3.8% PEO (10:1)</td>
<td>3.8% PEO</td>
<td>350</td>
<td>350</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>15% PLA/3.8% PEO (10:1)</td>
<td>3.8% PEO</td>
<td>350</td>
<td>350</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>15% PLA/3.8% PEO (10:1)</td>
<td>3.8% PEO</td>
<td>350</td>
<td>350</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>15% PLA/3.8% PEO (10:1)</td>
<td>3.8% PEO</td>
<td>350</td>
<td>350</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>15% PLA/3.8% PEO (10:1)</td>
<td>4.5% PEO</td>
<td>350</td>
<td>620</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>16% PLA/4.5% PEO (10:1)</td>
<td>3.8% PEO</td>
<td>620</td>
<td>350</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>15% PLA/3.8% PEO (10:2)</td>
<td>3.8% PEO</td>
<td>350</td>
<td>350</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

PLA = polylactic acid; PEO = polyethylene glycol.

Based on preliminary data, we hypothesized that the discrepancy of viscosity and flow rate between the core solution and sheath solution would influence the formation of concentric dual-material fibers in electrospinning. We also hypothesized that the morphology of nanopore on the microfibers would change after the water bath treatment by adding water-soluble PEO into PLA as the sheath solution. To investigate the effect of solution viscosity, flow rate, and the ratio of PEO in the sheath solution, we designed a fractional factorial experiment as follows (Table 1). Two levels of viscosity (350 mPa·s and 620 mPa·s), two levels of solution pump rate (1 mL/h and 2 mL/h), and three levels of PLA-to-PEO ratio (by volume) in the sheath solution (10:0, 10:1, and 10:2) were taken. A total of 7 groups of experiments were conducted.

2.3. Post-Processing of Microtubes

A schematic illustration of processing the electrospun microtubes is shown in Figure 1. By adopting the coaxial spinneret, PLA/PEO and PEO solutions were electrospun simultaneously into microfibers with a core-sheath structure. The porous microfibers were collected by aluminum foil and immersed in deionized water for 2 h to dissolve the PEO. After the water bath, the core was removed and the sheath was thinned, resulting in porous microtubes.
Figure 1. Schematic illustration of the porous microtube fabrication process. PLA = polylactic acid; PEO = polyethylene glycol; DI water = deionized water.

2.4. Characterization of Microtubes

To observe the tubular structure, the air-dried PLA microtubes were attached to thin glass slides and immersed in liquid nitrogen for 2 min. The frozen samples were then broken to form cross-sections. The surface and cross-sections of the microtubes were examined under scanning electron microscopy (SEM, Phenom ProX, NanoScience, Alexandria, VA, USA). Fiber diameter and nanopore size were analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA). The core-sheath concentricity rate was calculated by examining fibers in SEM images. Thirty fibers and 30 pores were selected randomly from each sample.

2.5. Cell Attachment

Green fluorescent protein expressing human dermal microvascular endothelial cells (HDMVE, cAP-0005GFP) were purchased from Angioproteomie (Boston, MA, USA). Cells were grown in endothelial growth medium (cAP-02, Angioproteomie) with 0.1% penicillin-streptomycin-amphotericin B solution (ATCC, Manassas, VA, PCS-999-002) until 90% confluency. Electrospun microtubes were cut into $2 \times 2$ cm and placed in a 6-well culture plate. The microtubes were sterilized by 70% ethanol and rinsed by deionized water for 3 times. Afterward, the microtubes were air dried in the biosafety cabinet with UV light on for 1 h. Cells were detached from the culture flask and resuspended in fresh medium. Approximately $5 \times 10^5$ cells were added to the surface of the microtubes and cultured at $37^\circ$C, 5% CO$_2$, in an incubator for 7 days. The cell attachment was examined under a fluorescence microscope (EVOS M5000, Thermo Fisher Scientific, Waltham, MA, USA).

3. Results

The SEM images of the porous microtubes are shown in Figure 2. Since DCM is highly volatile, the rapid evaporation of DCM during the electrospinning caused a sudden temperature decrease on the surface of the fibers, and the thermodynamic instability induced phase separation of the solution. Meanwhile, the condensation of humidity on the surface of the spinning jets could facilitate nonsolvent-induced phase separation. Both phenomena contributed to the dent formation on the microfiber surface. When the mixture of PLA and PEO was used as the sheath material, a large portion of the dents became pores after the dissolution of PEO. For example, in Group 2 (Figure 2b), the wall
of the microtubes was thinned so that there were many penetrating nanopores on the PLA sheath.

Figure 2. Scanning electron microscopy (SEM) images for all seven groups after water immersion. (a–g). Groups 1–7; and (h) cross-section image of Group 4. Scale bar = 10 μm.

The consistency between the core solution and the sheath solution played a critical role in generating a good tubular structure in the core-sheath electrospinning. In Groups 3, 5, and 6, many of the microtubes showed defects or a non-concentric structure. The most common defect was that the sheath failed to fully wrap the core so that core-sheath fibers ended up with a half-round tube structure after the water bath treatment (Figure 2c,e,f). For Group 3, the flow rate of the core solution was higher than that of the sheath solution. The inconsistency in solution flow during the electrospinning caused the structural defect. For Groups 5 and 6, the viscosities of the core and sheath solutions were different. This caused instability of the dual-material jet in the whipping. On the other hand, in Groups 2, 4, and 7, most of the microtubes showed a complete tubular structure without large openings on the wall. In all these groups, the viscosity was kept the same for the core solution and the sheath solution. In Group 4, the flow rate of the sheath solution was higher than that of the core solution, and the electrospun microtubes maintained a good tubular structure. Figure 2h shows the cross-sections of the microtubes in Group 4. It shows that a moderate increase in the flow rate for the sheath solution did not damage the final tubular structure of the microtubes.

The diameter analysis is shown in Figure 3, and the pore size analysis is shown in Figure 4. The outer diameter of microtubes ranged from 0.9 to 7.6 μm, and the size of the nanopores ranged from 130 to 820 nm. It should be noted that not all the factors had a linear impact on the microtube diameter or the nanopore size. For example, using different pump rates for the sheath and the core solutions generated larger microtubes compared to using the same pump rate; however, a higher pump rate of the sheath solution led to the largest microtube diameter (Figure 3b). The microtube diameter also increased when the viscosity of the core solution increased (Figure 3c). Regarding the nanopore size, the addition of 10% PEO to the PLA sheath solution resulted in a larger pore size, but more PEO (20%) did not increase the pore size (Figure 4a). Having inconsistent viscosities between the core solution and the sheath solution contributed to larger nanopore sizes, and the microtubes showed the largest nanopore size when the sheath solution had a higher viscosity (Figure 4c).
Endothelial cells were successfully attached to microtubes in 3D space after 24 h. Figure 5 shows the fluorescent cells after 3 days of culturing. Most cells were randomly distributed among the microtubes. Some cells grew along the microtubes to form a continuous line. Because the microtubes were distributed in a 3D space instead of on a 2D surface, some objects were out of focus. Confocal microscopy will be used in the future to capture the 3D images. This preliminary test shows that the electrospun microtubes were compatible with human endothelial cells; therefore, they can be used as scaffolds for capillary vessel engineering.

Figure 3. Diameter analysis. Effects of (a) PEO ratio in sheath solution; (b) pump rates; (c) sheath viscosity/core viscosity.

Figure 4. Pore size analysis. Effects of (a) PEO ratio in sheath solution; (b) pump rates; (c) sheath viscosity/core viscosity.

Figure 5. GFP human dermal microvascular endothelial cells (HDMVE) attached on the electrospun microtubes. (a) Cells randomly distributed on the microtubes, scale bar = 400 µm; (b) cells grown along a single microtube, scale bar = 200 µm.
4. Discussion

Electrospinning has been utilized for nanofiber fabrication for many decades. Scaffolds made of micro-nanofibers have shown great potential for tissue engineering. In our previous research projects, a novel divergence electrospinning strategy was developed to scale up traditional 2D nanofiber mats to 3D nanofiber scaffolds with gradient microstructures along the vertical direction [32–36]. The scaffolds' thicknesses ranged from 2 to 10 cm. Human fibroblasts were cultured in the nanofiber scaffolds and grew along the parallel fibers in 3D space. In this research, nanoporous microtubes were fabricated by core-sheath electrospinning to resemble capillary vessels. The outer diameter of the microtubes ranged from 1 to 8 µm and the average size of the surface nanopores ranged from 100 to 800 nm. The fabrication time of a millimeter-size microtube scaffold in height only took 3–5 min. The incorporation of micro-to-nano fibers into tissue engineered scaffolds has been widely studied in the past decades. For example, Xu et al. prepared poly (lactic-co-glycolic acid) based triblock copolymer microtubes for noninvasive monitoring of bone regeneration [37]. The microtube embedded hydrogel possessed an ideal sustained drug release property. After implanting the composite hydrogel into the tibial defect of rats, the results showed that the hydrogel scaffold was completely degraded after 4 weeks and the tibial defect was repaired after 6 weeks. In another review conducted by Nakielski et al., the challenges and impact of nano- and microfiber morphology on tissue engineering applications such as hemostatic agent was also discussed [38]. It was found that increasing fiber dressing porosity can ease blood absorption and increase clotting factor concentration. Other methods such as surface functionalization can trigger the activation of platelets and lead to faster clot formation. Therefore, our microtubes could be of high potential in the vascularization applications of tissue engineered scaffolds.

In our core-sheath electrospinning process, surface pore formation occurred in the phase separation phenomenon due to the rapid evaporation of the solvent (DCM). DCM has been widely used in the fabrication of porous micro-to-nano fibers using electrospinning. For example, Nguyen et al. obtained porous polycaprolactone (PCL) fibers with DCM and acetone mixture as a solvent for CaP particles coating in bone tissue engineering applications [39]. The polymer-rich phase formed a fibrous matrix, while the mixture solvent-rich phase formed the spherical pores. It was also found that the solvent mixture rate contributed to the formation of the pores. Similarly, Cao et al. fabricated PLA nanoporous fibers by adjusting the composition ratio of DCM and N,N-dimethylformamide (DMF) solvent mixture rate [40]. Natarajan et al. found that higher relative humidity and its miscibility/interaction with DCM solvent might contribute more to the generation of surface porosity [41]. In our study, the viscosity gradient between the core and the sheath solution showed a significant influence on pore size. In addition, the PEO ratio in sheath solution also played an important role in both the wall thickness and the pore size after the water bath treatment. In our experiment, the highest average pore size was obtained when the shear viscosity was 620 mPa·s and core viscosity was 350 mPa·s. Rezabeigi et al. showed that in the PLA-DCM-hexane electrospinning system, a range of viscosity allowing for the production of porous spherical microfibers exists. Lee et al. also found that in the core-sheath coaxial electrospinning process, the higher viscosity of polymer solutions resulted in higher pore size.

Our diameter analysis showed that the pump rates (solution flow rates), solution viscosity levels, and PEO ratio in the sheath solution had significant influences on the microtube diameter (p < 0.05). The PEO ratio in sheath solution and the viscosity difference between the core solution and the sheath solution showed a significant influence on the nanopore size (p < 0.05). Both the diameter and the nanopore size increased when the mixture solution of PEO and PLA was used as the sheath solution. Other research projects also showed results that are consistent with our analysis. For example, Duan et al. also found that higher flow rates promoted the formation of larger fibers, since more solution coming out from the nozzle and a larger amount of polymers would form larger fibers [42]. Similarly, Yu et al. suggested that by only increasing the sheath flow rate,
the fiber diameter could increase significantly [43]. As for the effect of viscosity, Chen et al. found that higher solution viscosity in core-sheath electrospinning contributes to larger fibers [44]. Given that higher viscosity makes the fiber stream more difficult to be elongated, electrospun fiber is expected to be larger. Additionally, since the diameter was measured by the outer layer of PLA fibers, the effects of sheath solution viscosity would be larger than the core solution. In this study, the wall thickness of the microtubes was not quantitatively analyzed due to the limitation of resources. In the future, fluorescent stain can be added to the sheath solution, and the wall thickness can be measured by confocal microscopy.

In the future, solvent mixtures with other types of solvents, such as dimethylformamide (DMF) [45], will be used in our core-sheath electrospinning process. The ratio of solvent mixtures in both core and sheath solutions will be investigated. The scaffolds can also be incorporated into a hydrogel matrix with endothelial cells for engineering vascularized tissues.

5. Conclusions

In this paper, a novel core-sheath electrospinning process was adopted to fabricate nanoporous microtubes for mimicking human fenestrated capillary vessels. The average diameter of these microtubes was on the same scale as the minimum diameter of human capillaries. The results show that the ratio of water to dissolvable polymer in the sheath solution, pump rate, as well as the viscosity gradient between the sheath and the core solution had significant impacts on the microtube diameter. The PEO ratio in the sheath solution and the viscosity gradient substantially influenced the pore size. These nanoporous microtubes can be incorporated into tissue engineering scaffolds to promote angiogenesis and tissue vascularization.

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References


42. Duan, G.; Greiner, A. Air-Blowing-Assisted Coaxial Electrospinning toward High Productivity of Core/Sheath and Hollow Fibers. Macromol. Mater. Eng. 2019, 304, 1800669. [CrossRef]
Exposure of Biomimetic Composite Materials to Acidic Challenges: Influence on Flexural Resistance and Elastic Modulus

Andrea Scribante *, Simone Gallo *, Stefano Scarantino, Alberto Dagna, Claudio Poggio and Marco Colombo

Department of Clinical, Surgical, Diagnostic and Paediatric Sciences—Section of Dentistry, University of Pavia, Piazzale Golgi 2, 27100 Pavia, Italy; stefano.scarantino01@universitadipavia.it (S.S.); alberto.dagna@unipv.it (A.D.); claudio.poggio@unipv.it (C.P.); marco.colombo@unipv.it (M.C.)
* Correspondence: andrea.scribante@unipv.it (A.S.); simone.gallo02@universitadipavia.it (S.G.)

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Abstract: Acidic conditions of the oral cavity, including soft drinks and cariogenic bacteria, represent a damage for restorative biomimetic composite materials. The aim of this study is to assess the influence of two different acidic challenges on the flexural strength and elastic modulus of five composites: x-tra fil (Group 1, XTF), GrandioSO x-tra (Group 2, GXT), Admira Fusion x-tra (Group 3, AFX), VisCalor bulk (Group 4, VCB), and Enamel Plus HRi (Group 5, EPH). Thirty samples for each group were randomly divided and assigned to three different treatments: storage in distilled water as the controls (subgroups 1a–5a), 3 weeks distilled water + 1 week Coca-Cola (subgroups 1b–5b), and 4 weeks Coca-Cola (subgroups 1c–5c). For each subgroup, the flexural strength and elastic modulus were measured using an Instron universal testing machine, and data were submitted to statistical analysis. Considering subgroups B, no material showed a significant difference in the flexural strength with the controls (p > 0.05), whereas for subgroups C, only GXT and VCB showed significantly lower values (p < 0.05). AFX reported the lowest flexural strength among the materials tested. As regards the elastic modulus, no material showed a significant variation after acidic storages when compared with the respective control (p > 0.05). AFX and EPH reported the lowest elastic modulus compared to the other materials. All composites tested showed adequate flexural properties according to the standards, except for AFX. This biomimetic material, along with EPH, might be indicated for V class (cervical) restorations considering the lowest values of elasticity reported.

Keywords: dentistry; conservative; restorative; materials; nanohybrid; resin composites; ormocer-based composites; acidic drink; acid; flexural strength; elastic modulus

1. Introduction

Along with periodontitis, dental caries represents the most common oral disease and the major cause of tooth loss [1]. It consists of an infective process leading to the dissolution of hydroxyapatite, the mineral component of the hard tissues of tooth and bone [2]. In particular, orthodontic patients might be more susceptible to demineralization around brackets and bands, due to plaque accumulation [3]. However, non-carious lesions might also affect the integrity of dental elements and, within this group, erosions, abrasions, attrition, and abfractions are included [4]. During dental erosion, the same fade of the tooth’s hydroxyapatite occurs as reported for decay, this time without the action of acidogenic bacteria but following the exposition to both intrinsic (e.g., gastroesophageal reflux, eating disorders associated with vomiting) or extrinsic (e.g., acidic drinks, bleaching procedures) acidic factors [5,6]. Focusing on acid drinks, they have become more and more popular in recent years, especially among...
the young. For example, it has been shown that between 56% and 85% of children daily consume such beverages [7]. The demineralizing action of acids on the teeth consists of an attack exerted by hydrogen ions (H\(^+\)) against the anions of the enamel crystals (CO\(_3^{2-}\) e PO\(_4^{3-}\)) with a consequent erosion. If remineralization does not occur by means of saliva or a remineralizing agent, a definitive loss of the outer enamel takes place, while the layer below becomes softer [8]. In addition to the effect on the dental structure, these acid substances might also have consequences on general health considering, for instance, the major risk of gastritis to which consumers are exposed, along with the risk of overweight, obesity, and type 2 diabetes [9].

With the aim of restoring the dental structure, the infected dental tissue, if present, must be completely removed and cavities generally filled with biomimetic composites in order to simulate the anatomical condition [10]. Considering the masticatory loads, especially in the posterior segment of the dental arches, restorative materials should guarantee good mechanical properties and much research has been conducted in this regard [11–13]. However, even restorative materials are subjected to the action of acids of the oral cavity with a corrosion and a subsequent alteration of their initial characteristics. For instance, in vitro microhardness has been shown to be significantly decreased after acidic exposure because of a degradation of the polymer network of the composite and the falling out of the resin, with an eventual risk of secondary decay [14].

Properties such as flexural strength have been largely investigated in orthodontics as regards fiber-reinforced composites retainers both in vitro [15–18] and clinically [19,20]. Conversely, to date, fewer studies have been reported that assess the variation of these parameters after exposure to acidic beverages [21–23].

Accordingly, the purpose of the present research is to assess the flexural strength and elastic modulus of common restorative composites after different acidic storages. The null hypothesis is that for each material tested there is no significant difference in the flexural strength and elastic modulus for the five different composite resins neither after a three-week acid challenge (followed by a one-week storage in distilled water) nor after a four-week acid challenge when compared to controls stored in distilled water during the whole experimentation.

2. Materials and Methods

2.1. Specimen Preparation

Five different composites were considered for this study and subdivided into respective groups: x-tra fil (Voco, Cuxhaven, Germany) (Group 1, XTF), GrandioSO x-tra (Voco, Cuxhaven, Germany) (Group 2, GXT), Admira Fusion x-tra (Voco, Cuxhaven, Germany) (Group 3, AFX), VisCalor bulk (Voco, Cuxhaven, Germany) (Group 4, VCB), and Enamel Plus HRi (Micerium, Genova, Italy) (Group 5, EPH). The characteristics of the materials tested are shown in Table 1.
<table>
<thead>
<tr>
<th>Group</th>
<th>Material</th>
<th>Code</th>
<th>Type</th>
<th>Composition</th>
<th>Filler</th>
<th>Lot Number</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>x-tra fil</td>
<td>XTF</td>
<td>Light-curing posterior filling material</td>
<td>Matrix: dimethacrylate (Bis-GMA, TEGDMA, UDMA)</td>
<td>Inorganic filler (Bariumaluminium silicate, fumed silica, pigments)</td>
<td>86 (w/w)</td>
<td>1906144</td>
</tr>
<tr>
<td>2</td>
<td>GrandioSO x-tra</td>
<td>GXT</td>
<td>Aesthetic nanohybrid bulk restorative material</td>
<td>Matrix: Bis-GMA, Bis-EMA, aliphatic dimethacrylate</td>
<td>Inorganic filler, organically modified silica</td>
<td>86 (w/w)</td>
<td>1907626</td>
</tr>
<tr>
<td>3</td>
<td>Admira Fusion x-tra</td>
<td>AFX</td>
<td>Nano-hybrid ORMOCER®-based material</td>
<td>Matrix: ORMOCER®</td>
<td>Filler: glass ceramics, silica nanoparticles, pigments</td>
<td>84 (w/w)</td>
<td>1904427</td>
</tr>
<tr>
<td>4</td>
<td>VisCalor bulk</td>
<td>VCB</td>
<td>Termoviscous bulk-fill composite (Nano-hybrid composite)</td>
<td>Matrix: Bis-GMA, aliphatic dimethacrylate</td>
<td>Inorganic filler</td>
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<td>76292</td>
</tr>
<tr>
<td>5</td>
<td>Enamel Plus HRi</td>
<td>EPH</td>
<td>Nano-hybrid composite</td>
<td>Matrix: Diurethandimethacrylate, BisGMA, 1,4-butandiol dimethacrylate</td>
<td>Filler: surface-treated nano zirconium oxide particles, glass</td>
<td>74% (w/w)</td>
<td>2018004910</td>
</tr>
</tbody>
</table>
Thirty samples were considered for each group, a sample calculation test was performed using the software Sample Size Calculator (Calculator.net, 2203 Timberloch PI, Suite 252, The Woodlands, TX 77380, USA) [24].

Each material was inserted into thirty rectangular prism-shaped stainless-steel molds (25 mm length × 2 mm depth × 2 mm height), with a surface-to-volume ratio of 2.08 mm$^{-1}$ [25]. Before its application, the VisCalor bulk was preheated with the preheating device (Caps Warmer, Voco, Cuxhaven, Germany) at 68 °C for 15 min, in accordance with the operating instructions, due to its thermoviscous behavior.

Each mold was completely filled, and a polyester matrix strip (Mylar strip, Henry Schein, Melville, NY, USA) was positioned above to form a flat surface and to prevent oxygen from interfering with the polymerization of the most superficial layer of the composite [26]. Samples were then photopolymerized for 3 min [16] into a light-curing oven (Spectramat, Ivoclar Vivadent AG, Schaan, Liechtenstein) with a light intensity of 1200 mW/cm$^2$, a wavelength of 430–480 nm, lamp socket R7s, a lamp diameter of 13.5 mm, and a lamp length of 160 mm.

The bars of composite obtained were removed from the molds and stored in water for 48 h in the dark, at 37 °C and 100% humidity [27].

Subsequently, the thirty specimens of the 5 groups were randomly divided into three subgroups (A, B, and C) of 10 specimens each, with an assigned storage for each one as here listed:

1. subgroups A (1a–5a): 4-week storage in 50 mL distilled water (control subgroups);
2. subgroups B (1b–5b): 3-week storage in 50 mL distilled water + 1-week storage in 50 mL soft drink;
3. subgroups C (1c–5c): 4-week storage in 50 mL soft drink.

The times of the acid challenges are based on a previous study reported in the literature considering 1 and 4 weeks in soft drinks [28].

Both distilled water and the soft drink used (Coca-Cola, Coca-Cola Company, Milano, Italy) were at room temperature (18 ± 1 °C). Specimens were immersed singularly in the 50 mL solutions, and in the case of subgroups C, the acidic drink was changed weekly [29]. This solution had a pH value of 2.52 which was measured before each immersion of the specimens. Moreover, it was checked before testing in both subgroups B and C. No remarkable variations of the pH value occurred during the various measurements.

2.2. Three-Point Flexural Test

Each sample was positioned inside an aluminum support having a distance of 21 mm between the two arms. A universal testing machine (Model 3343, Instron Corporation, Canton, MA, USA) was used to apply a compressive load on the middle of the specimens with a crosshead speed of 1.0 mm per minute until the failure (Figure 1) [30]. Flexural strength ($\sigma$) and elastic modulus (E) were calculated as follows [31]:

$$\sigma = \frac{3FL}{2BH^2}$$  \hspace{1cm} (1)

where F is the maximum load (Newtons), L is the distance between the arms (millimeters), B is the width of the samples (millimeters), and H is the height (millimeters).

$$E = \frac{FL^3}{4BH^3d}$$  \hspace{1cm} (2)

where F is the maximum load, L is the distance between the arms, B is the width of the samples, H is the height of the specimen, and d is the deflection (in millimeters) corresponding to the load F.
Figure 1. Mechanical tests performed on the samples (span length: 21mm): left, sample before the fracture; right, sample at the moment of the fracture.

Statistical analyses were performed with computer software (R version 3.1.3, R Development Core Team, R Foundation for Statistical Computing, Wien, Austria). Descriptive statistics were calculated (mean, standard deviation, and the minimum and maximum value). The normality of the distributions was assessed with the Kolmogorov and Smirnov test. Nonparametric analysis of variance (Kruskal–Wallis method) was applied to determine the presence of significant differences among the various groups considered [32]. The Mann–Whitney post hoc test was applied. Significance for all statistical tests was predetermined at \( p < 0.05 \).

3. Results

3.1. Flexural Strength

Descriptive statistics of the various groups are shown in Table 2 and Figure 2.

Table 2. Descriptive statistics of flexural strength (MPa) for each group/subgroup.

<table>
<thead>
<tr>
<th>Material Code</th>
<th>Group-Subgroup</th>
<th>Mean (*)</th>
<th>Standard Deviation (%)</th>
<th>Minimum</th>
<th>Median</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>XTF</td>
<td>1a</td>
<td>149.66 a</td>
<td>6.18</td>
<td>132.30</td>
<td>150.22</td>
<td>166.95</td>
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<tr>
<td>XTF</td>
<td>1b</td>
<td>144.78 a</td>
<td>11.22</td>
<td>123.24</td>
<td>143.13</td>
<td>172.86</td>
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<tr>
<td>XTF</td>
<td>1c</td>
<td>142.18 a</td>
<td>2.26</td>
<td>123.24</td>
<td>141.16</td>
<td>172.46</td>
</tr>
<tr>
<td>GXT</td>
<td>2a</td>
<td>139.31 a</td>
<td>7.48</td>
<td>122.06</td>
<td>138.60</td>
<td>154.74</td>
</tr>
<tr>
<td>GXT</td>
<td>2b</td>
<td>131.91 a</td>
<td>4.73</td>
<td>121.67</td>
<td>131.71</td>
<td>140.18</td>
</tr>
<tr>
<td>GXT</td>
<td>2c</td>
<td>118.09 b</td>
<td>8.84</td>
<td>100.80</td>
<td>120.09</td>
<td>129.94</td>
</tr>
<tr>
<td>AFX</td>
<td>3a</td>
<td>69.58 c</td>
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<td>59.06</td>
<td>70.68</td>
<td>75.99</td>
</tr>
<tr>
<td>AFX</td>
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<td>4a</td>
<td>147.18 a</td>
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<tr>
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<td>111.43</td>
<td>121.67</td>
<td>135.06</td>
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</tbody>
</table>

(*) Superscript letters (a, b and c) have been used to indicate statistical results: different letters indicate the presence of significant differences in flexural strength among the groups.
Figure 2. Mean flexural strength (MPa) for each group/subgroup. Legend: 1: x-tra fil; 2: GrandioSO x-tra; 3: Admira Fusion x-tra; 4: VisCalor bulk; 5: Enamel Plus HRi. Subgroups A (1a–5a): 4-week storage in 50 mL distilled water (control subgroups); Subgroups B (1b–5b): 3-week storage in 50 mL distilled water + 1-week storage in 50 mL soft drink; Subgroups C (1c–5c): 4-week storage in 50 mL soft drink. n.s.; non-significant; *: asterisks indicate the presence of significant differences among the subgroups.

The Kruskal–Wallis method showed significant differences among groups ($p < 0.0001$). Post-hoc showed that for groups 1, 3, and 5, a statistical difference occurred among the corresponding subgroups ($p < 0.05$) but not within the three subgroups of each material ($p > 0.05$). Subgroups 2a and 2b as well as subgroups 4a and 4b showed no significant difference with group 1 ($p > 0.05$) but, conversely, subgroups 2c and 4c showed significant lower values ($p < 0.05$), comparable with those assessed in group 5 ($p > 0.05$).

### 3.2. Elastic Modulus

Descriptive statistics of the various groups are shown in Table 3 and Figure 3.

<table>
<thead>
<tr>
<th>Material Code</th>
<th>Group-Subgroup</th>
<th>Mean (*)</th>
<th>Standard Deviation (%)</th>
<th>Minimum</th>
<th>Median</th>
<th>Maximum</th>
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</table>

(*) Superscript letters (a, b and c) have been used to indicate statistical results: different letters indicate the presence of significant differences in elastic modulus among the groups.
In the field of restorative dentistry, biomimetic composite materials have been undergoing a rapid evolution considering the number of products proposed. These are extremely more appreciated if compared to other ones previously proposed, like amalgam [33]. However, the acidic conditions of the oral cavity, such as the consumption of soft drinks or acidic foods and the presence of acidophile bacteria, constitute a danger not only for teeth but even for filling materials [34]. In particular, a recent study considering four of the five products tested in the present report (x-tra fil, GrandioSO x-tra, Admira Fusion x-tra, VisCalor bulk) stated that their microhardness is significantly reduced when stored in an acidic beverage, both for 1 day and for 1 week, with respect to the samples kept in water [14]. In the literature it has been supposed that, when exposed to low pH conditions, the filler of the resin tends to fall out and the matrix tends to decompose [35]. The dissolution of both enamel and restorative dental composites has been shown to take place under a pH value of 4 [36].

Figure 3. Mean elastic modulus (MPa) for each group/subgroup. Legend: 1: x-tra fil; 2: GrandioSO x-tra; 3: Admira Fusion x-tra; 4: VisCalor bulk; 5: Enamel Plus HRi. Subgroups A (1a–5a): 4-week storage in 50 mL distilled water (control subgroups); Subgroups B (1b–5b): 3-week storage in 50 mL distilled water + 1-week storage in 50 mL soft drink; Subgroups C (1c–5c): 4-week storage in 50 mL soft drink. n.s.; non-significant; The Kruskal–Wallis method showed significant differences among groups ($p < 0.0001$). Post-hoc showed that the highest elastic modulus was reported for groups 1, 2, and 4, with no statistical difference neither among the corresponding subgroups ($p > 0.05$) nor among the three subgroups of each material ($p > 0.05$). Values reported for groups 3 and 5 were significantly lower ($p < 0.05$) but, even this time, without significant differences neither among the corresponding subgroups, nor among the three subgroups of the two materials ($p > 0.05$). Finally, the values of subgroups 4c and 5a showed no statistical difference among them ($p > 0.05$).

4. Discussion

Among the mechanical properties, the flexural strength and elastic modulus have been studied until now in orthodontics [37], endodontics [38], and prosthodontics [39], particularly for fiber-reinforced composites. The flexural strength is a relevant index to identify the capacity to support masticatory loads, whereas the elastic modulus guides clinicians to choose the right material for the specific clinical case. Despite previous reports that have been conducted on nonfiber-reinforced composites, the variation of the abovementioned parameters after immersion into acidic solutions has not been widely investigated. Accordingly, the purpose of the present report was to evaluate the influence of different acidic storages on various resin composites and on an ormocer-based material commonly used in a clinical setting.
The first null hypothesis of this study has been partially accepted. No significant difference has been detected for XTF, AFX, and EPH ($p > 0.05$), independently of the acidic challenge (3 weeks in water + 1 week in Coca-Cola vs 4 weeks in Coca-Cola) and with respect to the controls stored in water for 4 weeks. Conversely, both GXT and VCB showed a significant difference with the controls when storing samples only in Coca-Cola, but not when the acidic challenge was preceded by 3 weeks in distilled water. Therefore, the erosive action of Coca-Cola exerted for 1 week did not significantly affect the flexural strength. Conversely, the 1-month acidic challenge was able to significantly decrease this parameter for only two materials tested, which was not dependent of the percentage of their filler content, considering that it was similar with that of other materials that did not report a significant variation. As well, it was not dependent on the fact that these two materials are bulk-fill composites, since some of the other materials not showing the abovementioned behavior belong to bulk-fill category as well. Therefore, this might be justified by the different concentrations of the chemical components which are not disclosed by the manufacturers.

On the basis of the results obtained during this first part of the experimentation, it should be emphasized that, however, standard deviations of the mean values of the flexural strength for each group/subgroups were almost always higher than 5%, which is the maximum value according to ISO 178/2010 [40].

As regards subgroups A and B, it is not possible to establish whether the immersion into distilled water might have altered the flexural strength, since no comparison has been carried out with dried controls. Contradictory results have been reported in the literature [41,42].

In the only previous study dealing with this topic, the nanofilled composite Filtek Supreme XTE, despite the highest initial values, was the only one to show a statistical reduction in the flexural strength when comparing the one-week storage in Cola with the controls kept in water for 24 hours; on the contrary, the other materials tested did not significantly vary their flexural properties, even after 1 month in acidic drink [25]. These outcomes partially disagree with the results obtained for the materials tested in this report, since we have not even found a material reporting a significant decrease in flexural strength after one week. Conversely, we stated that there were two composites whose values were significantly lower after the 1-month acid challenge.

Admira Fusion x-tra is the material reporting the lowest value of the flexural strength. Similarly, the previous study assessed an analogue characteristic for Admira Fusion [25]. Moreover, in another report, Admira Fusion x-tra was the one reporting the lowest microhardness value of both the external and internal sites after polymerization, as well as the highest mean percentage reduction in microhardness after acidic storage for both 1 day and 1 week in Coca Cola [14]. Both Admira Fusion and Admira Fusion x-tra belongs to a particular group of materials which differ from the other ones tested. In fact, they are based on the technology called Ormocer, an acronym of “organically modified ceramics”, which consists of inorganic-organic co-polymers in addition to the inorganic silanated filler particles [43]. Ormocers have improved biocompatibility compared to resin-based restorative dental materials [44] but, according to the systematic review and meta-analysis of Monsarrat et al., [45] the first generation of ormocers shows a worse clinical behavior than conventional composites, in particular after long-term aging. The ISO 4049/2009 [46], subsequently revised by The Academy of Dental Materials [47], establishes a minimum value of 80 MPa to consider polymer-based restorative materials adequate for filling occlusal surfaces. Neither Admira Fusion x-tra in the present report nor Admira Fusion in the previous one has exceeded this ideal value, not even the control samples. Conversely, according to our results, all the other materials tested were largely above this limit, independently of the storage condition, and therefore they might represent adequate materials for high-stress bearing areas.

The second null-hypothesis has been accepted. In fact, for none of the materials tested a variation of the elastic modulus occurred after the two different acidic storages if compared to controls, which is in accordance with the previous study mentioned [25]. The highest values of elastic modulus were reported for x-tra fil, GrandioSO x-tra and Viscador bulk, with no significant difference among them. On the opposite, both Admira Fusion x-tra and Enamel Plus HRi showed significantly lower values.
This latter had the lowest percentage of filler content which justify the low values of flexural strength reported in the three different conditions: an increase of filler content has been shown to be related to higher values of elastic modulus [48]. It might be deduced that Admira Fusion x-tra and Enamel Plus HRi are more reliable when used for V class (cervical) restorations, since their higher elasticity can absorb the indirect stresses generating at the cervical zone of the tooth [25]. Conversely, the higher elastic modulus of x-tra fil, GrandioSO x-tra and VisCalor bulk might justify their use in occlusal areas requiring stiffer composites.

In a previous study, the materials corresponding to groups 1 to 4, after being cured for 20 seconds with a LED unit having an output irradiance of 1000 mW/cm², have shown an adequate depth of cure represented by a hardness ratio greater than 0.80 [14]. Since the conditions of photopolymerization considered in the present report appear to be more extreme (3 min into a light-curing oven with a light intensity of 1200 mW/cm²), it can be supposed that the leaching of monomers into the media with the activation of enzymatic degradation probably did not occur and therefore there is no alteration of the effect caused by the acidic environment on the mechanical parameters here considered.

The main limitation of this report is that it has been conducted in vitro, therefore the buffering capacity of saliva, which contrasts the erosive action of acids [49], has not been considered. Controls were not stored in a dry environment but in distilled water which might have partially influenced the parameters studied. Actually, a previous study found no significant alteration of the flexural strength for composites stored in distilled water for 7 days when compared to the controls [41]. However, in our report, the samples of subgroups A were kept in water for 4 weeks, which is a longer time and an alteration of the parameters studied might have really occurred. Therefore, it would be interesting to further confirm our preliminary results by measuring not only flexural strength and elastic modulus after storage in water (positive controls), but even before (negative controls). As well, considering the eventual action exerted by distilled water, the experimentation should also provide a weekly change of the medium, as done in this study for the acid solution.

As regards the acidic challenges, the experimentation was conducted under extreme conditions represented by storages in Coca-Cola for an entire week (after 3 in water) and for 4 weeks. However, this continuous exposure to soft drink simulates a long-term exposure in the oral cavity: immersion in Coca-Cola for 1 day is comparable to an in vivo exposure for a month [50]. Moreover, chemical erosion might change the physical dimensions of the exposed samples, therefore this variation should be considered to correct the calculations of both the flexural strength and elastic modulus. Finally, it would be of interest to measure further parameters besides the two here considered, such as the surface roughness at the tension side of the beam detecting any correlation with the strength decrease, as well as to complement the results with images from optical and SEM microscopy. Further in vivo studies are required to confirm the results obtained, and other biomimetic restorative materials such as compomers and glass ionomers should be tested because of their higher susceptibility to an acidic storage media, due to a buffering action exerted towards it [51].

5. Conclusions

Under the limitations of this in vitro study, we can conclude that the flexural strength was significantly affected only for GXT and VCV after storage in Coca-Cola for an entire month. None of the different acidic exposures considered in this study have significantly altered the elastic modulus of the biomimetic materials tested.

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References


23. BinMahfooz, A.M.; Qutub, O.A.; Basunbul, G.I. E


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Biomimetic Catalysts Based on Au@ZnO–Graphene Composites for the Generation of Hydrogen by Water Splitting

Abniel Machín 1,*, Juan C. Arango 2,*, Kenneth Fontánez 2, María Cotto 2, José Duconge 2, Loraine Soto-Vázquez 3, Edgar Resto 3, Florian Ion Tiberiu Petrescu 4, Carmen Morant 5 and Francisco Márquez 2

1 Arecibo Observatory, Universidad Ana G. Méndez-Cupey Campus, San Juan, PR 00926, USA
2 Nanomaterials Research Group, School of Natural Sciences and Technology, Universidad Ana G. Méndez-Gurabo Campus, Gurabo, PR 00778, USA; jcarangolozano@hotmail.com (J.C.A.); kenneth.fontanez@gmail.com (K.F.); mcotto48@uagm.edu (M.C.); jduconge@uagm.edu (J.D.); fmarquez@uagm.edu (F.M.)
3 Materials Characterization Center Inc., Molecular Sciences Research Center, University of Puerto Rico, San Juan, PR 00926, USA; sotol6@uagm.edu (L.S.-V.); edgar.resto@upr.edu (E.R.)
4 IFToMM-ARoTMM, Bucharest Polytechnic University, 060042 Bucharest, Romania; tiberiuflorianion@gmail.com
5 Department of Applied Physics, Autonomous University of Madrid, 28041 Madrid, Spain; c.morant@uam.es
* Correspondence: machina1@uagm.edu

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Abstract: For some decades, the scientific community has been looking for alternatives to the use of fossil fuels that allow for the planet’s sustainable and environmentally-friendly development. To do this, attempts have been made to mimic some processes that occur in nature, among which the photosystem-II stands out, which allows water splitting operating with different steps to generate oxygen and hydrogen. This research presents promising results using synthetic catalysts, which try to simulate some natural processes, and which are based on Au@ZnO–graphene compounds. These catalysts were prepared by incorporating different amounts of gold nanoparticles (1 wt.%, 3 wt.%, 5 wt.%, 10 wt.%) and graphene (1 wt.%) on the surface of synthesized zinc oxide nanowires (ZnO NWs), and zinc oxide nanoparticles (ZnO NPs), along with a commercial form (commercial ZnO) for comparison purposes. The highest amount of hydrogen (1127 µmol/hg) was reported by ZnO NWs with a gold and graphene loadings of 10 wt.% and 1 wt.%, respectively, under irradiation at 400 nm. Quantities of 759 µmol/hg and 709 µmol/hg were obtained with catalysts based on ZnO NPs and commercial ZnO, respectively. The photocatalytic activity of all composites increased with respect to the bare semiconductors, being 2.5 times higher in ZnO NWs, 8.8 times higher for ZnO NPs, and 7.5 times higher for commercial ZnO. The high photocatalytic activity of the catalysts is attributed, mainly, to the synergism between the different amount of gold and graphene incorporated, and the surface area of the composites.

Keywords: hydrogen production; ZnO; gold nanoparticles; graphene; water splitting

1. Introduction

If you were asking to identify one of the many challenges that our world is facing right now, you would probably say climate change, energy production, or sustainability. Most of these problems are directly related to the continuous growth of the world population and the use of fossil fuels as our primary energy source [1].
In the case of finding a clean and renewable energy source, multiple candidates have been proposed over the years. One of them is hydrogen. Some of the properties that make hydrogen a good candidate to replace fossil fuels are its abundance, high energy yield, storage capability, and environmental compatibility [2]. Hydrogen is the most abundant element in the universe and can be found in water and biomass. If compared to hydrocarbons, hydrogen can produce 2.75 times more energy, can be stored as a solid, liquid, or gas, and if it is combined with oxygen, no harmful and toxic gases such as nitrogen oxides (NO\textsubscript{x}) or sulfur oxides (SO\textsubscript{x}) are released to the atmosphere [2].

Unfortunately, the vast majority of hydrogen that is produced in the world, comes from a process known as natural gas reforming [3]. As the name suggests, this process uses natural gas, methane (CH\textsubscript{4}), as the source to obtain hydrogen. This process releases carbon dioxide (CO\textsubscript{2}) to the atmosphere, increasing the amount of this greenhouse gas and exacerbating global warming [3].

Photosynthesis offers an excellent model for designing an artificial solar energy conversion system for clean fuel generation. In nature, electrons are provided to the reaction center of the photosystem-II in four consecutive proton-coupled electron transfer steps, and ultimately appear as reduced carbon derived products that form the basis of biological activity. Inspired by natural principles, for decades there has been a continuous effort to design artificial photosynthetic assemblies based on the use of solar energy to generate oxygen and hydrogen by water splitting [4–6]. Over the years, multiple candidates such as titanium dioxide (TiO\textsubscript{2}), zinc oxide (ZnO), tungsten trioxide (WO\textsubscript{3}), cadmium sulfide (CdS), among others [7–11] have been used to produce hydrogen via water splitting [12,13]. From all these photocatalysts, TiO\textsubscript{2} has been extensively studied over the years mainly due to its chemical stability, abundance, non-toxicity, and high hydrogen yield [12].

Similar to titanium oxide, ZnO has also been demonstrated to be chemically stable, easy to produce, non-toxic, abundant, and environmentally-friendly [9,10], although unlike titanium dioxide, ZnO has been widely used for the degradation of organic pollutants and energy storage [14–16]. Some authors [17,18] consider that ZnO shows some disadvantages for the production of hydrogen by water splitting, especially the recombination of photogenerated electron–hole pairs, fast backward reaction, and the inability to use visible light. To try to solve these limitations, different approaches have been implemented over the years. One of them has been the incorporation of noble metal to the surface of the catalysts [17,18]. Among noble metals, gold has gained much attention since the 1980s because of its wide range of applications, including electronics, photodynamic therapy, delivery of therapeutic agents, sensors, probes, diagnostics, and catalysis [19,20]. Multiple pathways to incorporate gold nanoparticles (Au NPs) can be found in the literature. Methods such as coprecipitation [21], chemical reduction [22], phytochemical reduction [23], and the hydrothermal approach [24] have been successfully implemented over the years. All these synthesis procedures take into account parameters such as the preparation procedure, gold loading (percentage of gold weight on the material), particle size, dispersion (percentage of support surface covered by gold), and shape of the particles.

Recently, another approach that has drawn a lot of attention is the use of graphene as a co-catalyst for the production of hydrogen via water splitting. Graphene has unique properties such as high thermal conductivity, excellent mobility of charge carriers, large surface area, and good mechanical stability [25]. As a co-catalyst, graphene has significant advantages, including that it (i) provides a support for anchoring well-dispersed metallic or oxide nanoparticles; (ii) works as a highly conductive matrix for enabling good contact throughout the matrix; (iii) induces an easy electron transfer from the conduction band of the semiconductor to graphene because of the large energy level offset formed at the interface, leading to an efficient charge separation; and (iv) acts as an efficient co-catalyst for H\textsubscript{2} evolution because of its large specific surface area and superior electron mobility [26].

There are several approaches reported on the literature to prepare graphene–ZnO composites. For example, Tien and group [27] used a microwave-assisted solvothermal process, whereas Ong and group [28] reported the preparation of the composites using a chemical deposition–calcination approach.

There is very limited information in the literature on the production of hydrogen via water splitting by combining graphene and ZnO. Haldorai and Shim [29] reported the production of hydrogen via
water splitting by employing a supercritical fluid mediated synthesis. They reported that the composites exhibited enhanced photocatalytic activity, because the ZnO particles on the graphene sheets captured light energy and acted as electron mediators.

To our knowledge, no results have been reported on the incorporation of graphene and gold nanoparticles on the surface of ZnO for the production of hydrogen by water splitting. The information found in the literature is very limited and focuses on the degradation of dyes and nitrobenzene under visible and UV light. For example, Zeng et al. [30] and Wang et al. [31] reported high photocatalytic activity for the degradation of rhodamine B and methylene blue, respectively. They found that the combination of gold and graphene on the surface of ZnO allows the system to use visible and UV light, and more importantly, greatly improves the degradation percentage compared to pure ZnO and Au–ZnO. On the other hand, Roy et al. [32] reported on the efficient reduction of nitrobenzene under UV and visible light, in which the catalyst reduced 97.8% of the original compound.

Due to the lack of information on the production of hydrogen by water splitting using Au@ZnO–graphene composites, the objectives of this research focused mainly on (i) synthesizing ZnO with different morphologies (nanoparticles and nanowires); (ii) incorporating different amounts of gold nanoparticles (1 wt.%, 3 wt.%, 5 wt.%, and 10 wt.%) and graphene (1 wt.%) to the surface of the as-synthesized catalysts and to its commercial form; and (iii) characterizing the photocatalytic activity of the compounds by studying the production of hydrogen by water splitting under UV–vis radiation. Au@ZnO–graphene-based catalysts were characterized using HRTEM, UV–vis spectroscopy, BET surface area, XRD, XPS, Raman spectroscopy, and GC–TCD.

2. Materials and Methods

2.1. Reagents

All reagents were used as received and all the solutions were prepared using deionized water (Milli-Q water, 18.2 MΩ cm−1 at 25 °C), Zn(CH₃COO)₂•2H₂O (98+%, ACS Reagent), HAuCl₄•3H₂O (ACS Reagent, 49.0+% Au basis), ethanol (95%), and NaBH₄ (+99.9%) were provided by Sigma Aldrich (Milwaukee, Wisconsin USA). NaOH (98+%) and ZnO (99.99%) were acquired from Alfa Aesar (Ward Hill, Massachusetts USA). Graphene (99%) was provided by Cheap-Tubes (Grafton, Vermont USA). For photocatalytic experiments, Na₂S (99.9+%) and Na₂SO₃ (98+%) were obtained from Sigma Aldrich (Milwaukee, Wisconsin USA) and used as sacrificial reagents.

2.2. Synthesis of Nanomaterials

ZnO nanowires (ZnO NWs) were obtained according the method described elsewhere [33]. Zinc oxide nanoparticles (ZnO NPs) were synthesized following the method used by Nejati et al. [34]. The deposition of Au NPs and graphene on the surface of ZnO NWs, ZnO NPs, and the commercial ZnO (commercial ZnO) was based on the method described by Naldoni et al. [35], later modified by Wang et al. [15]. In a typical synthesis, 200 mg of the product (ZnO NWs, ZnO NPs, and commercial ZnO) containing the gold nanoparticles was dispersed in a solution containing 10 mL of ethanol and 40 mL of deionized water, and the mixture was vigorously stirred for 30 min. Subsequently, 2 mg of graphene was added, and the suspension was kept under stirring for 1 h. After that, the product was collected and centrifuged 3 times with deionized water and dried overnight to 60 °C. Finally, the product was collected, sealed, and stored at room temperature. The different Au@ZnO–graphene composites were identified as x%Au@ZnO–graphene. The numbers (x%) correspond to the weight percentage of Au NPs in the sample. In all cases, the amount of graphene was 1 wt.%.

2.3. Characterization of the Catalysts

The catalysts were characterized by high resolution transmission electron microscopy (HRTEM), using a JEOL 3000F. XPS measurements were performed on an ESCALAB 220i-XL spectrometer, using the non-monochromated Mg Kα (1253.6 eV) radiation of a twin-anode, operating at 20 mA and 12 kV
in the constant analyzer energy mode, with a PE of 40 eV. Brunauer Emmett Teller (BET) specific areas were measured using a Micromeritics ASAP 2020, according to N\textsubscript{2} adsorption isotherms at 77 K. Raman (DXR Thermo Raman Microscope, employing a 532 nm laser source at 5 mW power and a nominal resolution of 5 cm\textsuperscript{-1}) and X-ray diffraction (Bruker D8 Discover X-ray diffractometer, operating at 40 kV and 40 mA in the range of 30–75° at 1° min\textsuperscript{-1}) were also used. UV–vis spectroscopy (Shimadzu UV-2401PC) was used as a complementary technique to determine the absorption edge of the catalysts.

2.4. Photocatalytic Experiments

The production of hydrogen via water splitting was measured by adding 50 mg of the x\%Au@ZnO–graphene catalyst into 100 mL of deionized water and transferring this suspension to a 250 mL quartz reactor. Then, solutions of 0.02 M Na\textsubscript{2}SO\textsubscript{3} and 0.4 M Na\textsubscript{2}S were added as sacrificial reagents. After that, the solution was thermostatted at 20 °C and purged for 30 min with nitrogen (N\textsubscript{2}). Finally, the reaction mixture was irradiated with UV–vis light for 2 h using different filters to select the appropriate wavelength (280 nm, 320 nm, 400 nm, and 500 nm). The produced hydrogen was quantified by gas chromatography (GC), using a thermal conductivity detector (GC–TCD, Perkin-Elmer Clarus 600) [18].

3. Results and Discussion

3.1. Characterization of Catalysts

The characterization of the different ZnO supports and Au@ZnO-based catalysts was shown in our previous research [18]. On these catalysts, 1 wt.% graphene was incorporated. Figure 1 shows the HRTEM images and the selected area electron diffraction (SAED) patterns of the 10%Au@commercial ZnO–graphene (Figure 1A), 10%Au@ZnO NPs–graphene (Figure 1B), and 10%Au@ZnO NWs–graphene (Figure 1C) composites. The 10%Au@commercial ZnO–graphene composite consisted of non-homogenous particles with different sizes (lengths and diameters greater than 50 nm) and shapes. Homogeneous spherical gold nanoparticles, with diameters of less than ca. 10 nm, were distributed on the surface of the catalyst. Graphene sheets of different sizes were also distributed unevenly through the sample. According to Wang et al. [15], it is believed that close and homogeneous contact between Au, support, and graphene favors the transfer of photogenerated electrons between them, thus improving charge separation and photocatalytic efficiency. As in the case of the commercial catalyst, the 10%ZnO NPs–graphene catalyst showed non-homogeneous particles of different sizes and shapes, with lengths and diameters greater than 50 nm. The non-homogeneous gold nanoparticles were unevenly distributed throughout the sample, presenting a spherical morphology with diameters of less than 10 nm. Graphene was also unevenly distributed throughout the sample and served as a support for ZnO particles and gold nanoparticles. In the case of the 10%Au@ZnO NWs–graphene catalyst, the incorporation of graphene and gold considerably modified the pristine material. The catalyst consisted of non-homogeneous wires, with an estimated length greater than 300 nm and diameters above 50 nm. Gold nanoparticles, with spherical morphology and diameters of less than 10 nm, were distributed throughout the sample. Graphene also appeared to be unevenly distributed in the sample, but had intimate contact with the gold nanoparticles and the support. SAED patterns of synthesized gold–graphene-based composites were characteristic of monocrystalline materials.

Table 1 shows the BET surface area results of the different Au@ZnO–graphene composites. The incorporation of the different amounts of Au NPs (1 wt.%, 3 wt.%, 5 wt.%, and 10 wt.%), along with graphene (1 wt.%), increased the surface area of all the catalysts when compared to the unmodified supports [18]. This enhancement suggests an intimate contact between the incorporated materials and the support [35]. Graphene, as explained above, has a very high surface area (~2000 m\textsuperscript{2} g\textsuperscript{-1}), which can contribute to increasing the surface area of composites. However, since the amount of graphene, when compared to gold, was minimum, the enhancement of the surface areas of the catalysts could be
primarily attributed to the Au NPs. The highest surface area of the commercial support was measured to be 65 m² g⁻¹ and was obtained with the 10%Au@ commercial ZnO–graphene. This represents an increase of 47 m² g⁻¹ if compared to the unmodified commercial ZnO support. The highest surface area of the Au@ZnO NPs–graphene composites was 117 m² g⁻¹ and was measured in the 10%Au@ZnO NPs–graphene catalyst, showing a difference of 50 m² g⁻¹ if compared to the result obtained with the unmodified ZnO NPs. For the Au@ZnO NWs–graphene composites, the highest surface area was 247 m² g⁻¹, obtained by the 10% Au@ZnO NWs–graphene catalyst. This represents a difference of 80 m² g⁻¹ if compared to the surface area of the unmodified ZnO NWs (167 m² g⁻¹).

![High resolution transmittance electron microscopy (HRTEM) images](image)

Figure 1. High resolution transmittance electron microscopy (HRTEM) images of 10%Au@commercial ZnO–graphene (A), 10%Au@ZnO NPs–graphene (B), and 10%Au@ZnO NWs–graphene (C). The red and yellow arrows indicate the presence of graphene and gold, respectively, and the insets correspond to the selected area of electron diffraction (SAED) patterns.

<table>
<thead>
<tr>
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<th>Commercial ZnO (m² g⁻¹)</th>
<th>ZnO NPs (m² g⁻¹)</th>
<th>ZnO NWs (m² g⁻¹)</th>
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<tr>
<td>Unmodified</td>
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<tr>
<td>10%Au–Graphene *</td>
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<td>117</td>
<td>247</td>
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* The amount of graphene in all the catalysts was 1 wt.%

The XRD patterns of the different composites with a gold loading of 10 wt.% are shown in Figure 2. The characteristic peaks of wurtzite crystalline phase (ca. 32.0° (100), 34.8° (002), 36.0° (101), 47.5° (102), 56.2° (110), 62.8° (103), 66.0° (200), 67.5° (112), 68.8° (201)) were observed in all the catalysts [16]. In all cases, reflections at 38.2° and 44.4° were observed, which have been associated with Au (111) and (200), respectively [20], indicating that Au³⁺ had been reduced to Au⁰, with the usual fcc structure. Au NPs showed other characteristic peaks of lower intensity at 64.7° (220) and 77.8° (311), which could
not be identified in the catalysts. Applying the Scherrer formula [36], an estimate of the mean size of the gold nanoparticles could be made, providing a value of ca. 15 nm in all cases. This value is very close to that determined by HRTEM (<20 nm). Graphene, on the other hand, has characteristic peaks at ca. 24.5° and 44.2° [24]. These peaks were not identified in any of the composites. The amount of graphene incorporated to the Au@ZnO–graphene composites was very low when compared to the amount of the support and gold loading, and it is possible that the signal emitted by graphene was very weak and not able to be detected by the instrument.

The Au@ZnO–graphene composites containing gold loadings of 5 wt.% and 10 wt.% were characterized by Raman spectroscopy (see Figure 3). Graphene has two characteristic peaks at ca. 1350.0 cm$^{-1}$ and 1595.0 cm$^{-1}$, known as D- and G-bands, respectively [37]. The D-band (1350 cm$^{-1}$) has been related to the defects and structural disorder in graphene sheets, whereas the G-band (1595 cm$^{-1}$) has been ascribed to the stretching of the $sp^2$ hybridized carbon–carbon bonds [37]. These two bands were observed in all the gold–graphene composites, including those with gold loadings of 1 wt.% and 3 wt.. The ratio of the intensity between the D- and G-band is a measure of the degree of disorder in graphene [38]. The narrow strong band at ca. 437.0 cm$^{-1}$ ($E_2$ modes) is present in all the composites and it has been ascribed to motion of Zn in the wurtzite phase [16]. No gold bands were found for any of the composites.

Au@ZnO–graphene composites, with gold loadings of 5 wt.% and 10 wt.% were also characterized by UV–vis spectroscopy (Figure 4). All the catalysts presented a similar absorption range between 325 nm and 400 nm, showing a maximum at ca. 370 nm. Interestingly, despite the introduction of graphene and gold, all the composites had almost the same absorption edge as the unmodified catalysts, indicating that there was a consistent band gap of nanocrystalline ZnO within the Au@ZnO–graphene composites. This suggests that no carbon species were incorporated into the lattice of ZnO. Because the impurity level would have shifted the absorption edge to a higher wavelength [39]. No gold peaks (~520–580 nm) were detected for any of the gold loadings incorporated. This might be attributed to the high dispersity of the Au NPs through the samples.

![Figure 2. X-ray diffractometer (XRD) diffraction patterns for 10% Au@commercial ZnO–graphene (a); 10%Au@ZnO NPs–graphene (b); and 10%Au@ZnO NWs–graphene (c).](image)
The catalysts were also characterized by XPS. Figure 5 shows the most relevant spectra of 10%Au@ZnO NWs–graphene and 10%Au@ZnO NPs–graphene. In both systems (Figure 5a,e), the O1s showed a main peak at ca. 530.2 eV, which was assigned to O²⁻ ions in the Zn–O bonds, and a shoulder around 531.5 eV, assigned to O²⁻ ions in the oxygen deficient regions, respectively [18]. As observed, the contribution of this secondary peak was clearly greater in ZnO NWs than in ZnO NPs. As it will be shown later, the highest reactivity was observed in catalysts based on ZnO NWs, so this behavior could be justified thanks to the existence of crystalline defects, as already described in previous works [18]. In fact, surface defects in crystalline ZnO affect its electrical properties, increasing electrical conductivity, which undoubtedly could have positive effects on photocatalysis with these materials. In both catalysts, the Zn2p3/2 spectra showed a single component that was unambiguously assigned to Zn²⁺ in ZnO (see Figure 5b,f). The presence of metallic gold (Au⁰) was evidenced by the presence of a doublet in the emission peak at ca. 84.0 eV (4f7/2) and 87.7 eV (4f5/2) (Figure 5c,g) [40]. No components were observed that could show the presence of Au³⁺, coming from the precursor (HAuCl₄ • 3H₂O), which evidenced the complete reduction of gold. Figure 5d,h shows the transition...
corresponding to C1s. The main peak observed at ca. 284.6 eV was assigned to the carbon backbone of aliphatic/aromatic (sp3/sp2) carbons, while the component indicated by an arrow, around 286.0 eV could be attributed to carbon in C–O and C–O–C groups [41,42], and to contamination by adsorption of oxidized species (CO, CO2).

**Figure 5.** X-ray photoelectron spectroscopy (XPS) spectra of Zn 2p3/2, O 1s, Au 4f, and C1s taken from as-grown Au@ZnO NWs–graphene (a–d), and Au@ZnO NPs–graphene (e–h).

### 3.2. Photocatalytic Hydrogen Production Via Water Splitting

Figure 6 shows the photocatalytic hydrogen production via water splitting of the different catalysts under irradiation at 280 nm (Figure 6a), 320 nm (Figure 6b), 400 nm (Figure 6c), and 500 nm (Figure 6d). The maximum hydrogen production of the unmodified ZnO catalysts was 442 μmol/hg and was obtained with ZnO NWs by irradiation at 280 nm. This high hydrogen production from ZnO NWs was not expected, especially when compared to the maximum hydrogen production of ZnO NPs (86 μmol/hg) and the commercial ZnO (94 μmol/hg). According to a study by Zhang et al. [16], incorporation of noble metal or metal ions, they exhibit a negative shift in the Fermi level that implies a greater degree of electron accumulation in Au-loaded. Thus, such a shift in the Fermi level improves the composite system and enhances the efficiency of the interfacial charge-transfer process. These improvements are in turn associated with a considerable enhancement of the electric charge oscillation known as surface plasmon resonance (SPR) in the presence of light irradiation [26]. Different studies [45–47] have demonstrated that when co-catalysts such as Au and graphene improve a promising material for solar cells due to the fast electron transport, with reduced recombination loss, and its ease of crystallization [16].

Under irradiation at 320 nm, the hydrogen production of ZnO NWs (365 μmol/hg) decreased when compared to that obtained at 280 nm, but then increased again (427 μmol/hg) at 400 nm. This was not expected either since the wide band gap energy of ZnO (3.37 eV for wurtzite) does not favor the production of hydrogen under visible light. Different studies [16,43,44] have found that surface defects and oxygen vacancies in photocatalysts can play a significant role in their photocatalytic activity. Crystalline defects in ZnO nanowires exist primordially due to oxygen vacancies. Even more, these studies have found that nanoparticles with crystalline defects can exhibit visible light activity even without doping them with transition metals.

Both ZnO NPs and the commercial ZnO obtained similar results in all the wavelengths that were evaluated. At 500 nm, the hydrogen production of the unmodified catalysts was almost zero, with the exception of ZnO NWs that obtained a high value of 350 μmol/hg, showing high catalytic activity. Incorporation of gold and graphene greatly increased hydrogen production in both the UV and visible regions of all the composites. The presence of co-catalysts such as Au and graphene improve the charge separation and suppresses the recombination of excited photogenerated carriers, resulting in a better evolution of H2 [26]. Different studies [45–47] have demonstrated that when semiconductors, such as ZnO, are doped with noble metal or metal ions, they exhibit a negative shift in the Fermi level that implies a greater degree of electron accumulation in Au-loaded. Thus, such a shift in the Fermi level improves the composite system and enhances the efficiency of the interfacial charge-transfer process. These improvements are in turn associated with a considerable enhancement of the electric...
near-field [45]. This activity relates strongly to the size and shape-dependent surface charge oscillation known as surface plasmon resonance (SPR) in the presence of light irradiation [26]. Furthermore, the incorporation of graphene on semiconductors creates the p–n junction, which also improves the separation of photogenerated charges [26,48]. The photogenerated holes that were created are then scavenged by the sacrificial agent (S²⁻/SO₃²⁻), and the electrons are excited to the conduction band. Electrons transferred from the conduction band of the semiconductor are injected into the graphene because graphene has a slightly lower redox potential than the semiconductor conduction band [26,48]. Graphene has a high charge carrier transfer and mobility as a result of its π-conjugated structure, and hence Au nanoparticles dispersed on the graphene can also accept electrons and act as active sites to react with adsorbed H⁺ ions for H₂ evolution [26,48].

According to other authors [37,49], some conduction electrons can be transferred directly to the Au NPs deposited on the surface of the semiconductor by ohmic interconnection or to carbon atoms on the graphene, and the electrons then react with the adsorbed H⁺ ions to form H₂. Thus, the synergistic effect between both co-catalysts, plasmonic Au nanoparticles and graphene, can effectively suppress photogenerated charge recombination, enlarge the active adsorption sites and reaction space, and consequently enhance the photocatalytic activity for H₂ evolution [37,49]. In this regard, Wang et al. [49] reported that Au@TiO₂–graphene composites had significantly increased the visible light absorption and enhanced the photocatalytic H₂ production activity compared to the Au@TiO₂. Luo et al. [37] found that by combining graphene and gold nanoparticles on TiO₂–P25, the hydrogen production via water splitting increased nine times more than bare TiO₂–P25.

In this research, the highest hydrogen production of the Au@commercial ZnO–graphene catalysts was 709 μmol/hg and was obtained by 10%Au@ZnO commercial graphene under irradiation at 400 nm. This enhancement represents a difference of 615 μmol/hg when compared to the highest amount obtained by the unmodified commercial ZnO catalyst (94 μmol/hg), and the fact that the maximum production of the commercial catalyst was obtained at 400 nm (visible light) is an indication that the Au NPs are allowing the use of visible light [18,30,37]. On the other hand, appropriate visible light irradiation can induce the SPR effect on the gold nanoparticles and greatly enhance the electron capture capacity [37]. Both reasons affect the generation and separation of charges in photocatalysis, which results in the improvement of photocatalytic properties. The highest amount of hydrogen obtained with 1%Au@commercial ZnO–graphene, 3%Au@commercial ZnO–graphene, and 5%Au@commercial ZnO–graphene catalysts was 405 μmol/hg, 529 μmol/hg, and 589 μmol/hg, respectively, under irradiation at 400 nm.

In the case of Au@ZnO NPs–graphene catalysts, the highest hydrogen production measured was 759 μmol/hg, representing a difference of 673 μmol/hg when compared to the maximum hydrogen production of the unmodified ZnO NPs catalyst (86 μmol/hg), and was obtained with the 10%Au@ZnO NPs–graphene catalyst at 400 nm. The highest hydrogen production for 1%Au@ZnO NPs–graphene, 3%Au@ZnO NPs–graphene, and 5%Au@ZnO NPs–graphene catalysts was 537 μmol/hg, 622 μmol/hg, and 728 μmol/hg, respectively. These results confirm once again that the presence of Au NPs allows the use of visible light to produce hydrogen.

Au@ZnO NWs–graphene catalysts showed the highest hydrogen production (1127 μmol/hg) with a gold loading of 10 wt.% at 400 nm, representing a difference of 685 μmol/hg when compared to the unmodified ZnO NWs catalyst (442 μmol/hg). The highest amount of hydrogen produced at 400 nm with the 1%Au@ZnO NWs–graphene, 3%Au@ZnO NWs–graphene, and 5%Au@ZnO NWs–graphene catalysts was 701 μmol/hg, 828 μmol/hg, and 944 μmol/hg, respectively.

Under irradiation at 500 nm (Figure 6d), the maximum hydrogen production of the Au@commercial ZnO–graphene, Au@ZnO NPs–graphene, and Au@ZnO NWs–graphene catalysts was 628 μmol/hg, 735 μmol/hg, and 1079 μmol/hg, respectively, with a gold loading of 10 wt.%. These high hydrogen productions under low energy irradiation are an indication of the high photocatalytic activity of the composites, especially considering the high band-gap energy (3.37 eV) of ZnO. At wavelengths above
400 nm, the water splitting depends mainly on the Au NPs, due to lack of energy to promote electrons from the valence band to the conduction band of ZnO.

In all cases, the highest amounts of hydrogen reported in this investigation were obtained with the catalysts with the highest surface area (65 m² g⁻¹ for 10% Au@ commercial ZnO–graphene; 117 m² g⁻¹ for 10% Au@ZnO NPs–graphene; 247 m² g⁻¹ for 10% Au@ZnO NWs–graphene). Materials with high surface areas can be attained either by fabricating small particles or clusters where the surface-to-volume ratio of each particle is high, or by creating materials where the void surface area (pores) is high compared to the amount of bulk support material [50]. Multiple studies have demonstrated that the synthesis of high surface area catalysts lead to an increment in the hydrogen production due to the availability of more sites for the interaction of the water molecule with the catalyst [49,50]. In our research, this increase in surface area is primarily achieved by incorporating Au NPs and graphene on the surface of semiconductors.

In the case of Au/graphene–TiO₂, over the years different possible mechanisms have been proposed for the production of hydrogen by water splitting (see Figure 7). One of the most widely accepted is that when compounds are irradiated with UV light (Figure 7A), a direct photoexcitation of TiO₂ with photons with energy larger than the bandgap (λ < 380 nm) leads to the generation of electrons in the conduction band, and electron holes in the valence band of the semiconductor [51]. The electron in the conduction band will move to the Au NPs, acting as electron buffers and catalytic sites for hydrogen generation [52]. When irradiated with visible light (λ > 500 nm) photoexcitation of Au NPs occurs, and electrons from the Au NPs are injected into the TiO₂ conduction band leading to the generation of holes in the Au NPs and electrons in the TiO₂ conduction band [51,52]. Then, the water molecule gains the electrons in the conduction band and hydrogen is produced. Evidence of the proposed mechanism is the fact that the photocatalytic response for hydrogen generation is consistent with the absorption of the Au surface plasmon band. The incorporation of graphene creates a p–n junction, which improves...
the separation of photogenerated charges, and the electrons are excited to the conduction band [26]. The electrons transferred from the conduction band of TiO$_2$ are injected into the reduced graphene in a graphene/TiO$_2$ system because the graphene/graphene redox potential is slightly lower than the CB of TiO$_2$ [26]. In addition, some conduction electrons of TiO$_2$ likely transfer directly to the Au NPs deposited on the surface of the semiconductor by ohmic interconnection or to carbon atoms on the graphene sheets, and the electrons then react with the adsorbed H$^+$ ions to form H$_2$ [25,26]. This creates a synergistic effect between both co-catalysts, and they can effectively suppress photogenerated charge recombination, enlarge the active adsorption sites, and consequently enhance the photocatalytic activity [16,25,26]. When irradiated with visible light ($\lambda > 500$ nm) (Figure 7B) photoexcitation of Au NPs occurs, and electrons from the Au are injected into the ZnO conduction band, leading to the generation of holes in the Au NPs and electrons in the ZnO conduction band [18]. The water molecule gains the electrons in the conduction band and hydrogen is produced. This proposed mechanism is an oversimplification since different studies [18–20] have determined that, due to the gold/semiconductor interfacial contact, the conduction band of the semiconductor undergoes shift toward more negative potentials [18]. Thus, the charge distribution between the Au NPs and the semiconductor causes a shift of the Fermi level toward more negative potentials [18,19].

![Figure 7](attachment:image.png)

**Figure 7.** Possible mechanism of hydrogen production under ultraviolet (A) and visible (B) irradiation for the Au@ZnO–graphene systems.
Table 2 shows the highest amounts of hydrogen obtained with Au@ZnO–graphene catalysts under the evaluated parameters. As already mentioned, and to the best of our knowledge, no results have been reported so far on hydrogen production by water splitting using Au@ZnO–graphene catalysts. Therefore, the results of this research are the first reported on the production of H₂ using catalysts based on ZnO and graphene–gold. The materials studied in this research will have to be contrasted by other researchers to establish a much deeper knowledge that allows us to know the complex mechanism of hydrogen production with ternary compounds based on ZnO.

Table 2. Highest amounts of hydrogen production via water splitting obtained with Au@ZnO–graphene catalysts under UV–vis light.

<table>
<thead>
<tr>
<th>Author</th>
<th>H₂ Production (µmol)</th>
<th>Source (nm)</th>
<th>Irradiation Time (h)</th>
<th>ZnO Crystal Structure *</th>
<th>Reaction Mixture</th>
<th>Au (wt.% )</th>
<th>Graphene (wt.%)</th>
</tr>
</thead>
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<tr>
<td>This work ZnO</td>
<td>709</td>
<td>200 &gt; λ &gt; 400</td>
<td>2</td>
<td>W</td>
<td>Water: 0.5 M Na₂S, 0.03 M Na₂SO₃</td>
<td>10</td>
<td>1</td>
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<td>commercial</td>
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<tr>
<td>This work ZnO</td>
<td>759</td>
<td>200 &gt; λ &gt; 400</td>
<td>2</td>
<td>W</td>
<td>Water: 0.5 M Na₂S, 0.03 M Na₂SO₃</td>
<td>10</td>
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<tr>
<td>NPs</td>
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<tr>
<td>This work ZnO</td>
<td>1127</td>
<td>200 &gt; λ &gt; 700</td>
<td>2</td>
<td>W</td>
<td>Water: 0.5 M Na₂S, 0.03 M Na₂SO₃</td>
<td>10</td>
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<td>NWs</td>
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* W = Wurtzite.

4. Conclusions

Graphene and different amounts of gold nanoparticles were incorporated on the surface of synthesized ZnO supports (ZnO NWs, ZnO NPs catalysts), and on the commercial form (commercial ZnO). These catalysts were fully characterized by different techniques, and their photocatalytic activity was determined by measuring the hydrogen produced by water splitting under UV–vis irradiation.

The highest amount of the unmodified ZnO support was 442 µmol/hg and was obtained by the ZnO NWs catalyst under irradiation at 280 nm. This unexpectedly high hydrogen production may be attributed to the morphology (nanowires) and possible defects in the crystalline structure. The maximum hydrogen production for the ZnO NPs and commercial ZnO catalysts was 94 µmol/hg and 86 µmol/hg, respectively, at 280 nm.

The maximum hydrogen production obtained with the commercial ZnO composites containing gold and graphene was 709 µmol/hg at 400 nm and was obtained with a gold loading of 10 wt.%. The enhancement in the hydrogen production was 7.5 times higher than that reported by the commercial ZnO.

The higher hydrogen production for the Au@ZnO NPs–graphene catalysts was 759 µmol/hg at 400 nm and was obtained with 10%Au@ZnO NPs–graphene. The enhancement in the hydrogen production was 8.8 times higher than that reported by the ZnO NPs catalyst.

In the case of the Au@ZnO NWs–graphene composites, the higher hydrogen production (1127 µmol/hg) was obtained with the 10%Au@ZnO NWs–graphene under irradiation at 400 nm.

The catalysts did not show a reduction in the surface area nor in the hydrogen production with the increment in gold loadings and incorporation of graphene. These results suggest that the best graphene and gold loading for the Au@ZnO–graphene catalysts could be higher than 1 and 10 wt.%, respectively.

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References
15. Fahimi, Z.; Moradlou, O. Fabrication of ZnO@C foam: A flexible free-standing electrode for energy storage devices. Mater. Des. 2020, 189, 108525. [CrossRef]
20. Elahi, N.; Kamali, M.; Baghersad, M.H. Recent biomedical applications of gold nanoparticles: A review. Talanta 2018, 184, 537–556. [CrossRef]
34. Nejati, K.; Rezvani, Z.; Pakizevan, R. Synthesis of ZnO Nanoparticles and Investigation of the Ionic Template Effect on Their Size and Shape. Int. Nano Lett. 2011, 1, 75–81. [CrossRef]

38. Wen, Y.; Ding, H.; Shan, Y. Preparation and visible light photocatalytic activity of Ag/TiO2/graphene nanocomposite. Nanoscale 2013, 3, 4411. [CrossRef]


41. Liu, H.; Xu, Q.; Yan, C.; Qiao, Y. Corrosion behavior of a positive graphite electrode in vanadium redox flow battery. Electrochim. Acta 2011, 56, 8783–8790. [CrossRef]


Article

Characterisation of the Interaction among Oil-In-Water Nanocapsules and Mucin

Mar Collado-González *, Gurmeet Kaur, Yadira González-Espinosa, Rebecca Brooks and Francisco M. Goycoolea

School of Food Science & Nutrition, University of Leeds, Leeds LS2 9JT, UK; G.Kaur@leeds.ac.uk (G.K.); Y.GonzalezEspinosa@leeds.ac.uk (Y.G.-E.); fs16rahb@leeds.ac.uk (R.B.); F.M.Goycoolea@leeds.ac.uk (F.M.G.)

* Correspondence: M.D.M.ColladoGonzalez@leeds.ac.uk

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Abstract: Mucins are glycoproteins present in all mucosal surfaces and in secretions such as saliva. Mucins are involved in the mucoadhesion of nanodevices carrying bioactive molecules to their target sites in vivo. Oil-in-water nanocapsules (NCs) have been synthesised for carrying N,N'-di-(m-methylphenyl)urea (DMTU), a quorum-sensing inhibitor, to the oral cavity. DMTU-loaded NCs constitute an alternative for the treatment of plaque (bacterial biofilm). In this work, the stability of the NCs after their interaction with mucin is analysed. Mucin type III from Sigma-Aldrich has been used as the mucin model. Mucin and NCs were characterised by the multi-detection asymmetrical flow field-flow fractionation technique (AF4). Dynamic light scattering (DLS) and ζ-potential analyses were carried out to characterise the interaction between mucin and NCs. According to the results, loading DMTU changes the conformation of the NC. It was also found that the synergistic interaction between mucin and NCs was favoured within a specific range of the mucin:NC ratio within the first 24 h. Studies on the release of DMTU in vitro and the microbial activity of such NCs are ongoing in our lab.

Keywords: mucin; oil-in-water nanoparticles; chitosan; stability; ζ-potential; quorum sensing

1. Introduction

Dental caries is caused by the destruction of enamel, dentin, and dental pulp [1] due to state of dysbiosis among various bacterial and yeast species present in the buccal cavity. Streptococcus mutans has been reported as one of the primary cariogenic pathogens leading to the biofilm formation (dental plaque) further progressing into carious lesions (dental caries). Chronic dental infections lead to periodontitis, resulting in systemic inflammation that may manifest into chronic infective endocarditis [2] and rheumatoid arthritis [3], among other diseases [4].

In the oral cavity, bacteria and yeast are found in multispecies colonies that are called biofilms [1]. Such biofilms confer protection to the microbial organisms from various adverse environmental conditions such as antibiotics, thus resulting in the development of multi-drug-resistant bacterial species. Quorum sensing (QS) is among one of the various systems that is involved in the formation of biofilms by S. mutans. QS can be defined as cell-to-cell communication in bacteria through various small chemical moieties (in Gram-negative bacteria) and small peptides (in Gram-positive bacteria), resulting in expression of virulence (toxin production, competence development, biofilm formation, etc.) [2]. Inhibition of virulence through disruption of QS has been recognised among the potential ways to combat drug resistance without killing the pathogen and avoiding population stress in bacteria. This gives essential time to the host immune system to fight against the infection and serve as a means of additional therapy in combination with antibiotics. Additionally, disruption of the dental plaque (biofilms) can be potentially achieved by using antibacterial molecules loaded in delivery
nanosystems [1], applying strategies to promote the inhibition of biofilm formation by exploiting QS [5] or by the application of low-molecular-weight chitosan (CS), for example [1].

Due to the microbial resistance to antibiotics, the development of alternative therapies, such as inhibition of QS, is of utmost interest [6]. We have recently uncovered the potent QS inhibition activity of a newly synthetic molecule N,N’-(di-m-methylphenyl)urea, also known as 1,3-dim-tolylurea (DMTU) [2]. DMTU interacts with glutamine 11, one of the amino acids in the active site of the peptidase (PEP) domain of ComA, which acts as an ABC transporter involved in the QS system of *S. mutans*. Thus, DMTU blocks the expression of virulence factors controlled by the QS system, such as the biofilm formation [2]. Besides, according to in vitro assays, DMTU down-regulates some genes involved in the expression of various virulence factors regulated through QS in *S. mutans* [7].

Oil-in-water nanocapsules (o/w NCs) prepared by solvent displacement enhance the bioavailability of lipophilic drugs [8]. Besides, o/w NCs have been evaluated for treating oral infections when carrying different bioactive molecules because they can adhere to tooth and mucosa, thus releasing their payload [6,9,10]. Vidal-Romero and colleagues synthesised o/w NCs that were applied as pills that melt in the mouth, for treating patients diagnosed with periodontitis. According to their results, their o/w NCs loaded with chlorhexidine significantly reduced the oral plaque in comparison to chlorhexidine mouthwash [11].

It has been reported that o/w NCs interact with the mucosa and biofilms and that such interaction is enhanced by polycationic o/w NCs [12,13] as they could interact electrostatically with bacterial membranes that are negatively charged. Thus, different polycationic polymers have been used for coating nanostructures such as Eudragit® [13] or chitosan [14]. Chitosan (CS) is a family of polycationic biopolymers obtained from the deacetylation of chitin. CS can interact with mucin [15,16], the main glycoproteins in saliva and mucosal surfaces [15,17]. The structural characteristics of CS (degree of acetylation, DA, and molecular weight, MW) directly determine the ability to interact with mucin and reduce its viscosity [16]. NCs coated with lecithin and chitosan have been thoroughly studied as drug delivery platforms of biologics and lipophilic molecules [18,19] Recombinant hepatitis B surface antigen (HB) has been associated ionically at the surface of these systems and found to modulate the systemic immune response upon intra-nasal vaccination [20]. Yet, another asset of these systems is their stability in biological media determined by the careful selection of the DA and degree of polymerisation (DP) of chitosan [21]. We have shown that o/w NCs are an effective platform to load and deliver lipophilic drugs such as capsaicin [22] and triclabendazole [23]. In addition, it has been reported that CS has antibacterial activity on bacterial biofilms [1]. Thus, DMTU-loaded o/w NCs coated with chitosan might offer a synergistic effect against biofilms. Müller and colleagues reported the success of mouthwash formulations that showed antimicrobial activity while not impairing the cell viability of different oral cell lines in vitro [24]. Understanding how chitosan-based nanoparticle systems interact with mucosa is essential to their optimisation and fully realises their potential in drug delivery in various contexts.

Based on all the above, this study evaluated a potential new treatment against biofilms that consists of CS-coated DMTU-loaded o/w NCs and mucin. The reason behind this study is supported by three facts: First, in the oral cavity, o/w NCs interact first with saliva that covers all mucosal surfaces and teeth; second, mucins are the most abundant glycoproteins in the saliva [25]; and third, the nanosize and great stability offered by o/w NCs will guarantee their success for delivery. Thus, studying the evolution of size and stability is of utmost importance in the development of nanometric delivery systems. In this work, we evaluate the size of o/w NCs after the interaction with mucin molecules and the stability of the nanostructures developed. For this purpose, mucin type III from Sigma Aldrich has been used as a model of salivary mucin due to its structural and conformational similarities [25,26]. Despite there being two different types of porcine stomach mucin available at Sigma-Aldrich, mucin type III has been used in this work as it is obtained from mucin type II after purification.
2. Materials and Methods

2.1. Materials

Lecithin (Epikuron 145V) was kindly provided by Cargill. Miglyol 812 was purchased from Sasol GmbH (Hamburg, Germany). Hydrochloric acid, absolute ethanol, 1,3-di-m-tolylurea (DMTU, R395609-1EA), and mucins were purchased from Sigma Aldrich-Merck (Darmstadt, Germany). Chitosan was a sample of biomedical grade from Heppe Medical Chitosan GmbH (Halle, Germany) with the following specifications: Sample code 80/20 (batch Nr 212-201015-04), degree of N-acetylation \(\sim 20\%\) (as determined \(^1\)H NMR), molecular weight of \(\sim 97.7\) kDa, and polydispersity (PD) (PD = \(M_w/M_n\)) \(\sim 1.4\) (determined by Size Exclusion Chromatography-Multiangle Light-Detector Refractive Index, SEC-MALS-DRI).

2.2. Methods

2.2.1. Preparation of Oil-In-Water Nanocapsules

The synthesis of o/w NCs was performed following the methodology from Qin and colleagues with some modifications [27]. In brief, for preparing the organic phase, 400 \(\mu\)L of a solution of lecithin 100 g/L in ethanol, 530 \(\mu\)L of ethanol containing DMTU or absolute ethanol in the case of the preparation of blank o/w NCs, and 125 \(\mu\)L of miglyol 812 were all added in a beaker. After that, 9.5 mL of ethanol were added and mixed. This organic phase was poured on the top of 20 mL of chitosan solution. Then, the volume of the suspension was reduced to 10 mL by a rotary evaporator at 40 \(^\circ\)C and slow-speed gyration to allow the solvents to evaporate without boiling the suspension. Once at room temperature, suspensions were kept at 4 \(^\circ\)C until further use.

2.2.2. Preparation of Mucin Solutions

The hydrophilic mucins were isolated as explained elsewhere [16]. Briefly, commercially available mucins were dissolved at 5 mg/mL in MilliQ water by stirring overnight. Mucin solution was centrifuged at 25.000 \(\times\) g and 10 \(^\circ\)C for 50 min. Supernatants were collected and 0.02\% \(w/v\) sodium azide was added. The main goal of this process is to purify the free mucin particles in the mucin suspension. Then, mucin solution was aliquoted and freeze-dried. By subtracting the weight of the samples before and after freeze-drying, the amount of mucin was accurately determined. Then, lyophilised mucin was stored until further use. In that moment, the mucin sample was diluted up to 5 mg/mL and stirred at least 3 h to achieve full dissolution of the glycoprotein. Once dissolved, the mucin solution was serial-diluted by a factor of 2 until a concentration equal to 0.15625 mg/mL was reached. The range of mucin concentrations used included the mucin concentration in saliva, that is, 36\% \(w/w\) [28].

2.2.3. Interactions Nanocapsules—Mucin

For the analyses of the interaction between o/w NCs and mucin, 0.1 mL of NCs was added to 0.9 mL of a mucin solution at 5, 2.5, 1.25, 0.625, 0.313, or 0.156 mg/mL. To simplify the notation, the final mass of the o/w NCs and the mucin on the aliquots was calculated, and the samples are noted as a mucin:o/w NC ratio in \(w/w\). Thus, the mucin:o/w NC ratio in \(w/w\) corresponding to the mucin solutions indicated above are: 2.68, 1.34, 0.67, 0.34, 0.17, and 0.08, respectively.

Three different aliquots were prepared: One of them was analysed after adding o/w NCs in mucin solution and corresponds to 0 h, and the other two samples were kept at 37 \(^\circ\)C to be analysed after 1 and 24 h. The hydrodynamic size of the particles in all the samples was analysed by dynamic light scattering (DLS), while the \(\zeta\)-potential was measured only in samples analysed at 0 and 24 h.

2.2.4. Dynamic Light Scattering (DLS) Analysis

Hydrodynamic size and \(\zeta\)-potential were measured by using a Zetasizer Ultra (ZSU5700, Malvern instruments, Worcestershire, UK). Hydrodynamic size was measured by analysing the
back-scattered light ($173^\circ$) from the sample. The attenuator and the position of the photoreceptor was fixed by software in an automatic mode. The working temperature was fixed to 25 °C. Once the sample was introduced in the chamber, the time for the temperature stabilisation was 120 s. Then, the size was measured in triplicate, followed by the analysis of the $\zeta$-potential, with no extra time for equilibrating the temperature of the sample. The $\zeta$-potential was measured in triplicate. All the curves included in this paper correspond to the average of the measurements. The analysis of the particle size was performed by Multiple Narrow mode for mucin suspensions and General Purpose mode for NCs. The size distribution by intensity was used for the analysis of the samples. The polydispersity index was obtained by cumulant analysis and it is included for comparative purposes only. As some of the suspensions showed a higher polydispersity index than 0.5, the size included in this work results from the distribution analysis from the distribution by intensity.

2.2.5. Asymmetrical Flow Field-Flow Fractionation (AF4)—Multidetector Analysis

Physico-chemical characterisation of mucins was performed by asymmetrical flow field-flow fractionation, AF4, using an AF200 Multiflow system (Postnova Analytics UK Ltd. Malvern, UK) coupled [29] with an autosampler (PN5300), isocratic pump (PN1130), solvent organiser (PN7140), solvent degasser (PN7520), UV disinfection (PN7205), RI detector (PN3150), MALS detector (PN3621), and UV detector (SPD-20A). For facilitating ease of reading, the equipment will be denoted as AF4. The measurement was performed using a regenerated cellulose membrane (Z-AF4-MEM-612-10KD, Postnova Analytics UK Ltd. Malvern (Worcestershire, UK)) with a 10 kDa cutoff. The spacer was 350 µm and the temperature was set to 30 °C by a thermostat (PN4020). Data collection and analysis were performed using NovaFFF software version 2.0.9.9 Postnova Analytics UK Ltd. (Worcestershire, UK).

For the analysis of mucin, 13 µL of mucin type III at 5 mg/mL concentration of NaCl 0.01 M were injected using NaCl 0.01 M as a carrier liquid filtered through a 0.1 µm membrane. The system was set-up at a constant detector flow rate of 0.5 mL/min, the flow rate of the focus was 2.5 mL/min, and the focusing time was 3 min. The elution was set as follows: A constant elution at 2.5 mL/min of cross-flow for 0.2 min. Then, the cross-flow was reduced to 0.2 mL/min in a power decay mode with an exponent of 0.25 in 20 min and then reduced to 0.12 mL/min in 5 min with an exponent of 0.8. Finally, the cross-flow was reduced up to 0.09 mL/min in 5 min with an exponent of 0.8. The signal of a blank consisting of 13 µL of NaCl 0.01 M injection was recorded using the same method and, for analysis, subtracted from the recorded signals obtained from mucins.

For the AF4 analysis of mucin, $dn/dc$ was 0.144 according to Maleki and colleagues [30]. MALS angles included in the analysis corresponded only to those between 44° and 140°, as poor signals were detected from lower and higher angles. Data recorded were fitted to a random coil model.

The radius of gyration ($R_g$) can be obtained from MALS analysis while the hydrodynamic radius ($R_h$) can be obtained from DLS measurements. The conformation of the mucin molecules, therefore, can be determined from the shape factor ($\rho$) that is calculated as indicated in Equation (1).

$$\rho = \frac{R_g}{R_h}$$

Soft spheres are defined by $\rho = 0.5$, hard spheres by $\rho = 0.77$, coated spheres by $\rho$ close to 1, oblate spheroids by $\rho$ higher than 1, and prolate spheroids by $\rho$ higher than 2 [31,32].

3. Results

3.1. Mucin Characterisation

Dynamic light scattering (DLS) analyses of mucin type III from Sigma-Aldrich revealed the presence of populations with different hydrodynamic diameters. According to the size distribution by intensity (Figure 1, top), the smallest population appears in the range between 10 and 30 nm. The second population appears in the range from 30 to 100 nm, approximately. In addition, the third
population of mucin particles appears in the range from 100 to 1000 nm. It is important to note that decreasing the concentration of mucin solutions, below 0.313 mg/mL, resulted in the movement of populations toward higher hydrodynamic diameter ranges according to profiles of size distribution by intensity. The 0.078 mg/mL mucin solution can be described by the presence of three peaks. The main population centred around 846 nm of hydrodynamic diameter, the second one centred around 119 nm, and the last one centred around 39 nm of hydrodynamic diameter. The reduction in the mucin concentration resulted in a poor signal recorded due to the low number of molecules in the solution that resulted in a decrease in the correlation coefficient (Figure 2). According to the physics principles of the DLS technique and as it can be seen in the size distribution by number (Figure 1, bottom), the population of particles higher than 100 nm is negligible in any of the mucin solutions.

**Figure 1.** Hydrodynamic diameter of mucin type III from Sigma-Aldrich at 1.25 (blue), 0.625 (red), 0.313 (green), 0.156 (pink), and 0.078 mg/mL (grey). Size distribution by intensity (top) and by number (bottom).

**Figure 2.** Correlation function of mucin type III from Sigma-Aldrich at 1.25 (blue), 0.625 (red), 0.313 (green), 0.156 (pink), and 0.078 mg/mL (grey).
Mucin was analysed by AF4. The elution of the mucin molecules is determined by the MALS and UV absorbance at 220 nm signals while the RI signal gives an indication of the concentration of analyte present in the sample (Figure 3). The MALS signal associated with the size of eluting material shows two prominent peaks. The first one where the intensity of the RI signal is predominant indicates a higher concentration of eluting material. However, the intensity of the RI signal over the second peak of MALS is significantly low, indicating that there is a fraction of a bigger-size material but of negligible concentration. This can correspond to the presence of aggregates present in solution. Analysis for the calculation of MW was, therefore, determined over the region where the material was assumed to be soluble (region 1, as delimited by integration limits in Figure 3).

The analysis of regions 1 and 2 of the AF4 recording is depicted in Table 1. The low mucin recovery after the measurement could be due to possible interactions of the mucin with the membrane, even though the AF4 membrane was negatively charged. The interaction hypothesis is reinforced by the noisy signal from the MALS at low angles (not shown). The ρ was calculated from the gyration radii (Rg) obtained from AF4 and the Rθ obtained for each population of mucin at 5.0 mg/mL obtained by DLS (Table S1). The ρ of mucin molecules in region 1 of AF4 analysis, which corresponds to the population centred around 40 nm in hydrodynamic radii in DLS recordings, was 1.26, which corresponds to oblate spheroids particles. Meanwhile, the ρ for those particles found in region 2 in the

![Figure 3](image-url)
AF4 analysis and centred around 270.2 nm of hydrodynamic radii in DLS measurements was 0.93, which corresponds to a coated sphere. Such a change in the conformation of the mucin in the solution reinforces the hypothesis of the mucin aggregation.

| Table 1. Physico-chemical parameters of the mucin particles analysed by AF4. |
|-----------------|-----------------|
|                | Region 1            | Region 2 |
| Mn (g/mol)     | (2.24 ± 0.17) × 10^5 |          |
| Mw (g/mol)     | (1.21 ± 0.02) × 10^6 |          |
| PD (Mw/Mn)     | 5.44               |          |
| Rg (nm)        | 50.9 ± 1.3         | 252.0 ± 25.8 |
| Mass eluted (%)| 71.15              | 1.95     |

3.2. Characterisation of Oil-In-Water Nanocapsules

We have formulated o/w NCs for the delivery of DMTU, which shows antibiofilm properties, and it is of potential use for the prevention of dental caries.

The inclusion of the DMTU in the formulation resulted in the increment in the hydrodynamic size, as shown in distributions by intensity of the o/w NCs obtained (Figure 4). For comparison, blank o/w NCs (B o/w NCs) showed a z-average hydrodynamic diameter equal to (176 ± 2) nm with a polydispersity index (PDI) of 0.13, while DMTU-loaded o/w NCs (P o/w NCs) showed a z-averaged hydrodynamic diameter of (287.3 ± 6.4) nm with a PDI of 0.25 (Figure 4). Regarding the ζ-potential, both formulations were positively charged, indicating the deposition of chitosan in their outer part. The ζ-potential values were +40 and +54 mV for B o/w NCs and P o/w NCs, respectively.

Figure 4. Size distribution by intensity of blank o/w nanocapsules (NCs) (grey) and 1,3-di-m-tolylurea (DMTU)-loaded o/w NCs (black).

3.3. Interaction among NCs and Mucin

Due to the negative charge of the mucin molecules in solution, (−16 ± 1) mV, an interaction is expected between these and the chitosan that is coating the o/w NCs, via the electrostatic interactions, in addition to the likely existence of other interactions between both. In addition, we have analysed the effect of these interactions on the stability of the o/w NCs. We define stability as the maintenance of the nanometric size of the o/w NCs.

3.3.1. Interaction among Blank Nanocapsules and Mucin

The interaction among mucin and B o/w NCs resulted in nanocomposites (NCOMs) of different hydrodynamic diameters depending on the mucin:B o/w NC ratios (in w/w). The interaction among mucin and B o/w NCs at a mucin:B o/w NC ratio equal to or below 0.17 w/w resulted in a NCOM larger than the detection limit of the DLS technique as it could be interpreted from the sharp peaks in Figure 5a and the correlation functions in Figure 5b. In those functions, it is noteworthy that the
decrease in the intercept value, which can be interpreted as the decrease in the number of particles in the suspension, resulted from the aggregation of the NCOMs. Moreover, the low ζ-potential in absolute values of such NCOMs indicates that the electrostatic repulsive forces that keeps the NCOMs away from each other must be weak (Figure 6 and Table S2). At a higher mucin:B o/w NC ratio, two populations of NCOMs were obtained, one of them centred on hundreds of nm, which is the most abundant, and the second population centred on thousands of nm (Figure 5a).

![Graphs of size analyses](image)

**Figure 5.** Size analyses of the nanostructures obtained after adding blank o/w NCs to mucin solutions at mucin:B o/w NC ratios of 0.08 (w/w) (pink), 0.17 (w/w) (green), 0.34 (w/w) (red), and 0.67 (w/w) (blue): (a) Comparison of hydrodynamic diameter and (b) correlogram functions of the suspensions at time 0 h; (c) comparison of hydrodynamic diameter and (d) correlogram functions of the suspensions after 1 h of incubation at 37 °C; (e) comparison of hydrodynamic diameter and (f) correlogram functions of the suspensions after 24 h of incubation at 37 °C.
Then, including higher amounts of mucin in the formulations resulted in an increase in both the size and the polydispersity of the NCOMs obtained. At a mucin:P o/w NC ratio of 0.08 (w/w), the hydrodynamic diameter of the NCOMs obtained was smaller (centred on 200 nm) than that of the P o/w NCs. The hydrodynamic diameter increased when increasing the amount of mucin in the formulation. Thus, those NCOMs obtained at a mucin:P o/w NC ratio of 0.34 (w/w) showed two populations, one of them centred around 80 nm and the second one centred around 360 nm (Figure 5c). After 24 h, those NCOMs prepared at a mucin:B o/w NC ratio of 0.08 (w/w) were completely out of the detection limit, as it could be inferred from the correlogram function (Figure 5f) and the absence of peaks in the intensity distribution (Figure 5e), whilst the rest of the NCOM suspensions showed their main peaks in the same region of hydrodynamic diameter and did not show strong changes in the size of the main population with respect to the measurement at 1 h. However, it is important to point out that all the curves showed a second peak or a shoulder in the region of lower hydrodynamic size.

3.3.2. Interaction among DMTU-loaded o/w NCs and mucin

Similar to the results obtained previously, the interaction among mucin and P o/w NC yield NCOM depended on the mucin:P o/w NC ratios (in w/w). At mucin:P o/w NC ratios up to 0.17 (w/w), the hydrodynamic diameter of the NCOMs obtained was smaller (centred on 200 nm) than that of the P o/w NCs. The hydrodynamic diameter increased when increasing the amount of mucin in the formulation. Thus, those NCOMs obtained at a mucin:P o/w NC ratio of 0.34 (w/w) showed two populations, one of them centred around 80 nm and the second one centred around 360 nm (Figure 7a). Then, including higher amounts of mucin in the formulations resulted in an increase in both the size and the polydispersity of the NCOMs obtained. At a mucin:P o/w NC ratio of 0.67 (w/w), two populations of particles were recorded, one of them centred in the hundreds of nm and the second one centred in the thousands of nm. At a mucin:P o/w NC ratio higher than 0.67 (w/w), just one broad population spans from hundreds to the out-of-detection limit of the technique (Figure 7a). According to the correlation function of these systems, the measurements carried out had good quality (Figure 7b).
Regarding their ζ-potential measurements, NCOMs prepared at a mucin:P o/w NC ratio of 0.08 (w/w) had a ζ-potential lower than that of P o/w NCs (Figure 8 and Table S3), indicating that those NCOMs had enough mucin to modify the net charge of the P o/w NCs but not enough to revert their charge. Increasing the amount of mucin in the system up to a mucin:P o/w NC ratio of 0.34 (w/w) resulted in unstable NCOMs according to their ζ-potentials. Repulsive electrostatic forces in those nanoparticles with a ζ-potential lower than 25 mV in absolute value are weak and cannot avoid the
nanoparticles with a ζ-potential lower than 25 mV in absolute value are weak and cannot avoid the aggregation of the nanoparticles [33,34]. Interestingly, while the NCOMs obtained in the mucin:P o/w NC system with a ratio of 0.17 (w/w) were positively charged, those prepared at a ratio of 0.34 (w/w) were negatively charged, indicating that the charge balance among CS and mucin had been overcome in the latter. Then, increasing the amount of mucin in the system, the ζ-potential values of the P o/w NCs coated with mucin reached a plateau stage at a mucin:P o/w NC ratio equal to or above 0.67 (w/w) (Figure 8, Table S3, and Figure S1). The NCOMs prepared at mucin:P o/w NC ratios of 1.34 (w/w) and 2.68 (w/w) were removed from further analyses due to the impossibility of checking the evolution of these systems. The size of all the rest of the NCOM systems was checked after being incubated at 37 °C. NCOMs prepared at a mucin:P o/w NC ratio of 0.08 (w/w) showed no change in the hydrodynamic size even after 24 h of being synthesized (Figure 7c,e). The constant hydrodynamic size of these NCOMs could be related to the fact that although their ζ-potential decreased once the mucin was added, it was within the stability region (Figure 8 and Table S3). The hydrodynamic diameter of the NCOMs prepared at a mucin:P o/w NC ratio of 0.17 (w/w) showed a strong size increment after 1 h of incubation, reaching micrometric size, as can be seen in the size distribution by intensity and by the correlation function of this suspension (Figure 7c,d). The aggregation process of these NCOMs continued and, after 24 h of incubation at 37 °C, these NCOMs became larger than the limit of detection of the technique, as can be seen from the correlogram (Figure 7f) and from the absence of any peak in the hydrodynamic diameter distribution by intensity (Figure 7e). Regarding the rest of the systems, NCOMs prepared at a mucin:P o/w NC ratio of 0.34 (w/w) showed no change after 1 h of incubation. However, enlarged nanoparticles were found after 24 h of incubation at 37 °C. Such an increment in the NCOM size could be related to their low ζ-potential (Figure 8 and Table S3). NCOMs prepared at a mucin:P o/w NC ratio of 0.67 (w/w) showed a reduction in their hydrodynamic diameter along the experiment time (Figure 7c,e), while their ζ-potential did not change since the synthesis (Figure 8 and Table S3).

![Figure 8](image-url). ζ-potential and size (Z-average) of nanocomposites obtained after the interaction among mucin and DMTU-loaded nanocapsules at different ratios at time of synthesis (black) and 24 h (grey) after the preparation event.
4. Discussion

In this work, we have synthesised CS-coated DMTU-loaded o/w NCs for their potential use against biofilm-forming bacteria in oral treatment. Such a nanometric system can interact with mucin, the most abundant glycoproteins in saliva and mucosal surfaces [13]. Mucin type III from Sigma-Aldrich has been used as, in addition to its similarity with other mucin types, it offers homogeneity between mucin samples and constitutes the easiest way to obtain purified mucin.

To characterise macromolecules, it is important to work with free macromolecules in solution. Thus, it is important to work at a concentration below the overlapping concentration ($c^*$) for the macromolecular molecules at defined conditions. Different $c^*$ has been reported for mucin, from $c^* = 0.1$ [23] to 5 mg/mL [27]. Our results agree with the latter as our DLS results showed no difference in the size of the molecules at concentrations below 5 mg/mL.

Mucin molecules showed a $R_h$ around 40 nm according to our DLS analysis and $R_g$ around 51 nm according to our AF4. These results fit well with the $R_h$ values found in the literature that spans from 44 nm determined by DLS to 75 nm determined by AF4 in similar conditions [30,35]. The molecular weight (Mw) of the porcine gastric mucin, determined in this work to be $(1.21 \pm 0.02) \times 10^6$ g/mol, is in agreement with previous reports on mucin [36,37] but disagrees with the molecular weight reported by Maleki and colleagues [30]. Most of the particles in the solution are free oblate-shaped mucin molecules according to their $\rho = 1.27$, which is consistent with elongated structures. This result is in agreement with the findings in the literature, where free mucin molecules have been described as rod-like structures [38]. In our results, a negligible population of aggregated mucin molecules can be detected according to AF4 measurements and the decrease in the $\rho$.

In this work, commercially available mucin solutions have been prepared at concentrations between 0.15 and 5 mg/mL, as at this concentration, mucin is present as fully dissolved polymer (free mucin molecules). Moreover, similar concentration ranges have been previously reported in the literature on mucin interactions in binary systems [26,39].

Oil-in-water NCs, prepared by the solvent displacement method, showed a nanometric size as expected because of the use of Miglyol 812 as a lipid phase and lecithin as a surfactant [8], and the methodology used for their synthesis [12,27,40]. The electrostatic interaction among the polycationic CS and negative phosphatidic groups from the lecithin [8,41] results in the adsorption of CS on the surface of o/w NCs. According to previous studies in which equivalent nanocapsules were prepared, it was shown that more than 96.8% of CS was incorporated in the nanocapsules [21]. Therefore, the amount of free CS in the system and its interaction with mucin in the current work can be considered negligible. CS also adsorbs on other types of surfactant used for the preparation of o/w NCs, such as Tween 80. It has been reported that such o/w NCs had a $\zeta$ potential lower ($+17 \pm 2$) mV [40] than that of the o/w NCs prepared in the current research work in which the addition of DMTU resulted in a change in the $\zeta$ potential from ($+40 \pm 1$) to ($+54 \pm 1$) mV. In addition to the net charge change, the variation in the size of the o/w NCs after adding DMTU indicates a change in the conformation of o/w NCs.

The interaction among CS and mucin [42–44], as well as the interaction among CS-coated liposomes and mucin [45], has been reported, in addition to the favourable interaction of small-sized particles with mucosa [13]. Thus, the study of the interaction among the o/w NCs formulated and mucins continues to be of great interest to find out if such nanosystems are stable enough to resist their interaction with mucins. Therefore, they could be used as drug delivery systems in oral environments.

pH is an important factor in the interaction between polyelectrolytes such as chitosan and mucin. The final pH of the chitosan solution was 3.6 and the pH of the nanocapsules suspension was 2.5. On the other hand, the pH of the mucin solution was 7.0. Considering that the interaction between chitosan and mucin is favoured in the pH range from 2.4 to 6.5, and the pH of the interacting suspensions and the fact that only one population are found after their interaction, the most likely explanation is that the interaction between nanocapsules and mucin occurs via chitosan–mucin electrostatic attraction.
The resulting structures obtained after the interaction among mucin and o/w NCs (NCOM) depended on the mucin: o/w NC ratio (in w/w), revealing that the interaction among the nanostructures occurred through electrostatic interactions. According to the results, the higher the content of mucin, the lower the ζ-potential of the NCOMs in both systems, B o/w NCs, and P o/w NCs. There are differences in the behaviour of both systems that differ in the absence or presence of DMTU. The balance among the charges among mucin and the B o/w NCs occurs at a mucin:B o/w NC ratio of 0.08 (w/w), approximately. Thus, the NCOMs prepared at such a ratio aggregated almost in the time of the interaction among the species. In addition, after 24 h, the particles in the suspension showed a negative charge. This could be explained as if mucin molecules were acting as glue among different o/w NCs. On the contrary, the interaction at mucin:B o/w NC ratios equal to or higher than 0.17 (w/w) resulted in small NCOMs at the time of synthesis as a result of the increase in the elasticity of the polyelectrolytes after their interaction [46] and the appearance of a compact layer of a polyelectrolyte adsorbed onto a layer of polyelectrolyte of opposite charge, for example, a layer of CS on a layer of mucin and vice versa [47]. The evolution of the different systems depended on the ζ potential of the system. At a mucin:B o/w NC ratio of 0.17 (w/w), a micrometric size after 24 h of incubation was shown due to the weakness of the electrostatic repulsive forces in the system. However, those systems prepared at mucin:B o/w NC ratios higher than 0.17 (w/w) were electrostatically stable, as is proven by their higher ζ potential. Therefore, those NCOMs were of nanometric size. Interestingly, the higher the amount of mucin, the smaller the size of the final NCOMs obtained.

Regarding the P o/w NCs, the balance of charges occurs in mucin:P o/w NC ratios among 0.17 (w/w) and 0.34 (w/w). Unlike the previous case, those NCOMs prepared at a mucin:P o/w NC ratio of 0.08 (w/w) showed no significant change in its size after 24 h. In such a system, the presence of mucin reduces both the ζ potentials and the size of the NCOMs after the interaction between the reactant species. At a mucin:P o/w NC ratio of 0.17 (w/w), aggregation occurred in a slow kinetic process. After 1 h, the system showed micrometric particles that underwent further aggregation up to be not detectable by the technique after 24 h of incubation. Again, the electrostatic interactions seemed to be responsible for the results obtained, as the ζ potential at the time of synthesis was +13 mV. Surprisingly, NCOMs showed that a low negative ζ potential, −12 mV, did not walk the same path despite the weakness of the electrostatic repulsive forces. Thus, those NCOMs prepared at a mucin:P o/w NC ratio of 0.34 (w/w) showed a nanometric population of particles after 24 h of incubation. In that time, the NCOMs had a ζ potential of +8 mV. The simplest explanation for that result is the presence of another type of stabilising force, such as steric forces, appearing among mucin molecules that are adsorbed on the surface of the NCOMs. Likely, such steric forces avoid the aggregation of the NCOMs in the system prepared at a mucin:P o/w NC ratio of 0.17 (w/w), which had −7 mV at the moment of the synthesis and −19 mV 24 h later, and the particles were still detectable by the technique. The increase in the mucin content up to a mucin:P o/w NC ratio of 0.67 (w/w) resulted similarly to the system prepared with B o/w NCs at the same ratio (in w/w). That is, as time passes, the size of the NCOMs decreases as a result of the increase in the flexibility of the polyelectrolytes after their interaction [46]. At higher mucin:P o/w NC (w/w) ratios, the final system resulted in higher structures at the time of synthesis. For example, the system prepared at a mucin:P o/w NC ratio of 1.34 (w/w) showed a broad population that spans from hundreds of nm up to micrometric-size particles, which fall out of the detection limit of this technique.

Few similar research works are available in the literature. Ünal et al. (2015) prepared o/w NCs in a similar way to those proposed in this work but using Tween 80 as a surfactant instead and Protasan UP CL 113 chitosan (DA = 10–25%, Mw = 50–150 kDa). The CS used in that work had characteristics similar to those of the polymer used in this study (DA = 20%, Mw = 97 kDa), so it is expected that the behaviour exhibited with mucin is similar. In the work from Ünal et al. (2015), the interactions among mucin and the o/w NC were evaluated at a mucin:o/w NC ratio of 0.03 (w/w) (value determined based on their used methodology) and analysed by turbidity. According to their results, there was a significant interaction between chitosan-coated nanoparticles and mucin; however, no comparisons can be established, as no DLS measurements were carried out [40]. Sunoqrot and colleagues reported...
that methoxy polyethylene glycol-b-poly(ε-caprolactone) nanoparticles (NPs) showed interaction with mucin molecules at any mucin:NP ratio (w/w), from 0.25 (w/w) to 4.0 (w/w) via Schiff base and Michael addition reactions instead of electrostatic interactions, as the ζ potential of the mucin and NPs in the working conditions was close to neutrality. In such conditions, only bigger aggregates were formed at a mucin:NP ratio higher than 1.0 (w/w) [39]. Boya and colleagues also reported that the hydrodynamic size of metal NP coated with pluronic 127 increased 3 to 4 times after the interaction with mucin. However, no comparisons can be established with the results in the current work, as it was not possible to determine the mucin:metal NP ratio (w:w) used in the cited work [48].

The resulting interaction among mucin and o/w NCs found in this research work resembles the behaviour of other binary systems in which the ratio among the interacting species determines the size of the final nanostructures. Thus, at low and high interacting species ratios, bigger structures are formed, whilst there is an interacting species ratio range that results in the formation of nanometric structures [49]. Similarly, in this work, the o/w NCs can keep a nanometric size or aggregate to form higher structures depending on the mucin:o/w NC ratio. The fact that the resulting NCOM, which has mucin on their surface, can interact further with mucin molecules, can be related to the mucoadhesive properties of the formulation developed. Further studies that are currently ongoing in our research group will allow us to further elucidate the effectiveness and suitability of the developed o/w NCs as potential QS inhibition nanosystems.

5. Conclusions

1,3-dim-tolylurea (DMTU)-loaded CS-coated o/w NCs are proposed as a new oral antibacterial treatment because DMTU acts as a potent QS inhibitor that can be used to fight against bacteria biofilm formation, and CS has mucoadhesion and antibacterial properties. This study evaluated the stability of the proposed o/w NCs after the interaction with mucin molecules in suspension as unravelling the interactions between o/w NCs and mucins is essential for the rational design of drug delivery systems. The results show that o/w NCs are sensitive to the presence of mucin in the suspension. There is a range of mucin content in the media that results in the aggregation of the o/w NCs in a dynamic process. On the contrary, lower or higher mucin content in the media did not result in the aggregation of the o/w NCs, due to the appearance of repulsive electrostatic forces in their surfaces. The incorporation of DMTU to the o/w NCs resulted in a change in their conformation that resulted in the increase in their stability in the presence of mucin. Future work is required to evaluate the stability of the potential treatment developed in different conditions, such as simulated saliva, to determine the stability of the o/w NCs in a more realistic situation. It will be also important to study drug release from o/w NCs after interacting with mucin to evaluate the efficacy of the proposed drug delivery system.

Supplementary Materials: The following are available online at http://www.mdpi.com/2313-7673/5/3/36/s1, Table S1: DLS data analyses for mucin type III from Sigma-Aldrich; Table S2: ζ-potential and Z-average of NCOMs prepared at different mucin:B o/w NC ratios (in w/w) at time of synthesis (0 h) and 24 h after the synthesis event; Table S3: ζ-potential and Z-average of NCOMs prepared at different mucin:P o/w NC ratios (in w/w) at time of synthesis (0 h) and 24 h after the synthesis event; Figure S1. ζ-potential of NCOMs prepared at different mucin:o/w NC ratios (in w/w). B o/w NCs are plotted in grey and P o/w NCs are plotted in black. The ζ-potential values of the B o/w NCs and P o/w NCs are included at ratio 0.0.

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References


17. Pedersen, A.M.L.; Belstrom, D. The role of natural salivary défences in maintaining a healthy oral microbiota. J. Dent. 2019, 80, S3–S12. [CrossRef]


27. Qin, X.; Engwer, C.; Desai, S.; Sanjurjo, C.V.; Goycoolea, F. An investigation of the interactions between an E. coli bacterial quorum sensing biosensor and chitosan-based nanocapsules. Colloids Surfaces B: Biointerfaces 2017, 149, 358–368. [CrossRef]


36. Malmsten, M.; Blomberg, E.; Claesson, P.; Carlstedt, I.; Ljusegren, I. Mucin layers on hydrophobic surfaces studied with ellipsometry and surface force measurements. J. Colloid Interface Sci. 1992, 151, 579–590. [CrossRef]

37. Shi, L.; Caldwell, K.D. Mucin adsorption to hydrophobic surfaces. J. Colloid Interface Sci. 2000, 224, 372–381. [CrossRef]


44. Silva, C.A.; Nobre, T.M.; Pavina, F.J.; Oliveira, O.N. Interaction of chitosan and mucin in a biomembrane model environment. *J. Colloid Interface Sci.* 2012, 376, 289–295. [CrossRef]


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Enhancing Kidney Vasculature in Tissue Engineering—Current Trends and Approaches: A Review

Charlotta G. Lebedenko and Ipsita A. Banerjee *

Department of Chemistry, Fordham University, 441 East Fordham Road, Bronx, NY 10458, USA; clebedenko@fordham.edu
* Correspondence: banerjee@fordham.edu

Abstract: Chronic kidney diseases are a leading cause of fatalities around the world. As the most sought-after organ for transplantation, the kidney is of immense importance in the field of tissue engineering. The primary obstacle to the development of clinically relevant tissue engineered kidneys is precise vascularization due to the organ’s large size and complexity. Current attempts at whole-kidney tissue engineering include the repopulation of decellularized kidney extracellular matrices or vascular corrosion casts, but these approaches do not eliminate the need for a donor organ. Stem cell-based approaches, such as kidney organoids vascularized in microphysiological systems, aim to construct a kidney without the need for organ donation. These organ-on-a-chip models show complex, functioning kidney structures, albeit at a small scale. Novel methodologies for developing engineered scaffolds will allow for improved differentiation of kidney stem cells and organoids into larger kidney grafts with clinical applications. While currently, kidney tissue engineering remains mostly limited to individual renal structures or small organoids, further developments in vascularization techniques, with technologies such as organoids in microfluidic systems, could potentially open doors for a large-scale growth of whole engineered kidneys for transplantation.

Keywords: renal tissue engineering; vascularization; organoids; decellularized matrices

1. Introduction

As the gap between organ need and donor availability continues to widen, the need for alternative methods of tissue replacement or regeneration rises [1]. In addition to donor shortage, surgical transplant methods present significant limitations due to immunological rejection, post-transplant infection, and possible harm to the donor [2]. To circumvent these drawbacks, scientists have been developing new regenerative medicine methodologies such as tissue engineering (TE). The goal of TE is to create tissue replacements derived from the patient’s autologous cells and engineered biomimetic scaffolds that can be grown in vitro and implanted to promote in vivo regeneration of the original tissue [3]. TE requires the proper choice of the scaffold biomaterial, appropriate cell types, an assortment of growth factors, and biomimetic components that will encourage development and organogenesis. The ideal TE scaffold should successfully mimic the target organ’s natural extracellular matrix (ECM) by promoting cell attachment, migration, proliferation, differentiation, tissue vascularization, and the flow of nutrients and waste [4]. Furthermore, a biodegradable scaffold that breaks down into harmless byproducts as the engineered tissue replaces it with its own secreted ECM is needed to avoid future scaffold removal through additional surgeries, formation of bacterial colonization, and other immunological reactions [5,6]. Other vital features of the scaffold include ease of synthesis, tissue-specific mechanical properties, and a diminished immune response [7].

The field of TE has seen major advances in engineering organs such as skin, cartilage, cornea, bladder, and bone, but human applications remain limited due to high costs and the failure of grafts to thrive in vivo [3]. While the engineering of relatively simple, avascular tissue, such as skin and cartilage, has seen great success in TE, the engineering
of larger organs with intricate structures and vascular networks, such as the kidney, has seen limitations [8]. A major difference is that avascular tissues do not require extensive angiogenesis or vasculogenesis [9]. A majority of blood vessels within the peripheral, cerebral, cardiac, and renal vasculature are smaller in diameter (lower than 6 mm), and blood vessels in those organs have been shown to be more difficult to replace with synthetic polymer grafts due to a relatively inadequate match in mechanical properties, thrombus formation, and hyperplasia in the innermost membranes [10,11]. Without vascularization, the growth of any larger organ is impossible due to a lack of oxygen and nutrient delivery to the inside of the tissue causing graft necrosis [12]. The upper limit for avascular tissue is around 400 µm, so any tissue with a dimension larger than this will require almost immediate vascularization to prevent hypoxic conditions from killing inner cells [13]. Lack of proper vascularization both before and after implantation is one of the greatest obstacles to engineering large tissues, so the development of vascularization techniques for TE is of the utmost importance.

Chronic kidney disease causes over half a million patients to reach end-stage renal disease each year, and the only viable treatment options are dialysis and transplant, where the short half-life of the kidney often makes a second transplant necessary [14]. Consequently, the kidney is the single most sought after organ for transplantation [15]. The kidney has presented challenges because of its highly complex 3D structure, with significant vascularization, and a diverse array of cell types and physiological functions [16]. Since nephrons cannot regenerate on their own once damaged [17], the need to engineer a proper scaffold with sufficient stability, porosity, and biocompatibility that promotes kidney cell differentiation, nephrogenesis, and organ vascularization remains high [18]. This review delves into some of the biological cues with regard to renal structure and current methodologies including a comparison of top-down versus bottom-up approaches being explored for improving vasculature in kidney TE.

2. Renal Vascular Structure and Cellular Composition

The major structural and functional unit of the kidney is the nephron, whose components include the glomeruli, renal tubules, and Bowman’s capsule (Figure 1), all significantly different in their ECM and cellular compositions [19,20]. Mimicking the natural ECM when designing scaffolds is thus particularly difficult for kidney TE due to the diverse ECM compositions. Furthermore, the kidney consists of over twenty types of epithelial cells, vascular cells, mesangial cells, and interstitial cells, all of which are essential for proper kidney functioning [21]. One of the most difficult components to replicate through TE approaches is the kidney vasculature. Human kidneys receive between 20 and 25% of cardiac output, so the kidney vasculature must be able to withstand significant blood flow and support proper glomerular filtration [22]. Cells of the renal vasculature include endothelial cells, vascular smooth muscle cells, pericytes, mesangial cells, and fibroblasts [23]. Kidney capillaries are comprised of endothelial cells covered by pericytes which are key to angiogenesis and vascular survival [24]. Overall, the kidney vasculature is extremely complex, making it one of the greatest challenges to overcome in TE approaches.
Figure 1. (a) Structure of kidney, comprised of renal cortex, medulla and pelvis. (b) Structure of an individual nephron, which comprises the renal corpuscle (glomerulus and Bowman’s capsule) and the renal tubule. (Reproduced with permission from Elsevier, adapted from reference [25]).

The development of kidney vasculature entails vasculogenesis and angiogenesis. Vasculogenesis, or the de novo formation of blood vessels from endothelial progenitor cells, is active in early development of the kidney, while angiogenesis, or the outgrowth and remodeling of new blood vessels from existing ones, is active at a later developmental stage. Several studies have provided detailed reports of the development of kidney vasculature. Both angiogenic hemangioblast precursor cells from the embryo as well as vasculogenic endothelial precursors within the embryonic kidney itself are required for the development of kidney vasculature, suggesting that an artificially grown, in vitro, kidney will require factors from the surrounding environment to induce vascularization [26].

Some of the important considerations for vascularization in engineered tissues are the type of endothelial cells (ECs) used and their ratio to tissue-specific stem cells or other cell types [27,28]. For example, the co-culture of induced pluripotent stem (iPS) cell-derived ECs with mural cells such as pericytes formed human blood vessel organoids [29]. Similarly, mesenchymal stem cells enhanced the vasculogenic and pro-angiogenic activities of endothelial colony forming cells [30]. Additionally, M0 and M2 macrophages have been used in combination with ECs to enhance vascularization in a 3D hydrogel scaffold [31]. Endothelial progenitor cells (EPCs) have been used as an alternative as they are relatively easier to gather from the patient’s blood or bone marrow than organ-specific ECs [32]. In addition to the types of cells, the proangiogenic growth factors and the gradients in which they are present are essential for proper vasculature development [33]. The depth of tissue invasion by vasculature and the density of blood vessels formed are closely related to growth factor gradient magnitude [34].

3. Whole-Kidney Tissue Engineering—The Top-Down Approach

Approaches to kidney TE which utilize the entire native structure of the kidney and therefore require a donor organ include the use of decellularized ECM or vascular corrosion casts. These can be thought of as top-down approaches since the original donor organ is used to make a scaffold that preserves the original structural and mechanical properties of the organ and can then be repopulated with autologous cells for regenerative medicine applications.

3.1. Decellularized ECM

The repopulation of decellularized kidney scaffolds with autologous cells holds promise as a method for whole-kidney TE. Many in-depth articles have been published
on the use of decellularized ECM (dECM) [35]. Several decellularization methods that leave whole intact renal scaffolds with minimal damage to ECM mechanical integrity and biological activity have been described [36,37]. However, there is still no consensus on a standardized decellularization procedure. The main challenges for dECM include preserving the greatest amount of tubular structure and vasculature in dECMs, determining the appropriate cell types and culture systems for recellularization, and defining the appropriate maturation point that the graft can be transplanted into the patient. It has been shown that dECM maintains glomerular morphometry, vascular resilience, and critical growth factors [38]. While scientists have yet to successfully repopulate the entire scaffold with proper cell differentiation, major strides have been made towards the eventual clinical application of kidney dECMs. For example, rat kidney dECM seeded with epithelial and endothelial cells perfused in a whole-organ bioreactor produced rudimentary urine in vitro [39]. These grafts transplanted in rats were perfused by the host vasculature and produced urine in vivo. This section will focus on the decellularization and recellularization techniques for recreating kidney vasculature.

In order to optimize the scaffold’s vascular integrity, the method used for decellularization of kidney tissue is vital. Although the dECM may contain an intact vascular network, the decellularization process induces significant structural changes in this vascular network [40]. In a comparative study between decellularization protocols of SDS with DNase or SDS with Triton X-100, the SDS with Triton X-100 treatment of porcine kidneys was shown to better preserve microvascular structures such as the glomeruli [41]. This treatment also allowed for improved vascular function when re-endothelialized and perfused with blood in vitro. Figure 2 shows a comparison of the native kidney, SDS with DNase treated dECM, and Triton X-100 and SDS treated dECM decellularization methods, the vascular corrosion casts of each, and the vascular microarchitecture of each scaffold. The dECM treated with Triton X-100 and SDS showed similar vascular structures to those in the native kidney while the dECM treated with DNase and SDS showed defect areas that are marked by arrows in the figure.

![Figure 2](image-url)
It has been observed that in addition to chemical/biochemical agents used, other factors such as flow rate and temperature must also be optimized for decellularization methods. Furthermore, more research is needed to determine whether dECM from other animals, such as porcine dECM, can be used in human clinical applications. Although the biocompatibility and hemocompatibility of porcine dECM have been demonstrated with human cells, further investigation is needed to determine the most optimum source of dECM.

The most challenging aspect of using dECM in TE is the recellularization process. Particularly, the re-endothelialization of the kidney for a complete, functioning vascular network presents many challenges. An advantage of dECM approaches, however, is that in addition to matrix-to-cell signaling, cell-to-matrix signaling can also occur in which the original dECM is gradually remodeled or replaced by cell-derived, autologous ECM, making the application of xenografts in TE more feasible. It has been shown that pluripotent mouse embryonic stem cells seeded into rat kidney dECM endothelialized and remodeled the rat basement membranes, suggesting successful matrix-to-cell, cell-to-cell, and cell-to-matrix communication [42]. The cells lined the blood vessels of the glomeruli and produced endothelial lineage markers while the rat dECM basement membrane eventually incorporated mouse laminin and type IV collagen.

The recellularization methodologies used for kidney dECM are critical for achieving successful cell distribution and differentiation. In a recent study, it was shown that the infusion of human induced pluripotent stem cell (hiPSC)-derived endothelial cells from the renal artery and vein of rat kidney dECM resulted in the partial repopulation of all vasculature components [43]. As shown in Figure 3, endothelial cell localization in different parts of the kidney was observed. Furthermore, the glomerular capillaries contained fenestrated endothelia while the small arterioles contained strings of endothelial cells in a single layer covering the inner vessel wall. Overall, the glomeruli were successfully repopulated with hiPSC-derived endothelial cells forming a continuous network. This study achieved an 89% repopulation of all glomeruli, indicating that further improved methods for achieving more uniform and complete cell distribution are needed. Many efforts to remodel the dECM before recellularization in order to improve scaffold repopulation have been made. The preloading of both rat and human kidney dECM with VEGF and angiopoietin-1 allowed for successful re-endothelialization of the entire kidney vasculature with hiPSC-derived endothelial cells [44]. Normally, pericytes produce VEGF and angiopoietin-1 in the developing kidney to promote endothelial cell differentiation and vascularization, so the preloading of these growth factors in the dECM could serve as a replacement for pericytes which typically make the re-endothelialization of small capillaries difficult due to their large size. The growth factors were shown to promote hiPSC adherence and survival within the dECM scaffold as well as the eventual expression of endothelial differentiation markers. However, some obstructed afferent arterioles were still observed, highlighting the challenges of repopulating the intricate kidney microvasculature. This preloading of growth factors along with an arteriovenous delivery system of hiPSCs did result in improved vascular functioning of the graft. Thus, the simultaneous arteriovenous delivery systems have proven to be effective in kidney dECM recellularization. Other attempts at remodeling the kidney dECM before recellularization include glutaraldehyde cross linking [45] and heparin immobilization [46]. Glutaraldehyde cross-linking of dECM increases the overall mechanical strength and structural integrity of the dECM. When implanted in mice, these cross-linked scaffolds showed good histocompatibility, and when seeded with fibroblasts, they showed good cytocompatibility. The immobilization of heparin to collagen on kidney dECM using collagen-binding peptide provides significantly improved conditions for neovascularization by preventing thrombosis and promoting re-endothelialization. Normally, exposed collagen in dECM promotes coagulation of perfused blood, so the immobilization of heparin to exposed collagen was one attempt at overcoming these thrombogenic tendencies of kidney dECMs. Heparin immobilization ultimately showed an increased retention rate and attachment strength of endothelial cells as well as decreased thrombogenicity.
upon whole-blood reperfusion. The practice of remodeling the kidney dECM scaffold holds great promise for eventual whole organ TE applications.

Figure 3. Decellularized kidney scaffold obtained from adult male rat reseeded with iPSC-derived endothelial cells. (a) Cross-section of repopulated kidney showing homogeneous distribution of iPSC-derived ECs into glomeruli and vascular structures. Scale bar 1 cm. (a’–a’’) Images showing iPSC-derived ECs localization into glomerulus (a’), vascular network (a’’), and peritubular capillaries (a’’’). Scale bar 20 µm. (Reproduced with permission from Springer Nature under Creative Commons License, adapted from reference [43].)

Another use for kidney dECM scaffolds is the deconstruction of dECM architecture and the construction of dECM-derived hydrogels. The most common engineered scaffolds made from natural biomaterials incorporate kidney dECM hydrogels [47]. Hydrogels derived from kidney dECM have the advantage of preserving the native ECM protein ratios of the kidney while providing highly tunable mechanical stiffness through gelation [48]. Although dECM scaffolds with preserved kidney structures provide structural guidance for cell seeding, especially with vascularization, these scaffolds tend to lack structural flexibility and can often restrict cell migration and complete cellular repopulation. By further processing kidney dECM into hydrogels through homogenization, researchers have been able to preserve the proper biochemical cues of the scaffolds while creating more suitable mechanical robustness. dECM hydrogels were shown to support growth of conditionally immortalized human glomerular endothelial cells better than hydrogels of purified collagen I [49]. Human kidney peritubular microvascular endothelial cells cultured in decellularized kidney cortex ECM-derived hydrogels on a planar surface maintained a quiescent state as opposed to when cultured on a collagen I hydrogel [50]. Compared to collagen I hydrogels, these cells cultured on kidney dECM hydrogels exhibited reduced CD31 expression with less even distribution. Various dECM modifications, such as dECM scaffolds with added hypoxia-inducible factor-1α, have also been used in successful kidney cell culture [51]. While dECM hydrogels are widely used, a significant limitation of this approach is that ECM composition greatly varies between different kidney structures, so different hydrogels might need to be constructed for different sections of the kidney. Ultimately, a hydrogel scaffold, of synthetic or natural materials, with similar composition and potential for kidney cell support to dECM hydrogels should be developed to eliminate the need for donor kidneys.

Although decellularization of kidneys followed by recellularization or hydrogel formation has been investigated for many years with procedural improvements and novel
techniques emerging constantly, this approach has inherent limitations that may prevent widespread application. The ultimate goal of TE is to overcome the need for donor organs, and dECM approaches will always require donor organs from human or animal sources as well as large quantities of cells. However, due to the complex architecture of the kidney and particularly the highly specialized kidney vasculature with no other effective methods for developing those architectures de novo, dECM approaches might be the most effective means of engineering a whole kidney.

3.2. Vascular Corrosion Casts

Typically, vascular corrosion casts are used to visualize the vasculature of a scaffold, such as when examining a dECM scaffold to ensure the vasculature has been preserved following decellularization. However, Huling and co-workers recently showed that vascular corrosion casts can be utilized as a means for developing a biomimetic vascular scaffold for potential regenerative medicine strategies or even whole-kidney TE applications [52,53]. Similar to the dECM approaches where the entire kidney ECM structure is preserved and recellularized, this technique uses a vascular corrosion cast to provide a whole-organ structure on which cells can be seeded. The general procedure for developing these pre-vascularized scaffolds is shown in Figure 4. The cast is made by perfusing a polyɛ-caprolactone (PCL) solution into rat kidneys through the renal artery which is then followed by complete tissue digestion. The remaining PCL cast mimics the natural vascular structure of the kidney. Results showed that the vessels in the kidney cortex and glomerular capillaries remained intact in the PCL vascular casts. By coating the PCL cast with rat type I collagen and removing the PCL by dissolving in acetone, a hollow, biomimetic collagen-based scaffold that mimics the native kidney vasculature remained. The collagen scaffold is soaked in a crosslinking solution of EDC and NHS. Mile Sven-1 (MS1) endothelial cells were then coated on the collagen scaffold as a pre-vascularization technique. These endothelial cell-coated scaffolds were embedded in a type I collagen 3D hydrogel, and a heterogeneous population of human renal cells and renal growth medium were added to the hydrogel. Scaffolds were incubated in the cell-loaded hydrogel before implantation into rats with renal cortical defects. The cell-loaded scaffolds were split into smaller parts and placed within these renal defects where they were secured in place with fibrin glue.

The implanted partial renal tissue constructs showed strong evidence of anastomosis to host renal vasculature after two weeks. The vascular channels of the scaffolds were filled with nucleated cells and red blood cells from the host, suggesting successful blood flow between the rat kidney and the implanted scaffold that allowed for enhanced neo-vascularization and renal cell survival. Unexpectedly, the MS1 cells that had been used to pre-vascularize the collagen scaffolds did not remain attached to the scaffolds but rather scattered throughout the constructs to form microvascular channels with host-derived endothelial cells. Although the MS1 cells did not remain attached to the scaffolds, it was still demonstrated that the presence of the scaffold was necessary for MS1 cell survival, possibly due to the increased blood and oxygen supply through the prefabricated vessels. Future studies could attempt seeding the endothelial cells on the inside of the vascular corrosion casts to prevent migration away from the scaffold, but the small size of the kidney capillaries might introduce obstacles to a direct cell perfusion method. Ultimately, this study served as a proof of concept that partial renal implantation, and perhaps even whole-kidney TE, could someday be achieved with cell-seeded vascular corrosion casts, but many procedural adjustments must still be made. These in vivo scaffolds showed low efficiency of renal tubule structure formation as compared to in vitro cultures, suggesting the need for new techniques for improving integration into the host tissue.
4. Kidney Organoids—The Bottom-Up Approach

Development of stem cell-derived miniature organoids can be thought of as a bottom-up approach to TE. The process starts with individual stem cells that can be programmed to differentiate towards a renal lineage and eventually develop into kidney structures through self-assembly, similar to in vivo counterparts in human fetal development. Kidney organoids show great promise for future TE and regenerative medicine applications, and organoids cultured in microfluidic systems show potential for improved in vitro vascularization that will allow for an increase in the size of organoids for eventual clinical applications.

The development of in vitro kidney organoids is possible because of the extensive research that has gone into understanding the molecular and cellular basis of embryonic kidney development. Furthermore, the ability to reprogram terminally differentiated adult cells back to induced pluripotent stem cells (iPSCs) has completely transformed the field of regenerative medicine and enabled scientists to obtain large amounts of autologous hPSCs without the controversial use of human embryonic stem cells (ESCs) [54]. The ability to use iPSCs for patient-derived, tissue-specific organoids has sparked tremendous growth in the field of TE. By emulating the molecular signaling processes of embryonic kidney development, scientists can now induce hiPSCs to form complex kidney structures in vitro which eventually can form kidney organoids with functioning nephrons [55]. The comparison between kidney organoids and human fetal kidneys has shown that the organoids do indeed closely mimic the natural embryonic development of human kidneys, although the organoids remain as rather immature forms [56]. Several review articles have been published that highlight the recent developments and future applications of kidney organoids [57,58].
hPSC-derived nephron progenitor cells (NPCs) are multipotent stem cells that can develop into nephron-like structures comparable to their in vivo embryonic counterparts [59]. NPCs undergo mesenchymal-to-epithelial transition upon Wnt stimulation, which is the protocol used for most formation of kidney organoids [60]. Interestingly, iPSCs from patients undergoing hemodialysis due to a disease also can differentiate into NPCs with similar efficiency as iPSCs from healthy donors, indicating promising future applications for TE with the autologous cells of patients with kidney diseases. NPCs are, however, separate from the kidney stroma and vasculature, and non-nephron cells are also a necessary component of kidney organoids as they help establish a multi-compartment environment that can support vascularization, particularly in glomerular and tubule-interstitial structures. In their work, Morizane et al. reported a high-efficiency protocol for generating NPCs and kidney organoids from multiple lines of both hESCs and hiPSCs [61]. It has been proposed by Low and co-workers that a subset of NPCs may serve as a source of renal vasculature as well [62]. This subset of NPCs displays a vascular progenitor-like property and eventually differentiates into more mature endothelial cells. They reported in vitro vascularization of a de novo vascular network in organoids derived from a subset of NPCs. Through Wnt signaling, the proportion of proximal and distal segments were carefully controlled, resulting in glomerular podocytes producing sufficient VEGFA for a defined vascular network. The procedure did not require exogenous VEGFA, and when VEGFA signaling was disrupted by VEGF receptor inhibitors, the vascular network was notably diminished while the nephrons remained the same. When implanted beneath the renal capsule of immunocompromised mice, an increase in organoid size, maturity, and vascularization was observed. Particularly, podocytes developed elaborate cellular processes and glomeruli developed a more mature architecture with distinct capillary tufts perfused by red blood cells. A fenestrated endothelium and glomerular basement-like membrane also developed, contributing to the establishment of a glomerular filtration barrier capable of performing preliminary filtration and reabsorption. Although this study successfully induced in vitro vascularization of organoids, the eventual in vivo environment was nonetheless required for maturation of a functioning vascular network.

To prepare fully functional kidney organoids, a wide array of differentiation protocols has been developed, all of which possess different benefits and levels of complexity. The differentiation protocols reported by Takasato et al. include both hESC-derived and hiPSC-derived organoids that successfully model human nephrogenesis [63,64]. The differentiation protocols often involve alternating usage of CHIR, a small molecule Wnt activator, FGF9 (a fibroblast growth factor), and activin, a TGFB activator followed by a period without any growth factors. Recently, the Takasato protocol reports the formation of organoids with all anticipated kidney cell types, segmented nephrons, a renal interstitium, and an extensive endothelial network [65]. NPCs developed within nine days and organoids developed within twelve days. The protocol enables mass production of 3D kidney organoids by using 96-well, ultra-low-attachment plates. Guidelines for adjusting the protocol for different lines of hPSCs will allow for greater quality control between batches. While the nephrons were well characterized, cells in the interstitial space, including pericytes, endothelial cells, smooth muscle cells, and fibroblasts, were not. Another simple and inexpensive bioreactor-based method for growing kidney organoids in bulk from hiPSCs was reported by Przepiorski and co-workers but further research is required to minimize the fibrotic tendencies of these organoids [66]. The differentiation protocol included the use of CHIR and knockout serum replacement, but no FGF9, and spinner-flask bioreactors were used to improve oxygen and nutrient perfusion. A 21-day protocol using high-throughput screening for kidney organoid differentiation performed entirely by liquid-handling robots has also been proposed, but organoids produced by these extremely efficient methods are likely more applicable for use in toxicity and disease screening rather than regenerative medicine applications due to their small size and relatively immature nephrons [67]. Another cost-effective method for developing kidney micro-organoids resulted in 6–10 nephrons per organoid and all anticipated renal cell
types [68]. The differentiation protocol involves activation with CHIR, FGF9, and heparin in matrigel-coated monolayer cultures under low speed swirling using suspension culture approach. Although relatively immature, the nephrons were surrounded by stromal and endothelial cell types. These could serve as a good source of hPSC-derived kidney cells because the suspension culture approach produces up to four times as many cells as static culture.

In a recent study, Taguchi and co-workers showed that endothelial cells are not always required for the initial branching morphogenesis of in vitro kidney organoids [69]. Many reported kidney organoids contain very few endothelial cells [70,71], and even when kidney organoids do contain endothelial cells, the eventual vascular networks found within in vivo organoids are derived entirely from the host. Furthermore, most glomeruli within in vitro organoids are avascular, and vascularization is not achieved without in vivo transplantation. Although in vivo vascularization would have poor commercial and industry applications, in vitro vascularization of organoids is proving to be a significant challenge [72]. In some in vivo vascularization studies, the vasculature that developed after implantation was entirely derived from the host, and in other studies the organoids themselves contributed cells to vascularization. Ideally, a kidney organoid should be able to contribute as much as possible to the eventual vasculature since a pre-vascularized organoid could allow for much larger sizes before implantation. Early studies of relatively simple kidney organoids implanted in rats showed early vascularization of glomeruli and the differentiation of nephrons with transport capabilities [73]. Embryonic stem cell (ESC)-derived organoids co-cultured with embryonic spinal cord segments transplanted beneath the kidney capsule of immunodeficient mice developed vascularized glomeruli that connected to host circulation and contained red blood cells [74]. Co-culture with spinal cords was used instead of the more common Wnt stimulation differentiation procedure, and recent organoid protocols have used iPSCs instead of ESCs. Thus, in vitro organoids that possess a well-developed network of endothelial cells before implantation, are generally well vascularized and connected to the host circulation after implantation.

Several studies have reported a combination of both host-derived and organoid-derived vasculature upon transplantation. hPSC-derived kidney progenitors subcutaneously implanted in immunodeficient mice formed organ-like masses exhibiting functioning nephrons and vascularization [75]. The glomeruli were mature and perfused, containing human capillaries and podocytes, confirming the presence of hPSC-derived vasculature. Dextran uptake from glomerular filtrate was observed, and implanted kidney progenitors survived for over three months. In an attempt to evaluate the organoid versus host contribution to endothelial cells for vascularization after transplantation, Murakami et al. found that the majority of the renal vasculature in their organoids upon transplantation came from donor-derived rather than host-derived endothelial cells [76]. The organoids were developed from mouse embryonic kidney cells and implanted under the kidney capsule of immunodeficient mice. Although the organoid cells were the primary source of vasculature, the in vivo implantation was still necessary as organoids only developed minor vasculature in vitro. The donor cell contribution to the vasculature came from a newly identified set of renal endothelial precursors present in the embryonic mouse kidney that were able to reorganize the arteriolar structure more efficiently than the host-derived endothelial cells. They proposed that this new set of endothelial precursors could be an ideal target for induction from ESCs or hiPSCs for future organoid vascularization applications.

Although less common than in situ organoid vascularization, the transplantation of organoids for host-derived vasculature at sites other than the kidney, or ex situ, shows promising applications as well. In ex situ transplantation, the host body is used as a bioreactor in a secondary location prior to re-transplantation at the target site with a second surgery. One such experiment with kidney organoid vascularization was the growth of a kidney-in-a-lymph node as an in vivo pre-vascularization approach using the lymph node as a secondary location [77]. This study implanted human kidney progenitors from human
fetal kidneys into mouse lymph nodes. Single-cell suspensions failed to differentiate within the lymph node, but kidney progenitors allowed to first differentiate into organoids could subsequently mature in the lymph node’s in vivo environment. Those in vivo organoids exhibited excretory, homeostatic, and endocrine functions with a significant amount of host-derived vasculature. The lymph node has been shown to function as an effective bioreactor for many types of engrafted cells [78]. Previous studies have shown that even normal somatic cells derived from autologous tissues may cause immunological responses, so it has been suggested that the lymph node may be a potent transplantation region for reprogrammed somatic cells that can then be utilized for tissue regeneration. The structure of the lymph node is such that it provides immediate availability to endothelial capillaries and therefore critical nutrients and growth factors from the blood. Furthermore, lymph nodes contain fibroblastic reticular cells and other stromal cells that secrete chemokines to enhance cell recruitment, growth, expansion and survival [79,80]. Thus, it may be hypothesized that lymph nodes may be utilized for potential kidney tissue regeneration as well.

Various 3D microenvironments for kidney organoid culture that do not involve implantation into a host animal have also been reported. For example, Garreta et al. proposed chick chorioallantoic membrane (CAM) as a suitable environment for generating renal vesicles, nephron structures, and vasculature in kidney organoids from hPSCs [81]. CAM is a naturally highly vascularized extraembryonic tissue and provides a soft in vivo microenvironment that promotes organoid vascularization. They compared the organoids grown on CAM to organoids grown on 3D hydrogels fabricated from functionalized polyacrylamide of tunable stiffness designed to mimic the CAM mechanical properties in vitro. Organoids grown on both of the soft 3D microenvironments were compared to organoids grown in rigid conditions. Results showed that organoids cultured in rigid conditions possessed vascular endothelial cells surrounding the nephron structures but lacked a vascular network. However, blood vessels from the CAM invaded organoids, interacting with the glomerular structures, and chick blood was able to circulate through the organoids. The kidney organoid structures were also more mature than organoids not cultured on CAM, with enlarged BowmaN’s capsules and aligned podocyte-like cells. Organoids cultured on the engineered soft 3D polyacrylamide hydrogels showed better nephron structures and vascularization markers than organoids cultured on rigid conditions. However, these organoids still had to be eventually implanted onto CAM for vascularization to occur. RNA-sequencing analysis revealed that organoids cultured on these soft 3D microenvironments at day 16 transcriptionally matched human second-trimester fetal kidneys, validated by markers of nephron progenitors such as SIX2 and PAX2, markers of proximal tubules such as SLC3A1, and markers of glomeruli such as NPHS1 and PODXL via qPCR.

Kidney organoids hold incredible potential for engineering immunocompatible transplantable kidneys from a patient’s autologous cells, but their applications are far from reaching the clinical level. Problems include the reproducibility of the differentiation protocols, differences between hPSC lines, and the limited degree of maturity and functionality that can be obtained due to a lack of blood supply. Although cellular heterogeneity is the main feature that gives kidney organoids their primary advantage, it is also one of the most challenging aspects of kidney organoid-based approaches. With over twenty cell types that must be organized in precise, intricate structures, the kidney organoid is an extreme challenge from a manufacturing perspective. There is great variability between different batches of human kidney organoids [82]. Because kidney organoids are some of the most complex, the challenge of reproducibility is substantial. A thorough evaluation of variability in kidney organoids showed significant transcriptional variation between batches, with varying levels of maturation and off-target, or non-kidney, populations [82].

A potential limitation to applying kidney organoids in a clinical setting is similar to risks associated with other stem cell therapies. Stem cells have the risk of forming off-target populations. Certain kidney organoids may have fibrotic tendencies. Furthermore, some sub-capsular grafts will exhibit inosculation to the host vasculature, but the collecting duct
system does not connect to the host in order to facilitate urine outflow. Methods for the most effective inosculation of kidney organoids with host vasculature should be further explored to allow for the growth of organoids to more clinically relevant sizes in vivo [83]. Although kidney organoids are far from reaching clinical translation, these obstacles are by no means insurmountable. The speed at which this field progresses is promising, and the in vitro culture of kidney organoids under flow in microphysiological systems could serve as an alternative route to organoid vascularization.

**Microphysiological Systems**

Microphysiological systems, or organ-on-a-chip models, are essentially finely tuned cellular microenvironments that have biological applications in pharmacology, toxicology, and disease modeling. The ability to develop in vitro microscale technologies that regulate stem cell differentiation and model microvasculature in 3D organoids has promising applications for tissue engineering [84]. As discussed in the section on kidney organoids, inducing the vascularization of kidney organoids in vitro is challenging. While a majority of kidney organoid vascularization approaches involve in vivo implantation with host-mediated vasculogenesis and angiogenesis, microphysiological systems have been used as in vitro organoid vascularization approaches. Microfluidic design principles can be utilized to create functional vascular networks with tunable properties tailored to specific organs; the types of vascular cells, flow dynamics, lumen diameter, and vessel branching architecture can be precisely controlled [84]. Photolithographic techniques allow for unique 3D micropatterns that can mimic organ vasculature branching [85]. Ultimately, the importance of microfluidics in developing organ structure and vasculature is highlighted in microphysiological systems [86]. Vascular perfusion is needed to guide cell migration and differentiation, polarize cells, and regulate filtration and transport functions [87]. Flow-enhanced vascularization and maturation of kidney organoids on 3D-printed microfluidic chips has been the most promising method of in vitro organoid vascularization to date. The microfluidic chip consists of a gelatin-fibrin ECM coating on a 3D-printed chip which allows fluid flow between the ECM and chip surface. Flow over the top surface of organoids with high fluidic shear stress and co-culture with human endothelial cells resulted in vascular networks with significantly increased junctional density and vessel length. The percentage of the vascular surface area in contact with organoid tubules was threefold for high fluidic shear stress compared to low fluidic shear stress. Figure 5 shows the procedure for development of renal organoids that are placed on an engineered ECM, contained within a perfusable millifluidic chip, and exposed to different amounts of fluidic shear stress. Results showed that significantly enhanced vascular networks were observed with increasing fluidic shear stress. Glomerular structures were also vascularized under high fluidic shear stress while nearly avascular structures formed in low fluidic shear stress or static culture. With higher vascularization levels, more advanced morphogenesis of tubular epithelial cells was also observed.

In addition to the vascularization of organoids, microfluidics has the potential to further optimize the organoid differentiation protocol and mass produce highly controlled organoids in vitro. Glass et al. recently optimized a microbioreactor procedure for generating kidney organoids from hPSCs under perfusion [88]. The microfluidic device was fabricated by soft lithography on silicon wafers using layers of polydimethylsiloxane (PDMS), and organoids were developed from human ESCs. Growing the organoids within this finely tuned microphysiological system allowed for improved control over the complex differentiation process as well as the ability to closely analyze and better understand the differentiation process through the number of experimental protocols.

Rather than attempting the immense task of engineering a whole kidney, many groups have focused on smaller-scale engineering of individual kidney structures or isolated kidney functions within microphysiological systems for both in vitro and in vivo applications. These smaller-scale systems follow the principles of renal assist devices (RADs), or extracorporeal devices made of cells that can be used to enhance kidney function and used in
combination with dialysis [89]. Ideally, these devices would be implantable and augment the functions of one or more failing kidney structures. Additionally, the development of these individual kidney structures and functions allows for a better understanding of kidney TE with eventual whole organ TE applications. Petrosyan et al. demonstrated this concept with their glomerulus-on-a-chip using human podocytes and glomerular endothelial cells under bidirectional flow [90]. Seeded podocytes formed slit diaphragms, endothelial cells formed capillary-like structures, and the deposited basement membrane contained specific glomerular extracellular proteins. The glomerulus-on-a-chip was highly reproducible and maintained these kidney-specific phenotypes and functions for at least a month in vitro.

Another example of an isolated kidney function on an in vitro microfluidic chip is the reconstruction of the human renal vascular-tubular interactions [91]. Using type I collagen hydrogel membrane and overlapping fabricated vascular or tubular lumens containing human kidney peritubular microvascular endothelial cells (HKMECs) or human kidney epithelial cells, a microphysiological system was designed that demonstrated renal-specific functions such as selective reabsorption of glucose and albumin. The vascular and tubular microfluidic channels were fabricated using photolithographic, soft lithographic, and injection molding techniques, and continuous exposure to flow resulted in a remodeled collagen membrane that mimicked the native kidney matrix and basement membrane, flow-directed alignment of endothelial cells, and intact cellular junctions as confirmed by VE-cadherin staining. Human fetal kidney pericytes incorporated into the collagen matrix were shown to grow processes along the surfaces of both epithelial and endothelial channels. This ability to recreate such vascular-tubular interactions of the kidney within a highly tunable microfluidic system could potentially lead to clinical applications in the form of RADs or significant advances in the functioning of kidney organoids.

Microfluidic devices have been used particularly to examine and construct the kidney microvasculature. It has been shown that kidney peritubular microvessels are highly susceptible to injury and show limited regenerative capacity. The incorporation of HKMECs in various flow-directed microphysiological systems has enabled the construction of 3D kidney microvasculature networks and a better understanding of the roles flow and pressure play on endothelial cell heterogeneity. The flow-directed, three-dimensional kidney microvasculature model described by Ligresti et al. showed that the use of HKMECs over HUVECs (human umbilical vein-derived endothelial cells) resulted in kidney-specific phenotypes [92], as shown in Figure 6. The microvascular networks were also made in

![Figure 5. Formation of renal organoids placed on engineered ECM, in a perfusable millifluidic chip, under controlled fluid shear stress. Differentiation days and culture conditions are indicated in the middle and bottom parts of the panel. Organoids not drawn to scale. Reproduced with permission from Nature (adapted from reference [86]).]
collagen gel using lithographic processes. HKMECs from both adult and fetal kidneys under flow formed a thin endothelial membrane with fenestral diaphragms similar to native kidney structure and a functional permeability barrier. The endothelium was found to express CD31 and VE cadherin at regions of cell–cell contact, and a basement membrane consisting mostly of collagen IV was deposited along the microvessel walls by HKMECs. The results of immunohistochemistry studies of the microvessel junctions showed strong expression of F-actin and plasmallemma protein PV1. The fact that PV1 was so strongly expressed throughout the endothelium when HKMECs were used suggests that HKMECs are necessary to produce the proteins needed to form the diaphragms that bridge the endothelial fenestrae. Because the HKMEC microvessels possessed more fenestrae, the vessel permeability was also higher compared to those formed in the presence of HUVEC cells. Proper junctions were formed at cell–cell contacts, and numerous closed fenestrae throughout the microvessel walls were seen. The average size of these fenestrae was very similar to that of fenestrae found in kidney microvessels in vivo. Although these miniature kidney microvessel models represent a very small portion of the engineered kidney, the ability to recreate very kidney-specific phenotypes in vitro could potentially be applied to larger-scale kidney TE.

![Figure 6. Schematic illustration of 3D the microphysiological system: (A) 3D views; (B) cross-sections. (C) Formation of kidney microvessel networks. Red indicates CD31, and blue indicates nuclei. (D) Confocal microscopy image of engineered human kidney microvessel. Red indicates CD31, green indicates vWF. Scale bar, 50 mm. (E) Confocal image of human kidney microvessels at a junction of the network. Red indicates VE cadherin, and blue indicates nuclei. Scale bar, 25 mm. (Reproduced with permission from the American Society of Nephrology. Adapted from Reference [92].) 5. Conclusions and Future Directions

We have summarized the four major kidney tissue engineering methodologies discussed in this review in Table 1. Vascularization is one of the major challenges that remains in the engineering of large organs such as the kidney that require an elaborate network of arteries, veins, and capillaries to prevent necrosis in the innermost part of the tissue.
The demand for vascularization in renal TE particularly remains higher than ever. An alternative vascularization strategy that attempts to overcome the lack of organ-specificity in the inosculation of pre-vascularized tissues is in situ angiogenic remodeling.

Table 1. Comparison of developments and limitations of top-down and bottom-up approaches discussed in this review.

<table>
<thead>
<tr>
<th>Top-Down Approaches</th>
<th>Bottom-Up Approaches</th>
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<tr>
<td>dECM</td>
<td>Organoids</td>
</tr>
<tr>
<td>• Maintains native kidney architecture &amp; biochemical cues.</td>
<td>• Only requires a small amount of patient-derived stem cells.</td>
</tr>
<tr>
<td>• Problems with recellularization &amp; mechanical strength</td>
<td>• Difficulties with reproducibility</td>
</tr>
<tr>
<td>• Requires donor kidney</td>
<td>• Limited to small sizes due to lack of complete vascularization &amp; implantation sites.</td>
</tr>
</tbody>
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<thead>
<tr>
<th>Vascular Corrosion Casts</th>
<th>Microphysiological Systems</th>
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</thead>
<tbody>
<tr>
<td>• Maintains native kidney vasculature architecture</td>
<td>• Improved \textit{in vitro} vascularization of organoids due to microfluidics.</td>
</tr>
<tr>
<td>• Can be made from a variety of materials</td>
<td>• Precise control over chip dimensions and flow dynamics.</td>
</tr>
<tr>
<td>• Issues with cell-seeding and integration with host-tissue</td>
<td>• Limited to small sizes &amp; mostly 2D applications.</td>
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With angiogenic remodeling, host blood vessels grow into the engrafted engineered tissues to allow for a higher degree of organ-specificity in the regenerated vascular networks. However, the location of the kidney in the body makes in situ angiogenic remodeling a great challenge, so improvements to pre-vascularization strategies should be a main focus of kidney TE. Many scaffolds and cell types have been reported for pre-vascularization techniques, but more kidney-specific applications are needed. Other techniques such as photolithography have been used for TE, but there are limitations due to high costs, high temperature requirements, exposure of cells to UV light (causing DNA damage), and the etching process itself that may require toxic solvents. Moreover, photolithography has very little control over surface chemistry, particularly when not confined to planar surfaces. One area of technology that has seen great progress in the field of TE vascularization but has yet to be applied to whole-kidney TE is 3D bioprinting. In contrast to conventional tissue engineering methodologies, progress has been made in 3D and 4D bioprinting systems, which may allow for more accurate positioning and directionality between the individual structural elements of specific tissue but, as yet, applications for organs as complex as the kidney remain limited. With the improvement of 3D bioprinting techniques and kidney-specific bioinks, as well as 3D bioprinting approaches to graft vascularization, a more precise and reproducible method for constructing kidney organoids or even whole organs could be realized.

A majority of approaches to kidney TE currently employ dECM scaffolds, which can then be repopulated with the patient’s autologous cells. Decellularized rat, porcine, and human kidneys have been successfully seeded with epithelial and endothelial cells to produce grafts that produce rudimentary urine both \textit{in vitro} and \textit{in vivo}. Infusion of hiPSC-derived endothelial cells through the renal artery and vein of rat kidney dECM was found to restore vasculature components. Although dECM helps maintain kidney structure, vascular integrity, and biocompatibility, it possesses significantly reduced mechanical strength, and it is difficult to entirely repopulate with appropriately differentiated cells, and moreover does not eliminate the need for donor organs. Methods of improving dECM mechanical strength and cell repopulation should certainly be explored in the meantime, but alternative bottom-up strategies should be the ultimate goal of kidney TE.
Two relatively new approaches that show promise for regenerating kidneys are the use of organ-on-a-chip models and stem cell-derived miniature 3D organoids. While kidney-on-a-chip models are useful for modeling vasculature and growing isolated kidney structures, such as a glomerulus-on-a-chip, such organ-on-a-chip models are still far from functioning in replacement and regrowth of damaged kidney tissue in vivo. 3D kidney organoids made from directed differentiation of PSCs contain multiple cell types found in the human kidney and possess impressive hierarchical levels of organization with nephron-like structures and vascularization. Cost-effective methods have been developed for growing kidney organoids in bulk from hiPSCs. The asynchronous mixing of kidney progenitor cells at distinct stages of differentiation has been shown to promote nephrogenesis, vascularization, and eventual inoculation of kidney organoids upon transplantation into mice. While the advances seen with kidney organoids hold great promise, they are still too functionally immature, physically small, and under-vascularized to have direct clinical applications. Furthermore, there is great variability between different batches of human kidney organoids, so more reproducible differentiation protocols must be developed. As things stand today, it appears that the merging of the organ-on-a-chip and 3D organoid techniques by culturing kidney organoids under flow on microfluidic chips may hold the most promise for developing functioning, vascularized grafts for TE applications.

Thus far, the most effective attempts at mimicking the kidney vasculature have been through the use of flow-enhanced kidney organoids, re-endothelialized dECMs, or vascular corrosion casts to create a collagen-based biomimetic scaffold that preserves the natural kidney vasculature. In addition to kidney dECM or stem cell-derived organoid approaches, a wide variety of synthetic and natural biomaterials have been used to construct scaffolds that promote vascularization and nephrogenesis of engineered kidney tissue, but clinical applications remain limited. The combination of the top-down and bottom-up approaches discussed in this review with novel biomaterials designed specifically for kidney tissue growth, differentiation, and functioning will continue to improve the field of renal TE and progress closer to the realization of vascularized, functioning kidney grafts to replace organ transplants in clinical applications for chronic kidney disease.

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References


58. Islam, M.; Nishinakamura, R. How to rebuild the kidney: Recent advances in kidney organoids. *J. Biochem.* 2019, 166, 7–12. [CrossRef]


64. Takasato, M.; Er, P.X.; Chiu, H.S.; Maier, B.; Baillie, G.J.; Ferguson, C.; Parton, R.G.; Wolvetang, E.J.; Roost, M.S.; de Sousa Lopes, S.M.C.; et al. Kidney organoids from human iPS cells contain multiple lineages and mod-el human nephrogenesis. *Nature 2015*, 526, 564–568. [CrossRef]


Learning from Nature: Bioinspired Chlorin-Based Photosensitizers Immobilized on Carbon Materials for Combined Photodynamic and Photothermal Therapy

Lucas D. Dias * and Ivan S. Mfouo-Tynga

São Carlos Institute of Physics, University of São Paulo, São Carlos 13566-590, Brazil; tivansdavids2012@gmail.com
* Correspondence: lucasdanillodias@gmail.com

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Abstract: Chlorophylls, which are chlorin-type photosensitizers, are known as the key building blocks of nature and are fundamental for solar energy metabolism during the photosynthesis process. In this regard, the utilization of bioinspired chlorin analogs as photosensitizers for photodynamic therapy constitutes an evolutionary topic of research. Moreover, carbon nanomaterials have been widely applied in photodynamic therapy protocols due to their optical characteristics, good biocompatibility, and tunable systematic toxicity. Herein, we review the literature related to the applications of chlorin-based photosensitizers that were functionalized onto carbon nanomaterials for photodynamic and photothermal therapies against cancer. Rather than a comprehensive review, we intended to highlight the most important and illustrative examples over the last 10 years.

Keywords: bioinspired photosensitizer; cancer; carbon nanomaterial; chlorin; photodynamic therapy; photothermal therapy

1. Introduction

Photodynamic therapy (PDT) is a light-based therapy that uses photosensitizing molecules (PSs), light of an appropriate wavelength, and molecular oxygen (O_2) to impart cytotoxicity via oxidative reactions, which are able to combat cancer cells (solid tumor) [1,2] and infectious diseases [3–5]. Regarding its mechanism, a light-activated PS transfers its excited-state energy to the O_2 for generating reactive oxygen species (ROS) and singlet oxygen (¹O_2), which are able to destroy cancerous cells via oxidative pathways [6]. Among the PSs used so far, we highlighted the bioinspired tetrapyrrole macrocycle photosensitizers, such as porphyrin, chlorin, and bacteriochlorin derivatives [7]. These types of photosensitizers were bioinspired by vital natural molecules, for instance, heme, chlorophyll a, and bacteriochlorophyll a, and have been reported as highly active photosensitizers [8].

In nature, the interaction of light (solar energy) with a photosensitizing molecule (chlorophyll a) results in the production of sugar and O_2 via a photosynthesis process [9]. Similarly, in photodynamic therapy, the interaction of light (energy) with a photosensitizing molecule results in the production of other molecules, in this case, ROS. This is a classic example where people have been using bioinspiration in nature to generate benefits for society (Figure 1).

Moreover, in recent years, the combination of photodynamic and photothermal therapies (PTTs) by using photosensitizers and carbon nanomaterials combined (carbon nanotube, graphene, fullerene, carbon dot) have been widely studied/tested, showing excellent results [10–13]. These carbon-based nanomaterials are considered promising photosensitizers due to their optical properties, high biocompatibility, and low toxicity [14,15]. This strategy, which involves the combination of a carbon nanomaterial with other organic photosensitizers, utilizes covalent (more stable and
using specific linkage) or non-covalent (electrostatic forces, e.g., π–π stacking, van der Waals forces, hydrogen bonding, or hydrophobic interaction) linkages [16–20].

![Diagram of photosynthesis and photodynamic therapy](image)

**Figure 1.** (a) General photosynthesis process; (b) Representation of a simple photodynamic therapy protocol. ROS: reactive oxygen species.

Herein, we review the literature related to the applications of bioinspired chlorin-based photosensitizers that were immobilized onto carbon nanomaterials for photodynamic therapy against cancer. Rather than a comprehensive review, we intended to highlight the most important and illustrative examples from the last 10 years.

2. Photodynamic and Photothermal Therapy: Mechanism and Applications

2.1. Mechanisms of Photodynamic and Photothermal Therapy

After PS photoactivation, PDT induces tumor and vascular damage, and mainly leaves normal tissues unaffected, due to the improved selectivity, as seen with later generations of PSs. Light of a suitable wavelength and intensity reach the PSs to undergo a series of photochemical reactions [21]. Light energy as a photon is transferred to the inert PS, changing it into activated or excited PS. In this form, the PS can lose the received energy via various possible pathways. The fluorescent phenomenon may occur and lead to the detection and delineation of the tumor target site, but this method is commonly underutilized. An excited PS may lose energy via undergoing type I photochemical reactions, yielding destructive free radicals, or via a type II photochemical process (singlet oxygen).
(Figure 2). It is difficult to determine which of these mechanisms (type I or II) is more predominant; both types of reactions can happen depending on three singular features: (i) the presence of oxygen, (ii) photosensitizer concentration, and (iii) PS type [22]. The singlet oxygen species has a half-life span of about 10 mm to a few centimeters to cause ablation and killing via hyperthermia [30]. The extent of the induced damage depends on the physical-chemical characteristics of the agent, including photothermal conversion efficiency, low power density, and biocompatibility, as well as photostability and absorption in the NIR region [29]. Photothermal excitation initiates PTT via the direct generation of heat after the irradiation. NIR light offers a better light penetration depth into tumor tissue from about 10 mm to a few centimeters to cause ablation and killing via hyperthermia [30]. The extent of the induced damage depends on the distribution of the agent within the tumor. Certain nanomaterials (e.g., gold nanoparticles, plant extracts as a combination therapy against biofilms [26].

In clinical scenarios, a two-step procedure is performed, starting with systemic administration of PSs via intravenous injection or topical application. The second step is exposing the sensitized tissue to the light to induce a series of photochemical reactions, oxygen reactive species, and subsequent destruction of the tissue under treatment [25]. Antimicrobial photodynamic therapy (aPDT) is highly recommended for cases of increased antimicrobial resistance. There are studies in the literature that reported the inactivation of both Gram-positive and -negative bacteria (up to 7 log units) by using this light-based therapy [26,27]. Activation of PSs can considerably reduce the number of bacteria and represents a promising approach to antimicrobial treatment [26]. The bactericidal effect of aPDT can be further enhanced by applying aPDT after the employment of certain enzymes or plant extracts as a combination therapy against biofilms [26].

PTT is also a minimally invasive and localized modality that utilizes a PS, which is able to absorb and convert energy into heat. This photothermal effect (i.e., heat generation) arises as a result of non-radiative relaxation processes, as presented in Figure 2. The type of absorbing agent (PS), light wavelength, and delivery (interstitial or non-invasive) are all determinants for the energy absorption and subsequent induction of PTT in tumors. Photothermal reactions are enhanced by using photothermal transducers that exhibit strong absorption in NIR and ensure that the generated heat is selectively dissipated in the tumor tissue and not into surrounding tissue [28]. The light device should uniformly deliver heat to the PS that is pre-localized in the targeted tumor, thus increasing the temperature and leaving the surrounding tissues unaffected. Above 41 °C, the photothermal effects become detrimental, where the damaging effects propagate from the core of the tumor and create a thermal gradient at the edges. The mechanisms of PTT for complete or partial thermal energy generation depend on the physical-chemical characteristics of the agent, including photothermal conversion efficiency, low power density, and biocompatibility, as well as photostability and absorption in the NIR region [29]. Photothermal excitation initiates PTT via the direct generation of heat after the irradiation. NIR light offers a better light penetration depth into tumor tissue from about 10 mm to a few centimeters to cause ablation and killing via hyperthermia [30]. The extent of the induced damage depends on the distribution of the agent within the tumor. Certain nanomaterials (e.g., gold nanoparticles,
graphene oxide sheets, carbon nanotubes) are becoming famous for their roles as energy absorbers and heat-conversion mediators [28]. Necrosis and apoptosis are often cited as the main or predominantly stated induced damaging mechanisms when using high and low energy irradiation, respectively [31]. The induction of necrosis appears as the main response when using high light doses, while apoptosis seems to be predominant at a relatively low light dose. After PDT and mulling over different response probabilities of death, apoptosis and senescence are considered as the main physiological modes, while necrosis and stress-induced death are the major provoked pathological modes. Other death modes appeared as variant forms of those physiological and pathological responses [32]. The necrotic response seems to always be appearing, especially at the later stages of the flow cytometry analysis using Annexin-V PI staining detection. Optimal conditions need to be carefully determined in order to prevent the development of tumor resistance and recurrence. Finally, effective responses strongly depend on the used model (in vitro or in vivo), type of PS, and treatment protocol.

When used in combination, PTT resulted in more tumor-killing and enhanced therapeutic outcomes, which were associated with the use of 2D nanomaterials as photothermal agents. Photosensitizers used in PDT are able to generate reactive oxygen species that lead to tumor-killing but still possess limitations, including low selectivity, poor water solubility, and moderate delivery to targeted tumor locations. When combined with certain nanomaterials that act as PTT-mediating agents, enhanced theranostic effects can be achieved, as well as improved targeting and controlled delivery in targeted tumor sites [33]. The heat generated by PTT agents stimulates cell membrane permeability, drug delivery, and the subsequent better uptake of PDT agents by tumors, which are destroyed by PTT-induced hyperthermia and enhanced PDT effects (Figure 3). Combining therapies, such as PTT and PDT, could lead to synergic effects, resulting in effective and multifunctional theranostic applications [34,35].

![Figure 3](image-url)  
**Figure 3.** In combination therapy, both photodynamic and photothermal therapy (PTT) and photodynamic therapy (PDT) agents are irradiated and excited to an upper electronic level, where the PTT agent generates heat that facilitates cell membrane permeability and more PDT agent release and uptake into tumor tissue. Hyperthermia and free radical generation cause ablation and tumor damage and killing through necrosis and/or apoptosis. PS: photosensitizing molecule, NP: nanoparticle; NIR: near-infrared region.

### 2.2. Anticancer and Antimicrobial Applications of PDT

PDT-mediated tumor damage induces both programmed and non-programmed cell death pathways [36]. Carcinogenesis stimulates genetic mutations and the disturbance of life-sustaining mechanisms. To allow for better selectivity and damage to malignant tissues, certain mechanisms, such as receptor binding, lipid binding, uptake via tyrosine kinase or epidermal growth factor receptor, diffusion, and bio-distribution, are activated to mediate cellular damaging events, which are responsible for the initiation of cancer therapeutic action [37–39]. The PS organelle or subcellular localization is the initiation site of PDT-mediated damage and depends upon the kind of PS used.
A well-known derivative of porphyrin, Photofrin®, is taken up and preferentially accumulated in cell membranes and other organelle membranes, such as mitochondria. Some amphiphilic PSs also accumulate in mitochondria, while others have the Golgi apparatus and endoplasmic reticulum as their preferential localization sites [40,41]. At high light doses, the tumor tissues are rapidly destroyed through a non-programmed pathway following a necrotic manner. During this execution, sub-cellular membranes are destroyed, leading to the excessive release of calcium ions and metabolic byproducts, which causes multiple forms of dysfunction and damage beyond repair [42–44]. The release of apoptogenic molecules, cytokines, and ROS cause lethal damage in neighboring tissues, known as the bystander effect, which can propagate to a certain extent [45,46]. PDT may induce programmed cell death through the activation of autophagic cell death and the induction of apoptosis. A low light dose triggers sequential apoptosis events, which start to cease cellular functions and cause cell death in the end. No major bystander or immune effects are required, as no excessive photodamaging effects are usually produced with a low light dose. Apoptosis is a well-conserved mechanism that eliminates damaged cells to preserve the integrity and vitality of tissues. Apoptosis operates well in both tumor and normal cells, as well as in other species like bacteria [47]. However, cancer may promote anti-apoptotic events over proapoptotic ones, hence it is essential to strategically apply target or combined treatments to ensure the facilitation of proapoptosis [48,49]. PDT is lethal and principally affects tumor tissues, leaving surrounding tissues unaffected and the PSs are cleared out and not retained in normal cells.

With an increasing number of pathogens becoming resistant, the common antibiotics are losing their efficacy as the need for an innovative technique to inactivate pathogens without inducing any resistance continues to rise. aPDT is recommended for cases of increased antimicrobial resistance and offers many advantages, including a broader targeting range of actions (bacteria, protozoa, fungi), reduced adverse effects, and antibacterial effects against antibiotic-resistant strains [50]. aPDT is an effective modality for in vitro, in vivo, and clinical applications (e.g., dentistry field) [51,52], and its efficacy has also been proven for localized and superficial infections [53].

3. Bioinspired Photosensitizers

Natural molecules, such as chlorophylls, heme, and cobalamin, are considered the “gold standard” that inspires chemists to precisely modulate effective photosensitizers for PDT applications [54,55], photocatalysts/catalysts for chemical processes [56,57], and molecular electronics [58,59]. These tetrapyrrolic macrocycle types are commonly found in nature and provide a key biochemical role in many natural processes. An example of porphyrin in biological processes is the heme present in cytochrome P-450 monoxygenase enzymes [60], hemoglobin, and myoglobin for the transport and storage of O₂ [61]. Chlorophyll is responsible for the green color of plants, algae, and cyanobacteria, and bacteriochlorophyll a, a bacteriochlorin derivative, is involved in photosynthesis in some bacteria. Overall, these tetrapyrrolic macrocycles are considered the “pigments of life” [62] (Figure 4).

Regarding the PDT field, tetrapyrrolic macrocycles are one of the most applied photosensitizers, especially the porphyrin and chlorins ones. Their structural and optical characteristics are based on four pyrrole rings and methine bridges, resulting in an aromatic macrocycle [7]. The general UV-vis absorption spectra of porphyrin, chlorin, and bacteriochlorin are presented in Figure 5. The range of spectra between 650 and 850 nm, where light penetrates tissue up to 1–3 cm, has been termed the phototherapeutic window for systemic in vivo applications (e.g., solid tumors) [63], but for antimicrobial photodynamic therapy, absorption of light in the blue region is sufficient [64].

Porphyrin photosensitizers show an absorption band in the region of 400 nm (Soret band) and other small bands in the region of 630 nm [7,8]. Chlorin-type photosensitizers have one of its double bonds in the pyrrole ring reduced, which results in a strong absorption band in the violet-blue region (∼380–450 nm), also known as the B or Soret band, and a moderate band in the red region (∼600–700 nm), known as the Q band [7,8]. While bacteriochlorins have two reduced double bonds in the pyrrole rings, resulting in a strong absorption in the near-infrared region (NIR), this class of photosensitizers
shows the tendency toward rapid phototransformation, low stability, and conversion to chlorin and/or porphyrin precursors [65,66]. However, there are some examples in the literature describing highly active bacteriochlorins as photosensitizers in PDT protocols, e.g., redaporfin (LUZ11) [65,66]. One way to overcome this instability is via the presence of electron-withdrawing substituents, the insertion of appropriate metal ions into the macrocycle, and the presence of exocyclic rings in the macrocycle [66].

Figure 4. Tetrapyrrolic macrocycles that are involved in natural processes.

Figure 5. UV-vis absorption spectra of tetrapyrrolic macrocycles that are involved in natural processes. Reprinted from Pucelik et al. [8] with permission (open access) from Elsevier, Copyright 2020.
Among them, this review paper focuses on chlorin photosensitizers. This class of photosensitizers can be obtained via four main routes: (i) the isolation of naturally occurring chlorophylls; (ii) synthesis using porphyrin as precursors (via hydrogenation, annulation, cycloaddition, and breaking and mending); (iii) semi-synthesis of chlorins, beginning with naturally occurring chlorophylls; (iv) de novo synthesis of gem-dialkylchlorins, wherein the reduced ring is linked to the acyclic precursors of the chlorin [67]. As the most studied PS, emphasis will be put on chlorin e6 (Ce6) and its derivatives.

As a selected example, Uliana and co-authors described obtaining chlorin e6 from *Spirulina maxima* by using a simple extraction method (Figure 6) [68]. First, a methyl-pheophorbide a derivative was obtained through extraction from dried *Spirulina maxima*, followed by filtration, neutralization using sodium bicarbonate, and purification via silica gel flash chromatography. The methyl-pheophorbide a derivative was obtained in 0.8% yield from natural alga. Then, the chlorin e6 was obtained in 89% yield via the basic hydrolysis of methyl-pheophorbide in the presence of an aqueous NaOH solution and acetone as the solvent.

Another approach to obtaining chlorin photosensitizers is via synthetic modulation by using porphyrin as a precursor. In 2012, Pereira and co-authors reported a sustainable methodology for chlorin synthesis via the reduction of porphyrin precursors using *p*-toluenesulfonylhydrazide in the total absence of solvents and bases. In this method, the desired porphyrin precursors were mixed with *p*-toluenesulfonylhydrazide and heated up to 140 °C in an evacuated tube (0.1 bar) [69]. When the ratios of (30:1) and (8:1) of TsNHNH2:porphyrin were used, the bacteriochlorin and a mixture of porphyrin, chlorin, and bacteriochlorin were obtained. When a ratio of 15:1 was used, a chlorin derivative with 10–20% of bacteriochlorin was obtained. The authors also described that when using a mixture of H2O2 and FeCl3 as the oxidants, selectivity for chlorin was observed (Figure 7).
Overall, so far there are many methodologies for obtaining chlorin photosensitizers, as described by Lindsey in 2017 [67]. The choice of method and target chlorin molecular structure should be based on some factors: (i) the type of PDT application (antimicrobial or anticancer photodynamic protocol); (ii) cost of the process; (iii) appropriate photo, physical, and chemical properties (solubility, stability, log P, the quantum yield of triplet state, etc.).

4. Carbon Materials Applied in Photodynamic/Photothermal Therapy

Carbon is the most versatile element in the periodic table [70] and its ability to hybridize in sp, sp², and sp³ configurations opens the way for the existence of a number of allotropes. So far, there are three naturally occurring allotropes of carbon (diamond, amorphous carbon, and graphite), and the synthetic ones include graphene, carbon nanotubes, fullerenes, carbon nanohorns, and nanodiamonds [71] (Figure 8).

Figure 7. Methodology for the synthesis of chlorin via the hydrogenation of a porphyrin precursor by using p-TsNHNH₂. Red circle: pyrrole ring; Blue circle: pyrrole ring reduced.

Figure 8. General structures of some carbon nanomaterials.
The properties of carbon materials (dimensions, hybridization, electrical conductivity, Young’s modulus) make them applicable in many fields ranging from materials science to biomedical applications [72]. Among these advantages, these carbon materials show strong absorption in the visible-NIR regions, which require relatively lower energy and laser intensity for photoinduction and a large surface for the development of new generations of anticancer systems [73].

Upon light activation, these carbon materials are capable of generating ROS through type I and II photodynamic reactions (PDR). They are designated as potential candidates for PDT applications. Both carbon-nanotube- and graphene-mediated PDR generate a significant amount of heat, thus they are considered as PTT agents [74]. Carbon-based nanoparticles are extensively used for PDT applications due to their distinctive optical characteristics, good biocompatibility, and tunable systemic toxicity [11]. The discovery of fullerene corresponds to the beginning of the study in the field that focuses on carbon nanomaterials. Fullerene has a soccer-ball-like shape and truncated icosahedron with 12 pentagons with C5–C5 single bonds and 20 hexagons with C5–C6 double bonds [75]. The average diameter of fullerene derivatives is 8.5 nm wide and C60 is currently the smallest and most commonly studied fullerene derivative [76]. After light activation, fullerene derivatives can generate ROS and are considered as potential therapeutic agents for PDT [77]. They contain an extended \( \pi \)-conjugated system, which offers the ability to absorb visible light. After light absorption, they form singlet excited states that undergo intersystem crossing to the triplet state. In this form, they are readily quenched by molecular oxygen and generate singlet oxygen, while others form free radicals [78]. Fullerene derivatives require less photobleaching, are more photostable, and are more ROS-inducing through the type I pathway than the commonly used tetrapyrrolic PS [79]. The fullerene derivatives are more effective in hypoxic tumors, as they induce more cytotoxic effects than singlet oxygens, which are produced by most PSs through a type II pathway [80]. Carbon-based materials are insoluble in water but the stability of their dispersions in an aqueous environment may be increased by incorporating them into water-soluble structures, such as liposomes, micelles, dendrimers, cyclodextrins, and nanoemulsion systems [79]. This approach protects their hydrophobic cores from the potential modifying effects of solvating agents and allows for limited contact with oxygen, which might cause a decreased PDT efficacy [81]. The novel design and characteristics (photosensitivity) allow C60 to also be utilized as a drug-carrier to improve the delivery to targeted areas. Meanwhile, C60 can actively contribute to the synergic therapeutic effect of PDT, chemotherapy, PTT, and others [82]. The general properties, advantages, and drawbacks of carbon nanomaterials are presented in Table 1.

The use of two-dimensional (2D) nanomaterials (NMs) as theranostic (therapeutic and diagnostic) agents is becoming popular as it improves the PDT outcomes for better cancer therapy [86]. One of the most studied 2D NMs is graphene and its derivatives or graphene-based materials (GBM), including few-layer graphene (FLG), graphene oxide (GO), reducing graphene oxide (rGO), nanographene oxide (NGO), and graphene quantum dots (GQDs) [87,88]. Graphene is made up of a single layer of carbon atoms forming a honeycomb-like structure, which has a high surface area of 2.630 m²/g, optimal thermal conductivity of approximately 5000 W/mK, approximate optical transparency of 2.3% of visible light, and a good room temperature quantum effect for electrons and holes [89]. GBMs offer an excellent carrier capacity and mobility due to the delocalization of its 2D plane sp² hybridization that renders them potential candidates for improved delivery and theranostic applications [90]. In PDT, GBMs are mostly exploited due to their optimal loading efficiency and ability to absorb light in the near-infrared region of the visible spectrum; thus they are considered as promising anticancer agents, both in vivo and in vitro [91]. Besides the tumor-targeted drug delivery and high loading drug efficiency, they are considered as suitable materials for gene therapy using materials such as DNA, microRNA, short interfering RNA (siRNA), and anticancer agents [92]. With GBM-mediated PDT, cancer theranostics led to cancer-killing with limited effects on healthy tissues, where GBMs are considered as great therapeutic tools. Their supramolecular \( \pi-\pi \) stacking is extensively used in combination therapeutic approaches, as they are loading platforms for other agents, and thus the resulting systems enhance the therapeutic efficiency and synergetic killing ability [93,94].
Carbon nanotubes (CNTs) are closely related to GBMs as they are considered to be graphene-sheets rolled up into 1D hollow cylinders ranging between 1 to 100 nm wide. Many types of CNTs can be distinguished, where among them is the single-walled CNT (SWCNT) category and the two or more layers called multi-walled CNT (MWCNT) category. Due to the large π–π stacking systems present at the surfaces, CNTs may easily across various physiological barriers within the body to induce various responses, such as immunogenic and cytotoxic effects. As a result, they appear to have good phototherapeutic and anticancer activities [94,95]. Like graphene, CNTs have attracted attention and are widely studied due to their unique characteristics [73]. Similarly, they have strong light absorption in the NIR region, deep tissue penetration, and photothermal abilities. PTT acts via hyperthermia, which had been used for treating certain conditions by inducing selective cell death responses [96]. Therefore, CNTs can be utilized for PTT to damage cancer cells and reduce heat tolerance compared to normal cells. Cancer cells are highly proliferating and more prone to die from a shortage of supply of blood in the tumor [96]. In physiological conditions, CNTs are insoluble in water but oxidizing them with a strong acid generates carboxylic acid groups and CNT-derivatives that increase the stability of their dispersions in an aqueous medium. Among other advantages, CNTs are easily conjugated to other agents (e.g., hydrophobic drugs) in combination therapy to yield both PTT and PDT effects. Combining PTT with PDT or any other anticancer treatment could enhance the PTT-mediated anticancer effects with limited side effects to normal tissues through a controlled harsh thermal phenomenon, thus preventing inflammatory reactions and cancer metastasis. Using CNTs in combination therapy approaches is seen as a promising treatment that could induce better drug delivery and high therapeutic outcomes. Despite the therapeutic use and biomedical applications of CNMs, little is known about their induced-effects on different biological systems and cellular compartments.

Table 1. General properties, advantages, and drawbacks of some carbon nanomaterials [83–85].

<table>
<thead>
<tr>
<th>Entry</th>
<th>Carbon Nanomaterial</th>
<th>Properties</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Carbon dot</td>
<td>D = 0, strong optical absorption in the UV region (260–320 nm)</td>
<td>Low toxicity, excellent photoluminescence, good hydrophilicity, small size (below 10 nm), easy synthesis, good electrochemiluminescence, high stability in physiological media, good fluorescent property, biocompatible</td>
<td>Low solubility in physiological media, aggregation</td>
</tr>
<tr>
<td>2</td>
<td>Fullerene</td>
<td>D = 0, H^2 = mostly sp^2, E.S.A. = 80–90, T.C. = 4.9, E.C. = 10^{-10}, T = elastic, hardness = hard</td>
<td>Low toxicity, biocompatible</td>
<td>Low solubility in physiological media, aggregation</td>
</tr>
<tr>
<td>3</td>
<td>Carbon nanotube</td>
<td>D = 1, H = mostly sp^2, E.S.A. = −1300, T.C. = 3500, E.C. = structure-dependent, T = flexible, elastic, hardness = hard</td>
<td>Low toxicity, high conductivity, high chemical stability and sensitivity, high electron-transfer rate, biocompatible, strong NIR light absorption</td>
<td>Low solubility in physiological media, aggregation, low homogeneous size</td>
</tr>
<tr>
<td>4</td>
<td>Graphene</td>
<td>D = 2, H = sp^2, E.S.A. = −1500, T.C. = 4850–5300, E.C. = −2000, T = flexible, elastic, hardness = uppermost</td>
<td>Low toxicity, high sensitivity, large surface area, inherent size- and shape-dependent optical properties, unique physicochemical behavior, biocompatible</td>
<td>Low solubility in physiological media, aggregation</td>
</tr>
</tbody>
</table>

1 D = dimensions, 2 H = hybridization, 3 E.S.A. = experimental specific surface area (m^2 g^{-1}); 4 T.C. = thermal conductivity (W m^{-1} K^{-1}); 5 E.C. = electrical conductivity (S cm^{-1}); 6 T = tenacity.

So far, no clearance regarding the neurotoxicity, hepatotoxicity, nephrotoxicity, immunotoxicity, cardiotoxicity, genotoxicity, epigenetic toxicity, dermal toxicity, and carcinogenicity of CNMs had been reported [97]. However, several studies had shown that there is a certain level of toxicity associated with the use of 0D, 1D, and 2D CNMs in both normal and cancerous cells [98]. Like any effective therapeutic agent, CNMs have demonstrated capabilities in inducing cyto-damage responses, such as oxidative damage, immuno-dependent reactions, and cell death induction, all of which lead
to cellular and nuclear fragmentation and destruction [99]. The biocompatibility and toxicity issues raised by CNMs represent important impediments for further uses, despite their proven capabilities in various biomedical applications [100]. As it stands, much more research is needed in order to determine the therapeutic indices of CNMs, as well as their safety protocols on biological systems and cellular compartments.

5. An Update on Chlorin-Based Photosensitizers Immobilized on Carbon Materials for Photodynamic and Photothermal Therapy

5.1. Carbon Dots

In order to describe the efficiency of chlorin-based photosensitizers immobilized on carbon dots for photodynamic and photothermal therapy against cancer, different parameters have been reported (2010–2019), such as the description of carbon material, concentration, light dose, incubation time, and type of cancer cell lines, where the results obtained are presented in Table 2.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Carbon Material</th>
<th>Concentration</th>
<th>Irradiation</th>
<th>Incubation Time</th>
<th>Cancer Cell Lines</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chlorin e6-conjugated C-dots</td>
<td>0–50 µM</td>
<td>30 mW/cm² (3 min)</td>
<td>24 h</td>
<td>MGC803 cells</td>
<td>~10% (cell viability)</td>
<td>[101]</td>
</tr>
<tr>
<td>2</td>
<td>Chlorin e6-carbon dot</td>
<td>1 µM</td>
<td>100 mW/cm² (10 min)</td>
<td>4 h</td>
<td>B16F10 cells</td>
<td>~10% (cell viability)</td>
<td>[102]</td>
</tr>
<tr>
<td>3</td>
<td>Chlorin e6–polyethyleneimine-coated carbon nanodots</td>
<td>2.6 µg/mL</td>
<td>15.5 mW/cm² (60 min)</td>
<td>24 h</td>
<td>HeLa cancer cells</td>
<td>20% (cell viability)</td>
<td>[103]</td>
</tr>
<tr>
<td>4</td>
<td>Layered double hydroxides–chlorin e6-carbon dots</td>
<td>0–10 µg/mL</td>
<td>27 J/cm² (60 min)</td>
<td>24 h</td>
<td>HeLa cancer cells</td>
<td>9.8% (cell viability)</td>
<td>[104]</td>
</tr>
<tr>
<td>5</td>
<td>Chlorin e6-carbon dot</td>
<td>3.0 mg/kg body weight (b.w.)</td>
<td>0.5 W/cm² (10 min)</td>
<td>24 h</td>
<td>BALB/c athymic nude mice (A549 cells)</td>
<td>Volume tumor was decreased (up to 80%)</td>
<td>[105]</td>
</tr>
</tbody>
</table>

In 2012, Huang and co-authors [101] reported the synthesis of multifunctional chlorin-e6-conjugated C-dots and their evaluation against cellosaurus (MGC 803) cells (Table 2, entry 1). In this protocol, the authors used the following parameters: a range of concentrations (0–50 µM), irradiation of 30 mW/cm² for 3 min, and 24 h of incubation, obtaining a cell viability of 10%. In 2015, Beack and co-authors (Table 2, entry 2) reported the synthesis of a transdermal bioconjugate constituted of a carbon dot, chlorin e6 photosensitizer, and hyaluronate, and its application in a photodynamic protocol against melanoma skin cancer [102]. The authors prepared this bioconjugate using amide bond formation between a chlorin-e6-functionalized carbon dot and diaminohexane-modified hyaluronate in PBS as a solvent by using the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-N-hydroxysuccinimide sodium (EDC-NHS) methodology (Figure 9). The conjugation was confirmed using infrared spectroscopy [102].

For the in vitro photocytotoxicity assessment, a concentration of 1 µM, incubation of 4 h, and irradiation of 100 mW/cm² (at 660 nm) for 10 min were used in B16F10 melanoma cells. The bioconjugate showed better results compared to chlorin e6 and chlorin-e6-functionalized carbon dots, which may have been due to its more effective uptake. For in vivo studies, the authors described that when this bioconjugate (carbon dot–chlorin e6 photosensitizer) was applied, the tumor volume increased without the laser irradiation but was suppressed under laser irradiation at 660 nm due to the photodynamic effect. In sum, this approach using transdermal delivery is safer and more effective than systemic delivery because it is locally accumulated in melanoma skin cancer [102].
In 2016, Wang and co-authors (Table 2, entry 3) reported the covalent linkage of chlorin e6 on polyethyleneimine-coated carbon nanodots by using the EDC strategy and its application in an in vitro photodynamic protocol against HeLa cancer cells [103]. In this study, the authors used the following parameters: chlorin e6 functionalized on polyethyleneimine-coated carbon nanodots (CDot-PEI-Ce6) (the final concentration of chlorin e6 was 2.6 µg/mL), under red light irradiation (15.5 mW/cm²), for 60 min, where a loss of cell viability (over 80%) was observed. This CDot-PEI-Ce6 presented high water solubility, biocompatibility, and high cellular uptake, as determined using flow cytometry and confocal laser scanning [103].

In order to develop a fluorescence imaging material that also shows photodynamic therapy performance against cancer, Hu and co-authors reported the preparation of double hydroxide (LDH) ultrathin nanosheets as a delivery vehicle to load chlorin e6 and carbon dots (Table 2, entry 4) [104]. The in vitro PDT studies using these chlorin e6 and carbon dots functionalized on layered double hydroxide ultrathin nanosheets (0–10 µg/mL) against HeLa cancer cells showed a good result (viability up to 9.6%) when using an incubation time of 24 h and a light fluence of 27 J/cm² at 650 nm. Moreover, the authors found that this system (chlorin e6 + carbon dots + layered double hydroxides) presented satisfactory cellular imaging and a better photostability compared to chlorin e6 [104].

Aiming to reach the “ideal” balance between accumulation and clearance from the body, Liu and co-authors [105] reported the development of a new multifunctional nanomaterial: photosensitizer (chlorin e6)-loaded assembled carbon dots (Table 2, entry 5). Moreover, in this study, the authors used Gd³⁺ on the carbon dots’ surface for monitoring it via magnetic resonance and fluorescence imaging. For in vivo PDT studies, female BALB/c athymic nude mice at the ages of 6–8 weeks with weights of 18–22 g were used and the multifunctional nanomaterial synthesized was intravenously injected (3.0 mg/kg body weight (b.w.)) and irradiated at 633 nm using a power density of 0.5 W/cm² (10 min) at 24 h after injection. On the 21st day after the PDT protocol, the tumor (formed by A549 cells that were transplanted subcutaneously) was analyzed and showed better performance than chlorin e6 in inhibiting tumor growth. Moreover, the authors observed that carbon dots underwent degradation into ultra-small particles under the acidic conditions in the tumor, and consequently, were easily cleared from the body [105].

5.2. Fullerene

In order to describe the efficiency of chlorin-based photosensitizers immobilized on fullerenes for photodynamic and photothermal therapy against cancer, different parameters have been reported (2010–2019), such as descriptions of the carbon material, concentration, light dose, incubation time, and type of cancer cell lines, where the results obtained are presented in Table 3.
In 2016, Guan and co-authors [106] described the synthesis of a nanomaterial based on a tri-malonate derivative of fullerene (C70), with chlorin e6 as a photosensitizer and 1,10-diamino-4,7-dioxadecane (OEG2) as a linker (Table 3, entry 1). This nanocomposite based on a fullerene derivative was applied in an in vitro photodynamic procedure, where it was imaging-guided against human lung carcinoma/alveolar cell lines (A549 cells) using an incubation time of 3 h and irradiation (20 mW/cm² for 10 min) at 660 nm. From the analysis of Figure 10, the authors obtained a cell viability of ~10% at 0.2 mg/mL of the nanocomposite (chlorin e6–functionalized fullerene C70).

Moreover, the authors evaluated this nanomaterial in an in vivo photodynamic study using female BALB/c mice (16–20 g) and a tumor model formed via subcutaneous injection of luciferase-expressing murine mammary carcinoma (4T1-luc) cells. For this in vivo protocol, the authors used a solution of 1 mg/mL of the developed material in saline as a solvent (200 µL, corresponding to a relative dose of 0.2 mg) with 4 h of incubation; then, the tumor region was irradiated for 10 min (at 660 nm, 100 mW/cm²) [106]. Overall, the authors described some advantages of the prepared nanocomposite: (i) high chlorin e6 loading efficiency (up to ≈57 wt%), (ii) good efficient absorption (red/NIR region), (iii) good cellular uptake for in vitro and in vivo studies, (iv) monitoring of tumor via imaging, (v) good biocompatibility, and (vi) full clearance from the body [106].

Recently, in 2020, Rybkin and co-authors reported a material obtained by covalently linking chlorin e6 to fullerene, which was characterized by means of absorption spectroscopy, steady-state/time-resolved fluorescence spectroscopy, dynamic light scattering, and an assessment of its photocytotoxicity (light fluence of 23 mW/cm²) against HeLa cancer cells (IC₅₀ = 1.17 µM) (Table 3, entry 2) [107]. Moreover, the authors evaluated the influence of the linker and the presence of a Zn metal ion in the chlorin core on the physical/chemical properties and photodynamic efficiency; they observed that these functionalized carbon nanomaterials act via both type I and type II mechanisms. In this study, the authors also demonstrated that water-soluble fullerene derivatives, which were covalently linked to a hydrophobic dye, could be attractive for the creation of a highly active photosensitizer and as a platform for the synthesis of various nanoscale switch-off systems [107].

Table 3. Parameters used for chlorin-based photosensitizers immobilized on fullerenes as a photosensitizer against cancer.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Carbon Material</th>
<th>Concentration</th>
<th>Irradiation</th>
<th>Incubation Time</th>
<th>Cancer Cell Lines</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fullerene (C70)-chlorin e6</td>
<td>0.05–0.2 mg/mL</td>
<td>20 mW/cm² at 660 nm (10 min)</td>
<td>3 h</td>
<td>A549 cells</td>
<td>~10% (cell viability)</td>
<td>[106]</td>
</tr>
<tr>
<td>2</td>
<td>Fullerene–chlorin e6</td>
<td>10 mM</td>
<td>23 mW/cm² at 630 nm (30 min)</td>
<td>24 h</td>
<td>HeLa cancer cells</td>
<td>IC₅₀ = 1.17 µM</td>
<td>[107]</td>
</tr>
</tbody>
</table>

Figure 10. In vitro relative viabilities of A549 cells incubated with nanomaterial based on a tri-malonate derivative of fullerene (C70), with chlorin e6 as a photosensitizer and 1,10-diamino-4,7-dioxadecane (OEG2) as a linker at different concentrations, with 660 nm (20 mW/cm² for 10 min) laser irradiation (red) or in the dark (black).
5.3. Carbon Nanotubes

In order to describe the efficiency of chlorin-based photosensitizers immobilized on carbon nanotubes for photodynamic and photothermal therapy against cancer, different parameters have been reported (2010–2019), such as descriptions of the carbon material, concentration, light dose, incubation time, and type of cancer cell lines, where the results obtained are presented in Table 4.

Table 4. Parameters used for chlorin-based photosensitizers immobilized on carbon nanotubes as a photosensitizer against cancer.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Carbon Material</th>
<th>Concentration</th>
<th>Irradiation</th>
<th>Incubation Time</th>
<th>Cancer Cell Lines</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Single-wall carbon nanotubes–chlorin e6–chitosan</td>
<td>5–100 µg/mL</td>
<td>20 J/cm²</td>
<td>24 h</td>
<td>HeLa cells</td>
<td>~10% cell viability at 30 µg/mL</td>
<td>[108]</td>
</tr>
<tr>
<td>2</td>
<td>Multi-walled carbon nanotubes–mTHPC</td>
<td>8–20 µg/mL</td>
<td>125 mW/cm² at 650 nm (300 s) or 2.3 W/cm² at 808 nm (200 s)</td>
<td>3 h</td>
<td>Human ovarian carcinoma SKOV-3 cells</td>
<td>~10% (cell viability)</td>
<td>[109]</td>
</tr>
<tr>
<td>3</td>
<td>Chlorin e6 with albumin–single-walled carbon nanotube</td>
<td>1–50 mg/mL</td>
<td>0.15 W/cm² at 630 nm (1 min) and 1 W/cm² at 808 nm (2 min)</td>
<td>12 h</td>
<td>Mouse squamous carcinoma cell line SCC-7</td>
<td>10% cell viability</td>
<td>[110]</td>
</tr>
<tr>
<td>4</td>
<td>MnO₂-coated carbon nanotubes with chlorin e6</td>
<td>0.5–1 µg/mL</td>
<td>1.0 W/cm² at 660 nm (5 min)</td>
<td>24 h</td>
<td>HeLa cells</td>
<td>I₅₀ of 0.58 mg/mL</td>
<td>[111]</td>
</tr>
</tbody>
</table>

In 2011, Xiao and co-authors [108] reported the functionalization of single-wall carbon nanotubes with chlorin e6 and chitosan (Table 4, entry 1). This functionalized carbon nanomaterial showed a good efficiency (~10% cell viability at 30 µg/mL) against HeLa cells when a light dose of 20 J/cm² and 24 h of incubation were used.

In 2016, in order to investigate the fundamental mechanisms of cell death and intracellular signaling cascades activated by PTT, PDT, and a combined therapy, Marangon and co-authors [109] prepared a dual system using multi-walled carbon nanotubes loaded via π–π stacking with the approved photosensitizer mTHPC (or Foscan®) (Table 4, entry 2). For the PDT and/or PTT procedures, the authors used the following parameters: mTHPC-immobilized on carbon nanotubes at 0–20 µg/mL and at 650 nm (125 mW/cm²) for 300 s for PDT and/or at 808 nm (2.3 W/cm²) for 200 s for PTT. The authors found that the dual system prepared through multi-walled carbon nanotubes loaded via π–π stacking with the mTHPC made the photosensitizer more photostable outside the cells, reduced non-specific diffusion of the non-functionalized photosensitizer, promoted cellular uptake, and promoted photothermal activation using the 808 nm irradiation [109]. This paper described and discussed the molecular mechanisms of PDT, PTT, and their combination by using flow cytometry, proteomics, and genomic analysis for the first time [109]. Overall, different mechanisms of cell death were evidenced depending on the photosensitizer sub-localization and irradiation conditions (light dose), culminating synergistically to the apoptosis. Moreover, the authors studied the intracellular signaling cascades that were activated by PTT and PDT using cytometry, proteomics, and genomics. They demonstrated that single PDT, single PTT, and the combined treatment (PTT+PDT) elicited a programmed cell death that was instigated by oxidative stress [109].

In 2016, Xie and co-authors [110] described the combination of chlorin e6 with albumin, followed by its loading onto the surface of Evans blue-modified single-walled carbon nanotubes by exploiting the high affinity between albumin and Evans blue (Table 4, entry 3). For the in vitro PDT/PTT evaluation, cell viability of over 10% (mouse squamous carcinoma cell line SCC-7) was observed when PTT and PDT were combined when 630 nm (0.15 W/cm² for 1 min) and 808 nm (1 W/cm² for 2 min) was used. Moreover, for in vivo studies, at the same PDT/PDT parameters, tumors were virtually eradicated. Overall, the authors concluded that the use of combined PTT and PDT therapy can significantly improve treatment outcomes without recurrence, and Evans blue functionalization on carbon nanotubes can be easily extended to other nanomaterials [110].
In 2018, Yin and co-authors [111] developed a smart nanoplatform for cancer diagnosis and treatment via the functionalization of MnO$_2$-coated carbon nanotubes with chlorin e6 (CMC) (Table 4, entry 4). The photothermal properties of the prepared nanoplatform were studied using a laser at 808 nm, where the temperature reached more than 50 °C, which is enough to combat cancer cells. For in vitro experiments, the efficiency of MnO$_2$-coated carbon nanotubes with chlorin e6 was evaluated in HeLa cells using 24 h of incubation and irradiation at 633 nm, presenting an IC$_{50}$ of 0.58 mg/mL. Furthermore, the in vivo synergistic cancer therapy was evaluated using nude mice (with xenograft HeLa cell line tumors), at various concentrations (0–1 µg/mL), irradiated with a laser for 10 min (1.0 W/cm$^2$) at 24 h post-injection, and tumor volumes were determined every 2 days. For the CMC-treated group, the authors found that the tumors were eliminated (decrease of up to 100% of tumor volume). According to the authors, by combining photothermal and fluorescence imaging properties, the prepared nanoplatform presents great potential for dual imaging-guided synergistic PDT/PTT [111].

5.4. Graphene

In order to describe the efficiency of chlorin-based photosensitizers immobilized on graphene for photodynamic and photothermal therapy against cancer, different parameters have been reported (2010–2019), such as descriptions of the carbon material, concentration, light dose, incubation time, and type of cancer cell lines, where the results obtained are presented in Table 5.

In 2011, Tian and co-authors described the preparation of chlorin e6 loaded onto polyethylene glycol (PEG)-functionalized graphene oxide via supramolecular π-π stacking [93]. This nanomaterial was applied in a human nasopharyngeal epidermal carcinoma KB cell line by using the following parameters: concentration of photosensitizer (0.00138–0.011 mg/mL), 0.1 W/cm$^2$ (10 min) of irradiation, and 24 h of incubation. The authors obtained a cell viability of 10% at 0.011 mg/mL (Table 5, entry 1).

Furthermore, also in 2011, Huang and co-authors [112] described the functionalization of folic-acid-conjugated graphene oxide with chlorin e6 (Table 5, entry 2). This nanomaterial was evaluated in cellosaurus (MGC803) cells using a range of concentrations (0–100 µM), irradiation of 30 mW/cm$^2$ for 10 min, and 48 h of incubation, which resulted in a cell viability of ~10%.

Huang and co-authors [113] described the use of graphene oxide coated with polyvinylpyrrolidone and linked with a targeting peptide (Table 5, entry 3). Then, this nanomaterial was functionalized with chlorin e6 via the hydrophobic interactions of π-π stacking. The in vitro PDT evaluation of this nanomaterial (0–50 µM) was performed using human gastric mucinous adenocarcinoma (MGC803) cells, laser irradiation at 671 nm (30 mW/cm$^2$) for 3 min, and an incubation time of 24 h. As a result, the authors observed complete cell killing after illumination when the synthesized nanosystem was used. Moreover, this nanosystem was able to increase the accumulation of chlorin e6 in tumors when compared to non-functionalized chlorin e6 [113].

In 2015, Liu and co-authors [114] described a simple sonication method (liquid-phase exfoliation) for the functionalization of graphene with chlorin e6 (Table 5, entry 4). For the in vitro PDT experiments, the authors used HeLa cells and evaluated the free chlorin e6 and chlorin-e6-functionalized graphene in different concentrations (0–0.20 µg/mL), with an incubation time of 24 h and irradiation at 660 nm (power density of 0.14 W/cm$^2$) for 2 min. The authors found that free chlorin e6 exhibited negligible photocytotoxicity to HeLa cells when used in 0.20 µg/mL, while significant photocytotoxicity was observed in the presence of chlorin-e6-functionalized graphene at a Ce6 concentration of more than 0.050 µg/mL. In fact, this work described a simple method for graphene functionalization and functionalized graphene was presented as a promising nanomaterial for in vitro PDT against HeLa cells [114].

Furthermore, also in 2015, Zeng and co-authors [115] described the synthesis of polyethylene-glycol-functionalized graphene oxide followed by functionalization with branched polyethyleneimine (Table 5, entry 5). Then, this material was loaded with chlorin e6 via the hydrophobic interactions of π-π stacking. The authors found that this chlorin-e6-functionalized graphene showed a high PDT efficiency.
(up to 10% of viability at 2.0 µM) using HeLa cells, irradiation at 662 nm (0.2 W/cm²) for 5 min, and was also an excellent lysosome-targeting material [115].

Table 5. Parameters used for chlorin-based photosensitizers immobilized on graphene as a photosensitizer against cancer.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Carbon Material</th>
<th>Concentration</th>
<th>Irradiation</th>
<th>Incubation Time</th>
<th>Cancer Cell Lines</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Graphene oxide-polyethylene glycol-chlorin e6</td>
<td>0.00138–0.011 mg/mL</td>
<td>0.1 W/cm² (10 min)</td>
<td>24 h</td>
<td>Human nasopharyngeal epidermal carcinoma KB cell line</td>
<td>~10% cell viability</td>
<td>[93]</td>
</tr>
<tr>
<td>2</td>
<td>Folic-acid-conjugated graphene oxide-chlorin e6</td>
<td>0–100 µM</td>
<td>~30 mW/cm² (10 min)</td>
<td>48 h</td>
<td>MGC803 cells</td>
<td>~10% cell viability</td>
<td>[112]</td>
</tr>
<tr>
<td>3</td>
<td>Graphene oxide-polyvinylpyrrolidone-chlorin e6</td>
<td>0–500 µM</td>
<td>30 mW/cm² (3 min)</td>
<td>24 h</td>
<td>MGC803 cells</td>
<td>Complete cell killing</td>
<td>[113]</td>
</tr>
<tr>
<td>4</td>
<td>Graphene-chlorin e6</td>
<td>0–0.20 µg/mL</td>
<td>0.14 W/cm² (2 min)</td>
<td>24 h</td>
<td>HeLa cells</td>
<td>Up to 100% cell killing</td>
<td>[114]</td>
</tr>
<tr>
<td>5</td>
<td>Graphene oxide-polyethylene glycol-chlorin e6</td>
<td>0.25–2 µM</td>
<td>0.2 W/cm² (5 min)</td>
<td>24 h</td>
<td>HeLa cells</td>
<td>10% cell viability</td>
<td>[115]</td>
</tr>
<tr>
<td>6</td>
<td>Folic-acid-conjugated polyethylenimine-PEGylated graphene-chlorin e6</td>
<td>0.5–10 µg/mL</td>
<td>200 W/cm² (5 min)</td>
<td>24 h</td>
<td>HeLa cells</td>
<td>~15% cell viability</td>
<td>[116]</td>
</tr>
<tr>
<td>7</td>
<td>PEGylated nanographene-chlorin e6</td>
<td>0–2.0 µM</td>
<td>0.1 W/cm² (10 min)</td>
<td>24 h</td>
<td>4T1 cells</td>
<td>Complete cell killing</td>
<td>[117]</td>
</tr>
<tr>
<td>8</td>
<td>Graphene oxide-polyethylene glycol-chlorin e6</td>
<td>50 µmol/L</td>
<td>1.5 W at 808 nm</td>
<td>1 h</td>
<td>U87 cells</td>
<td>10% cell viability</td>
<td>[118]</td>
</tr>
<tr>
<td>9</td>
<td>Chlorin e6-PEG-conjugated graphene oxide</td>
<td>0–2.5 µg/mL</td>
<td>2.0 J/cm²</td>
<td>24 h</td>
<td>CCA cells</td>
<td>10% cell viability</td>
<td>[119]</td>
</tr>
<tr>
<td>10</td>
<td>Up-conversion nanoparticles-graphene oxide-chlorin e6</td>
<td>25–800 µg/mL</td>
<td>0.72 W/cm² (10 min)</td>
<td>24 h</td>
<td>HeLa cells</td>
<td>15% cell viability</td>
<td>[86]</td>
</tr>
<tr>
<td>11</td>
<td>Graphene oxide-chlorin e6</td>
<td>1.0 µM</td>
<td>~2 W/cm² (15 min)</td>
<td>3–24 h</td>
<td>A549 cells</td>
<td>IC₅₀ = 0.69 at 3 h</td>
<td>[120]</td>
</tr>
</tbody>
</table>

In 2016, Zeng and co-authors [116] described the preparation of folic-acid-conjugated polyethylenimine-modified PEGylated nanographene for the targeted delivery of chlorin e6 (Table 5, entry 6). The authors evaluated this nanocomposite against (HeLa cells) using a concentration of 0.5–10 µg/mL, irradiation of 200 W/cm² for 5 min, and 24 h of incubation. According to the authors, a cell viability of ~15% was obtained at 1.0 µM.

In 2016, Cao and co-authors [117] reported the synthesis of nanographene that was chlorin-e6-loaded and PEGylated, characterized using UV-vis, and applied in PDT, PTT, or a PDT/PTT combination (Table 5, entry 7). Moreover, a dual-modal MRI approach was applied for the monitoring and prognosis of phototherapies. For in vitro experiments, the authors evaluated three different groups against 4T1 cells: (i) PDT group—chlorin-e6-loaded onto PEGylated nanographene (0–2.0 µM), incubation time of 24 h, and irradiation at 660 nm (0.1 W/cm² for 10 min); (ii) PTT group—chlorin-e6-loaded onto PEGylated graphene (0–2.0 µM), incubation time of 24 h, and irradiation at 808 nm (1 W/cm² for 3 min); (iii) combined PDT and PTT group—chlorin-e6-loaded onto PEGylated graphene (0–2.0 µM), incubation time of 24 h, and irradiation at 660 and 808 nm (0.1 W/cm² for 10 min) and (1 W/cm² for 3 min). To evaluate this nanomaterial in the in vivo experiments, chlorin-e6-loaded and PEGylated nanographene (2 mg/mL per kg b.w.) was injected intravenously, followed by irradiation of the tumors (PTT group: at 808 nm, 1.5–2.5 W/cm²; PDT group: at 660 nm, 0.2 W/cm²; combination PDT + PTT: for 10 min at 808 nm followed by 10 min at 660 nm). Overall, for the in vitro and in vivo studies, the PDT/PTT group exhibited the highest cell death at all concentrations [117].
In 2017, Shim and co-authors [118] described the linking of 30-amino-acid claudin 4-binding peptide and Clostridium perfringens enterotoxin to chlorin e6 using poly(ethylene glycol) as a spacer and anchored to graphene oxide nanosheets (Table 5, entry 8). Regarding its application in PDT, the authors evaluated the effects on U87 cells, at a concentration of 50 µmol/L, an incubation time of 1 h, and using a laser at 808 nm (1.5 W). The authors observed a synergistic anticancer effect when the functionalized graphene oxide nanosheets were used to treat cells upon irradiation at two wavelengths (660 nm and 808 nm). Singlet oxygen production was observed to reach the highest value after 20 min of exposure at 660 nm (PDT procedure). The authors concluded that dual therapy (PTT and PDT) should be optimized to provide the highest therapeutic effect but it is a promising tool against different kinds of cell lines.

In 2018, Kim and collaborators described the synthesis of graphene oxide conjugated with chlorin e6 and methoxy poly(ethylene glycol) [119] (Table 5, entry 9). This nanomaterial was evaluated in a cellosaurus cell line using different concentrations (0–2.5 µg/mL), a light dose of 2.0 J/cm², and 24 h of incubation, resulting in a cell viability of 10%. Moreover, the authors observed that the chlorin e6 release from graphene oxide was faster in the presence of glutathione, indicating that graphene oxide displays redox responsiveness.

Furthermore, also in 2018, Gulzar and co-authors [86] reported the development of core–shell-structured upconversion nanoparticles with graphene oxide, which was then loaded with chlorin e6; their evaluation in HeLa cells used concentrations of 25–800 µg/mL, irradiation of 0.72 W/cm² (at 808 nm) for 10 min, and 24 h of incubation (Table 5, entry 10). The authors obtained a cell viability of ~15% at 800 µg/mL of the prepared nanomaterial.

In 2019, Kang and co-authors [120] promoted the functionalization of graphene oxide with a derivative purpurin-18-N-ethylamine chlorin e6 via non-covalent and covalent methodologies (Table 5, entry 11). The authors evaluated the PDT efficiency of functionalized graphene oxide (non-covalent and covalent) by using the following parameters: irradiation of 2 W/cm² for 15 min and an incubation time of 3–24 h against human lung adenocarcinoma (A549) cells. The functionalized graphene oxide (non-covalent and covalent) showed IC₅₀ values at 3, 12, and 24 h incubation of 0.69 and 0.22, 0.48 and 0.21, and 0.31 and 0.20 µM, respectively. Nevertheless, the non-covalently bound complex presented lower dark toxicity than the covalently bound complex [120]. According to the authors, the lower IC₅₀ values of the non-covalently bound complex may have been because of its faster delivery effect based on the rapid intracellular release of the derivative purpurin-18-N-ethylamine chlorin e6 from the graphene oxide complex [120].

6. Conclusions and Future Perspectives

Over the years, conventional therapies have failed to effectively treat cancer; as a direct consequence, the condition has become one of the major causes of mortality worldwide. New technological approaches and strategic targeting to achieve better management of the condition, as well as early and proper diagnosis, therapy, and limited side-effects, are all required. Certain treatments are effective at killing cancer but also causing damage to healthy, neighboring tissues.

PDT procedures are able to combine a high efficacy against cancer cells without any serious damage to patients. Furthermore, its handling should be as easy as possible, using a low molar concentration of photosensitizer, and a low light dose. In this review paper, we emphasized the use of chlorin photosensitizers, which is a type of bioinspired tetrapyrrolic macrocycle. In the 1980s, chlorins were introduced as photosensitizers in PDT since they showed good accumulation in tumors, absorption at longer wavelengths (NIR), and fast clearance from the body. Nowadays, a family of synthetic and natural chlorin photosensitizers has been applied in PDT against cancer and other diseases.

In this regard, the combination of chlorin-based photosensitizers (especially chlorin e6) with carbon nanomaterials (carbon dots, carbon nanotubes, fullerene, and graphene) has been demonstrated as an effective method for diagnosis, as nano delivery systems, and against tumors due to PDT and PTT actions. Moreover, the use of a photothermal approach can overcome the obstacle of oxygen dependence.
that accompanies by PDT. We can emphasize that the effect of PDT and PTT are dependent on the type of carbon material, different preparation methods, morphologies, and modification methods.

Besides the high efficiency of chlorin-functionalized carbon nanomaterials in PDT/PTT applications, some challenges need to be overcoming with future studies: (i) the toxicity of carbon nanomaterials needs to be systematically evaluated concerning their size, functionalization, purity, and purification methods; (ii) the biodistribution, pharmacokinetics, and clearance from the body need to be studied and compared using different carbon nanomaterials and chlorins; (iii) the influence of the functionalization of carbon nanomaterials surfaces on PDT/PTT efficiency and toxicity needs to be evaluated and compared; (iv) the development of simple synthetic methods for the functionalization of carbon nanomaterials with photosensitizers (combined therapy) is required. In this review paper, we have demonstrated that the application of combined therapy (photothermal and photodynamic) produces a synergic effect for combating cancer cells (in vitro and in vivo studies).


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**References**

32. Liu, X.D.; Yang, W.X.; Guan, Z.Z.; Yu, W.F.; Fan, B.; Xu, N.Z.; Liao, D.J. There are only four basic modes of cell death, although there are many ad-hoc variants adapted to different situations. Cell Biosci. 2018, 8, 12. [CrossRef] [PubMed]


34. Liu, T.; Wang, C.; Cui, W.; Gong, H.; Liang, C.; Shi, X.Z.; Li, Z.W.; Sun, B.Q.; Liu, Z. Combined photothermal and photodynamic therapy delivered by PEGylated MoS2 nanosheets. Nanoscale 2014, 6, 11219–11225. [CrossRef]


52. Diogo, P.; Mota, M.; Fernandes, C.; Sequeira, D.; Palma, P.; Caramelo, F.; Neves, M.; Faustino, M.A.F.; Goncalves, T.; Santos, J.M. Is the chlorophyll derivative Zn(II)e(6)Me a good photosensitizer to be used in root canal disinfection? *Photodiagnosis Photodyn. Ther.* 2018, 22, 205–211. [CrossRef] [PubMed]


67. Silva, G.A. Nanotechnology approaches to crossing the blood-brain barrier and drug delivery to the CNS. *BMC Neurosci.* 2008, 9, 4. [CrossRef]

68. Rauti, R.; Musto, M.; Bosi, S.; Prato, M.; Ballerini, L. Properties and behavior of carbon nanomaterials when interfacing neuronal cells: How far have we come? *Carbon 2019*, 143, 430–446. [CrossRef]


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Cationic Nanostructures for Vaccines Design

Ana Maria Carmona-Ribeiro * and Yunys Pérez-Betancourt

Biocolloids Laboratory, Instituto de Química, Universidade de São Paulo, São Paulo 05508-000, SP, Brazil; y.betancourt@usp.br
* Correspondence: amcr@usp.br

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Abstract: Subunit vaccines rely on adjuvants carrying one or a few molecular antigens from the pathogen in order to guarantee an improved immune response. However, to be effective, the vaccine formulation usually consists of several components: an antigen carrier, the antigen, a stimulator of cellular immunity such as a Toll-like Receptors (TLRs) ligand, and a stimulator of humoral response such as an inflammasome activator. Most antigens are negatively charged and combine well with oppositely charged adjuvants. This explains the paramount importance of studying a variety of cationic supramolecular assemblies aiming at the optimal activity in vivo associated with adjuvant simplicity, positive charge, nanometric size, and colloidal stability. In this review, we discuss the use of several antigen/adjuvant cationic combinations. The discussion involves antigen assembled to (1) cationic lipids, (2) cationic polymers, (3) cationic lipid/polymer nanostructures, and (4) cationic polymer/biocompatible polymer nanostructures. Some of these cationic assemblies revealed good yet poorly explored perspectives as general adjuvants for vaccine design.

Keywords: cationic nanoparticles; dioctadecyldimethylammonium bromide; poly (acrylates); biomimetic lipid/polymer nanoparticles; cationic polymer/biocompatible polymer assemblies; cationic adjuvants; cationic lipids; polycation

1. Introduction

Purified antigens in subunit vaccines usually lack the danger signals of full pathogens, resulting in poor immunogenicity [1]. Adjuvants then become essential components of modern vaccines, enhancing and guiding the immune response against each specific pathogen [2–5]. The only adjuvants licensed for human use worldwide are the aluminum-based salts like Al(OH)3. Their water dispersions consist of polydisperse and large aggregated particles poorly dispersed in water that are positively charged at the pH of water and can combine with negatively charged antigens such as peptides, proteins, nucleic acids, and RNA [6–8]. Other cationic adjuvants based on nanoparticles [9–12], liposomes [13–15], cationic bilayer fragments [9,16,17], supported cationic bilayers on polymeric nanoparticles (NPs) [10], silica [18], or cationic polymers on superparamagnetic iron oxide NPs have also been proposed as effective micro- or nanomaterials able to effectively interact with antigens and antigen-presenting cells (APC) [19].

The uptake of antigen/cationic assemblies depends on size [20,21]. Virus-like NPs (20–200 nm mean diameter) are taken up by endocytosis via clathrin-coated vesicles, caveolae, or their independent receptors and are preferentially ingested by dendritic cells (DC) [22]. Bacteria-like microparticles (500–5000 nm diameter) undergo phagocytosis and primary ingestion by macrophages. Vaccines administered as particles in dispersion are internalized efficiently by APC either by endocytosis or phagocytosis or a combination of both mechanisms [23,24]. Particles with diameters below 500 nm, in particular NPs (40–100 nm diameter), are more efficient to promote CD8 and CD4 type 1 T-helper cell responses than the microparticles (diameters above 500 nm). Similarly,
to Al(OH)$_3$, large particles usually induce good antibody responses from T-helper cells type 2 [23]. Cationic micro- and nanoparticles are effectively taken up both by macrophages and dendritic cells. After electrostatics promotes the binding of cationic particles and assemblies to APCs, subsequent internalization takes place [12,14,21,25]. Antigens of Mycobacterium tuberculosis [15,26], Chlamydia trachomatis [12], Neisseria meningitides [17,27], Taenia crassiceps [9,10], and Mycobacterium leprae [13] carried by cationic particles, liposomes, or bilayer fragments containing dioctadecyldimethylammonium bromide (DODAB) cationic lipid enhanced the cellular and humoral antigen-specific immune response [15,16,28]. Excellent reviews are available on the use of a variety of cationic lipids such as dimethylaminoethane–carbamoyl–cholesterol (DC-Chol) and derivatives [29], 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [30], DODAB [9,16,31], and others to present antigens [32].

In response to pathogens, the innate immune system recognizes the pathogen-associated molecular patterns (PAMPs) by means of the pattern recognition receptors (PRRs) on the surface and in endosomes of APCs [1,33]. As a second line of defense, the adaptive immune system developed by vertebrates consists in memory T and B cells that employ new synthesized antigen-specific receptors able to recognize pathogen-specific antigens when presented by major histocompatibility complexes (MHC) on the surface of an APC. The balance between activity of T or B cells relies on signals provided by the APC (such as co-stimulatory molecules and precise cytokines) in response to the priming by PAMPs [1]. In summary, the co-delivery of antigen and adjuvant to APCs in subunit vaccines results in up-regulation of co-stimulatory molecules essential for adequate T and B cells stimulation [1]. In order to formulate vaccines, a fundamental comprehension of innate and adaptive immune responses is required: the first PAMPS recognition is made via several receptors (innate immunity) leading to the responses able to activate and differentiate T helper cells with possible B cell (antibody-mediated) and CD8 T cell-mediated adaptive immune responses [33].

2. Assemblies from Cationic Lipids and Surfactants

Mimicking nature is a powerful approach for developing novel lipid-based devices for drug and vaccine delivery. Cationic biomimetic particles offered a suitable interfacial environment for adsorption, presentation, and targeting of antigens in vivo. Thereby, antigens can effectively be presented by tailored biomimetic particles for development of vaccines over a range of defined and controllable particle sizes [34]. Lipid supramolecular association with particles has been systematically studied on latex, silica, or drug particles over a range of experimental conditions in order to achieve optimal bilayer deposition onto each particle. The difficult step of vesicle disruption, especially for bilayers in the rigid gel state, was circumvented by using previously disrupted charged vesicles, namely charged bilayer fragments or disks (BF). BF, under appropriate conditions of the intervening medium, coalesced around particles for presentation of antigens to the immunological system [35].

Antigen loading in the vaccine can be driven in cationic assemblies by electrostatic attraction between the antigen and oppositely charged moieties of the adjuvant. For example, cationic DODAB bilayers in water dispersions are available as closed microstructures such as vesicles or open, nano-sized bilayer fragments (BF) obtained by ultrasonic disruption from closed vesicles [28,36–39]. These micro- or nano-structures efficiently combine with serum proteins [40], recombinant heat-shock proteins from micobacteria [13], purified extracts from parasites such as Taenia crassiceps [9,10], ovalbumin (OVA) model antigen [16], genetic material such as DNA [41–43], mono- or oligonucleotides such as CpG [44–46], and other oppositely charged biomolecules, drugs, nanoparticles, surfaces, or biological cells [28,41]. Several good reviews appeared on the use of a variety of cationic lipids and surfactants to formulate vaccines [21,32,47–49]. Examples of monocationic lipids are N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium trimethyl chloride (DOTMA), dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), dimethyl dioctadecylammonium bromide (DDAB or DODAB), dimethylaminoethane carbamoyl cholesterol (DC-Chol), 1,2-distearoyl-3-trimethylammonium-propane (chloride salt) (DSTAP), and
dimyristoyl-3-trimethylammonium-propane (chloride salt) (DMTAP). One example of a polycationic sphingolipid is \( N \)-palmitoyl \( d \)-erythro-sphingosyl-1-0-carbamoyl-spermine triacetate salt (CCS). Figure 1 shows the chemical structures of DOTMA, DOTAP, DODAB, CCS and DC-Chol.

**Figure 1.** Chemical structure of cationic lipids or surfactants used to formulate vaccines. They bear the cationic charges of primary or secondary amino groups or quaternary ammonium nitrogens in their structures which combine with oppositely charged antigens. CCS is \( N \)-palmitoyl \( d \)-erythro-sphingosyl-1-0-carbamoyl-spermine triacetate salt. The CCS chemical structure was reprinted from [50] with permission from Elsevier, Copyright 2006.
In particular, CCS is a polycationic surfactant with one primary and two secondary amine groups self-assembling as micelles in aqueous phase; CCS required “helper” lipids such as cholesterol to form liposomes before being combined with the hemoaglutinin/neuraminidase antigens of influenza virus to elicit both Th1 and Th2 responses in mice immunized via the nasal route [50,51].

Among the synthetic lipids, DODAB is possibly the less expensive and the most studied synthetic lipid from several points of view such as its self-assembly as closed or open bilayers in aqueous solutions, its physico-chemical properties in aqueous solutions, its ability to interact with several oppositely charged molecules, nanostructures, nanoparticles, surfaces, and cells with a controllable cytotoxicity against mammalian cells lines; its combinations with a large variety of antigens were able to induce a Th1 response but did not improve the Th2 humoral response [9,13,16,28,37,52]. Among the other cationic lipids, only those with more fluid bilayers due to their double bonds in the hydrocarbon chains or to short hydrocarbon chains such as DOTAP or DMTAP, respectively, were able to elicit both Th1 and Th2 responses combined with antigens [50]. The sphingolipid CCS has a large polycationic polar head and inverted-cone molecular shape self-assembling as micelles. Bilayers were formed combining CCS with other neutral lipids such as dioleoyl phosphatidylethanolamine (DOPE) or cholesterol (Chol), at a CCS/helper lipid mole ratio of 1/1 to 4/1; these cationic assemblies were explored by Barenholz and coworkers to deliver influenza virus antigens by the intranasal route [50,51].

The intranasal (i.n.) is an advantageous mucosal route that allows rapid administration for large populations in the case of pandemics. A good example is the i.n. influenza vaccine, based on CCS combining carrier and adjuvant activities, which elicits, in mice, strong systemic (serum) and local (lung and nasal) humoral and cellular immunity. Unsized liposomes of DC-Chol, DODAB, and DSTAP resulted in low serum and local responses, while two others (DMTAP- and DOTAP-based vaccines) induced both systemic and local vigorous Th1 and Th2 immune responses [50]. However, only the vaccine formulated with CCS was equivalent or superior to the commercial vaccine co-administered with cholera toxin as an adjuvant [50]. Innovative mucosal vaccines against influenza [53] or other diseases were recently comprehensively reviewed [54].

Figure 2 illustrates the possible events for mucosal immunization driven by an antigen/adjuvant nano-assembly as reprinted with permission from Bernocchi et al. 2017, reference [54]. The possible routes for an antigen/adjuvant nano-assembly are depicted from Figure 2A–E where there is direct capture by dendritic cell in Figure 2A, antigen diffusion through the cell junctions in Figure 2B, M cells performing the sampling of the antigen/carryer assemblies and directing them to appropriate cells in their M cell pocket in Figure 2C, epithelial cells performing endocytosis of the NP/antigen for further deliverance to local dendritic cells (DC) and T cells which are able to boost the immune response in Figure 2D. At last in Figure 2E, once in endosomes, NP could release antigens and be exocytosed as free, unloaded, NP (E1) and/or induce the endosomal escape of the antigens (E2) that would be processed as an endogenous antigen and presented by MHC-I (E3). In the other way, also on Figure 2E, NP could be degraded in endo/lysosomes (E4), and the released antigen be processed as exogenous and presented by MHC-II (E5). This could lead to DC and CD8+ T cell activation and/or priming. The activated DC from these pathways then migrate to germinal centers or directly to lymph nodes to activate CD4+ T cells that in turn activate B cells. They undergo an IgA+ phenotype switch, migrate by the blood flow to the effector sites and produce secreted IgA (sIgA) as IgA+ plasma cells.

Once NP/Ag undergo endocytosis by APC, the epitopes of processed antigens can be presented as complexes with either major histocompatibility complex I (MHC-I) or major histocompatibility complex II (MHC-II) [55,56]. In the case of cationic lipids, the versatile DODAB can be dispersed as nanosized, cationic bilayer fragments (BF) able to combine with oppositely charged antigens in general, driving the immune response to a cell-mediated one (Th1) [9,16]. In a certain sense, the DODAB BF are discoidal and open nanostructures instead of closed, vesicular bilayers or liposomes, and antigen is expected to become adsorbed from the electrostatic attraction all around the disk-like bilayer so that desorption and endosomal escape might allow MHC-I presentation after trafficking from the cytosol to the endoplasmic reticulum. This would agree with findings by Korsholm and coworkers [57]; they
studied the mechanism of adjuvanticity for DODAB/OVA liposomes and found that these liposomes did not affect the maturation of murine bone-marrow-derived dendritic cells (BM-DCs) related to the surface expression of MHC-II, CD40, CD80, and CD86 but enhanced the uptake of OVA by BM-DCs via endocytosis; intraperitoneal injection of DODAB/OVA liposomes also enhanced the uptake of the antigen by peritoneal exudate cells and targeted the antigen preferentially to antigen-presenting cells, leading to enhanced uptake and presentation of antigen [57].

![Diagram of mucosal immune induction in nasal and airways epithelia by antigen-loaded nanocarriers (NP/antigen).](image)

**Figure 2.** Mucosal immune induction in nasal and airways epithelia by antigen-loaded nanocarriers (NP/antigen). (A) Dendritic cells (DC) protruding arms, the transepithelial dendrites, directly capture the NP/antigen. (B) NP/antigen can also diffuse through epithelial junctions and reach the underlying DC. (C) The M cells create a pocket enriched in immune cells (DC, macrophages-Mϕ and lymphocytes T) and perform the sampling of the luminal antigens so that the immune cells contact the NP/antigen. (D) The NP/antigen can also enter cells by endocytosis and deliver the antigens of the nanovaccine into the cells. The endocytosis of NP/antigen by the epithelial cells triggers the production of cytokines, defensines, and chemokines involved in local immune cells recruitment (DC and T cells) able to boost the immune response. (E) The endocytosis of NP/antigen can also be the first step of antigen presentation by epithelial cells. In endosomes, NP could release antigens and be exocytosed as free, unloaded, NP (E1) and/or induce the endosomal escape of the antigens (E2) that will be processed as an endogenous antigen and presented by major histocompatibility complex I (MHC-I) (E3). In the other way, NP could be degraded in endo/lysosomes (E4), and the released antigen will be processed as exogenous and presented by major histocompatibility complex II (MHC-II) (E5). This could lead to DC and CD8+ T cell activation and/or priming. The activated DC from these pathways then migrate to germinal centers or directly to lymph nodes to activate CD4+ T cells that in turn activate B cells. They undergo an IgA+ phenotype switch, migrate by the blood flow to the effector sites and produce secreted IgA (sIgA) as IgA+ plasma cells. Reprinted from [54] with permission from Elsevier, Copyright 2017.

Nano-sized formulations enter the cells by endocytosis following the endo-lysosomal pathway before the protein is delivered and degraded in the endosomes; the resulting peptides are complexed with MHC-II and presented on cell surface for activation of CD4+ T helper cells, stimulating cytokine secretion and humoral antibody responses (Th2). When the nanostructure promotes the protein escape from the endosomes to the cytosol, the protein may be degraded in the proteasome with the peptidic products of the degradation carried by transporters of antigen processing to the endoplasmic
reticulum where they combine with MHC-I. Cellular expression of peptide-associated MHC-I activates CD8+ T cells and cell-mediated immunity [58–61]. For effective control of tumors and pathogens by the immune system, neoplastic and infected cells must be targeted and destroyed by cytotoxic T lymphocytes (CTLs). While MHC-I conventionally present endogenous cytosolic antigens, the alternative pathway, termed cross-presentation, also allows the presentation of peptides derived from exogenous antigens by MHC-I [62]. As tumor antigens and pathogen-derived proteins are often not endogenously produced by antigen-presenting cells (APCs), this exogenous pathway is crucial for the generation of CD8+ CTL responses against these cell-associated antigens [63]. Enhancement of the targeting of exogenous antigens to the cross-presentation pathway may help develop effective vaccines against tumors, parasites, intracellular bacteria, and viruses. In summary, there are distinct intracellular routes for antigen uptake and presentation to attain CD4 and CD8 T cell activation and ideal antigen adjuvant systems should activate both of these pathways, thereby also inducing cross-presentation [58]. Since subunit vaccines are not effective in cytotoxic T cells activation, the association with adjuvants becomes crucial [64]. Interestingly, the antigen encapsulation in nanostructures (nanoparticles and bilayer nanodisks included) may direct the antigen presentation towards a different or combined immune response. This orientation can be affected by multiple factors, such as the mechanism of uptake, and is dependent upon the nanostructure physical properties such as the size, the outer surface charge, and also the inner particle charge. In our group, we observed that cationic nanodisks of DODAB BF complexed with the model antigen ovalbumin induced in vivo a large Th1 response and very low or absent humoral response [9,13,16], whereas NPs of PDDA/OVA, where the antigen was entangled with the cationic polymer PDDA, elicited potent Th2 humoral response in absence of the cell-mediated one [65]. Therefore, the ideal adjuvant should combine the ability of offering the antigen to be degraded inside the endosome with the ability to allow the antigen endosomal escape. Should we mix DODAB BF/antigen with PDDA/antigen to achieve the right balance between Th1 and Th2 responses?

The progress in gene or siRNA delivery to cells contributed substantially to the development of novel cationic lipids [66–68]. A particularly interesting class of cationic lipids is the lipopolyamines synthesized by Byk and coworkers in the 1990s aiming at DNA transfer to cells [69]. They were recently explored by Pizzuto and coworkers as single-component adjuvants able to elicit both Th1 and Th2 responses in absence of toxicity in vivo [70]. Figure 3 illustrates the ability of these polyamines to activate Toll-like receptor 2 (TLR2) and 4 (TLR4) besides inducing, in combination with OVA antigen, both IgG1 and IgG2a; OVA alone or Alum induced exclusively IgG1, and lipopolyamines induced both IgG1 and IgG2a antibodies production [70]. Figure 3A shows the chemical structure of the lipopolyamines. Figure 3B illustrates the uptake of lipopolyamines alone or complexed with ovalbumin by cultured human cell lines transfected with Toll-like Receptors (TLRs), leading to (1) secretion of inflammatory and type-I interferon cytokines able to trigger a Th1 response (cell-mediated immunity); (2) secretion of the interleukin-1beta (IL-1β) able to induce a Th2 response (humoral immune response). Figure 3C shows that the uptake of lipopolyamines/antigen complexes in vivo by intraperitoneal macrophages induced secretion of interleukin-5 (IL-5) and humoral immunity plus tumor necrosis factor-alpha (TNF-α) and gamma-interferon inducible protein (IP-10) [71], typical inducers of Th1 response (cell-mediated immune response) by the cultured macrophages.

On Figure 3 reproduced from Pizzuto and coworkers are shown two lipopolyamines with 12 or 18 carbon atoms in their alkyl chains; however, only the lipopolyamines with 12 or 14 carbon atoms in their alkyl chains activated TLR2- and TLR4-transfected cells, whereas the C18-lipopolyamine with very similar or identical polar head group activated only TLR2-transfected cells. The hypothesis cast to understand this was related to the fusogenic behavior of the lipopolyamines, since those with shorter lengths of the carbon chains (as those with C12 or C14) would be more fusogenic than those with long chains (as those with C18); thereby, the former would be taken up more easily by the cells. The possible toxicity in vivo of the lipopolyamine–OVA complexes was evaluated from determinations of liver enzymes alanine transaminase (ALT), aspartate transaminase (AST), and the inflammatory
cytokine tumor necrosis factor-alpha (TNF-α) in the serum plus histological examination of liver slices of the injected mice post-injection; no toxicity was detected, neither in serum nor on liver slices. The TLR stimulation and secretion of pro-inflammatory and interleukin-1beta (IL-1β) cytokines suggested that the C12 or C14-polyamines would be promising one-component vaccine adjuvants eliciting both humoral and cell-mediated responses [70].

Aluminum adjuvants typically activate the inflammasome pathway and Th 2 response [72] so that alum combinations with TLR agonists are needed to induce the cell-mediated Th 1 response against pathogens [5]. Pizzuto et al. also demonstrated that lipopolyamines induced IP-10, IL-6, and IL-1β secretion in murine macrophages and TNF-α in murine and human macrophages. TNF-α and
IL-6 are pro-inflammatory cytokines typical of the NF-κB induction. IP-10 is instead the signature of Type I IFN antiviral and T cell-stimulating response and is typical of the IRF induction. Finally, IL-1β secretion demonstrates the concomitant activation of the NF-κB pathway, which expresses pro-IL-1β, and of the inflammasome pathway that cleaves pro-IL-1β. The activation of both TLR and inflammasome pathways combined with the carrier properties makes cationic lipid lipopolyamines excellent candidates as one-component vaccine adjuvants [70].

The mycobacterial cord factor trehalose-6,6′-dimycolate (TDM) present in the cell wall of mycobacteria and its synthetic adjuvant analog trehalose-6,6′-dibehenate (TDB) are glycolipids that trigger innate immunity. Bone-marrow-derived dendritic cells (BMDCs) stimulated with TDB induced Nlrp3 inflammasome-dependent IL-1β secretion; in vivo, in Nlrp3-deficient mice, recruitment of neutrophils by TDB was reduced, showing the essential role of the Nlrp3 inflammasome for the induction of an innate humoral immune response triggered by TDB [73].

In murine models of Mycobacterium tuberculosis (Mtb) infection, TDM administration drove the early pro-inflammatory M1-like macrophage response related to the granulomas of primary pathology; proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-12p40 were produced in lung tissue [74]. Furthermore, CD11b+CD45+ macrophages with a high surface expression of the pro-inflammatory CD38 and CD86 markers were found in lung lesions of mice at 7 days post-TDM introduction, but low phenotypic marker expression of anti-inflammatory M2-like markers CD206 and EGR-2 were present on macrophages. TDM played a role in establishment of the M1-like shift in the microenvironment during primary tuberculosis. Thus, the MTB cell wall cording factor TDM is a physiologically relevant and useful molecule for modeling early macrophage-mediated events during establishment of the tuberculosis-induced granuloma pathogenesis [74].

In order to improve fusion of cell membranes with DODAB bilayers, which are in the rigid gel state at room temperature [75], DODAB bilayer fluidity had to be increased by using DODAB combinations with other lipids and surfactants such as the glycolipid TDB [15,76], the surfactant monoolein (1-monooleoyl-rac-glycerol) [77], the C24:1 β-glucosyleramide [78] or the glycolipid de-O-acylated lipoooligosaccharide (dLOS) as a booster vaccine against tuberculosis [79]. In particular, intranasal immunization with DODAB/TDB combined with influenza antigen A (H3N2) induced superior humoral and cell-mediated responses; there was an effective facilitation of uptake by DC, DC maturation in vitro, increased mucosal IgA production, increased IgG, IgG1, and IgG2b antibody titers in comparison with other formulations using cationic lipids after intranasal administration in vivo [80]. Immunization of mice with a mycobacterial fusion protein in DODAB-TDB liposomes induced a strong, specific Th1-type immune response characterized by substantial production of interferon-gamma mediated by CD4 T cells and high levels of IgG2b isotype antibodies [15]. The combinations of DODAB and monoolein improved the fusion of the liposomes with cell membranes, thereby allowing their use for mammalian cell transfection [81] and in vitro gene silencing [82]. These combinations also induced strong humoral and cell-mediated immune responses, producing antibodies (IgGs) against specific cell wall proteins of Candida albicans (CWSP) useful for fighting fungus infections [77,83]. Figure 4 illustrates the use and activity of DODAB/monoolein vesicles as adjuvants as reproduced from reference [77]. One should notice the inverted hexagonal phase of monoolein inside the liposome.

Another important line of research for vaccines against pathogens has been the use of cationic liposomes or DODAB bilayer fragments (BF) as adjuvants for intranasal immunization. The cationic DOTAP/DC-Chol liposomes combined with ovalbumin (OVA) were intranasally administered eliciting enhanced production of IgG antibodies in the serum (Th2 response) in immunized mice as well as mucosal IgA [84]. Immune responses for DODAB BF and alum complexes with outer membrane vesicles (OMV) of Neisseria meningitidis B administered by intranasal and subcutaneous routes in mice were compared; intranasal immunization produced a mixed Th1 and Th2 response, while subcutaneous immunization exhibited a Th1 profile only [27]. Non-replicating, nanometric membrane vesicles (MV) released both by Gram-positive and Gram-negative bacteria contain proteins, lipids, and nucleic acids that are effectively able to stimulate the innate and adaptive immune system [85,86]. In this regard,
the cationic lipids can add extra adjuvanticity. Furthermore, multiple antigens can decorate these MV; for example, outer MVs from attenuated *S. typhimurium* was successfully decorated with one, two, or three antigens from *M. tuberculosis* (ESAT6, Ag85B, and Rv2660c) and major outer membrane protein epitopes from *Chlamydia trachomatis*; in vitro data showed that the antigen Ag85B delivered by outer MVs is able to be recognized and processed by dendritic cells and subsequently activate *M. tuberculosis*-specific T cells [87].

Figure 4. Activation of cell-mediated immunity and humoral response by DODAB/monoolein liposomes incorporating cell wall surface proteins of *Candida albicans* (CWSP). Reprinted from [77] with permission from Elsevier, Copyright 2015.

The development of effective intranasal vaccines is of great interest due to their potential to induce both mucosal and systemic immunity. Some oil-in-water nanoemulsion (NE) formulations containing various cationic and nonionic surfactants were used as adjuvants for the intranasal delivery of vaccine antigens. Association of NE droplets with the mucus protein mucin in vitro was important as were the cationic NE formulations that facilitated cellular uptake of the model antigen, ovalbumin (OVA), in a nasal epithelial cell line. NE-facilitated mucosal layer penetration and cellular uptake led to enhancement of the immune response [88].

In an interesting comparative study, several cationic lipids were evaluated regarding their effectiveness as humoral adjuvants while carrying the influenza antigen hemoagglutinin (HA) [89]. DDA or DODAB and other cationic lipids combined with a neutral lipid (DPPC) in a molar proportion of 1:1 were again evaluated as poor inducers of humoral response with exception of DC-Chol. The cationic liposomes contained a cationic compound (DDA or DODAB, 1,2-dipalmitoyl-3-trimethylammonium-propane DPTAP, DC-Chol, or 1,2-diacyl-sn-glycero-3-ethylphosphocholine (eDPPC) and a neutral phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and carried the influenza antigen HA; they were well characterized regarding hydrodynamic diameter, zeta potential, membrane fluidity, HA loading, and humoral immune response in subcutaneously immunized mice from the production of HA-specific antibodies by ELISA and HA-neutralizing antibodies by hemagglutination inhibition (HI) assay. Figure 5, reproduced from reference [89], shows that liposomes at 1:1 DC-Chol/DPPC combined with HA gave the inhibition of hemoagglutination.
titers that could be related to the highest IgG1 and IgG2a titers compared to the other liposomal HA formulations and HA alone. Moreover, increasing the proportion of cationic lipid increased the incorporation of HA and the immune response \[89\]. One should notice that the physical state of the cationic bilayers was the rigid gel state in all cases and the physical state of the DC-Chol/DPPC bilayers was not determined in reference \[89\].

Figure 5. Hemagglutination inhibition (HI) assay for neutralizing antibodies against hemagglutinin (HA) elicited by combinations of HA with DDA/DPPC, DPTAP/DPPC, or DC-Chol/DPPC where DDA is dioctadecyldimethylammonium bromide, DPTAP is 1,2-dipalmitoyl-3-trimethylammonium-propane, DPPC is 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, eDPPC is 1,2-diacyl-sn-glycero-3-ethylphosphocholine and DC-Chol is 3β-[N-(N',N'-Dimethylaminoethane)-carbamoyl] cholesterol. Reprinted from \[89\] with permission from Elsevier, Copyright 2012.

The mRNA technology for vaccines \[90\] has been recognized as representing a transformative technology to control infectious diseases \[91\] and to fight cancer \[92\]. For example, while constructing an mRNA vaccine against influenza, the mRNA encoding the HA antigen of influenza A H1N1 virus was delivered by cationic lipid nanoparticles (LPN) and induced protective immune responses in mice. The lipid nanoparticles comprised several lipids such as DOTAP, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) (DSPE-mPEG2000) (50:50:1 mol/mol) \[93\]. The system allowed also the covalent binding of mannose (Man) to the PEG moiety so that targeting of the mannose -cationic NPs (LNP-man) to the mannose receptors on antigen-presenting cells such as macrophages and dendritic cells improved the delivery efficiency of the assembly. These cationic lipid/mRNA NPs could protect their mRNA cargo from degradation by nucleases and deliver the m-RNA into cells by electrostatic adsorption and fusion with the cell membrane. LNP-Man contained DOTAP, DOPE, and DSPE-PEG-Mannose (50:50:1 mol/mol). This vaccine was properly tested from administration by the intra-nasal route and induced excellent protection against influenza. The important issue of lipid-based anticancer vaccines was recently reviewed \[94\].

In order to ascertain whether antigen depot or lymphatic targeting would benefit long-term immunological memory, OVA antigen was encapsulated with DOTAP cationic liposomes (LP) or DOTAP-PEG-mannose liposomes (LP-Man) to generate depot or lymphatic-targeted liposome vaccines,
respectively [95]; in vivo imaging showed that LP accumulated near the injection site, whereas LP-Man accumulated in draining lymph nodes (LN)s and spleen enhancing the uptake by resident antigen-presenting cells. LP vaccines with depot effect induced higher anti-OVA IgG production than LP-Man vaccines on day 40 after priming but failed to mount an effective B-cell memory response upon OVA re-challenge after three months. In contrast, lymphatic-targeted LP-Man vaccines elicited sustained antibody production and robust recall responses three months after priming, suggesting that lymphatic targeting rather than antigen depot promoted the establishment of long-term memory responses [95].

Small interfering RNAs (siRNAs) are able to recognize a homologous mRNA sequence in the cell and induce its degradation; each siRNA molecule can inactivate several target RNAs in a sequence-specific manner [96]. The main problems in the development of siRNA-based drugs and vaccines for therapeutic use are the low efficiency of siRNA delivery to target cells and the degradation of siRNAs by nucleases in biological fluids [67]. Among the approaches used to deliver RNA are those based on non-saturated double-chained cationic lipids [97]. These lipids were shown to facilitate fusion with cell membranes [98]. Figure 6 shows the encapsulation of self-amplifying RNA based on alphavirus genome, which contains the genes encoding the alphavirus RNA replication machinery but lacks the genes encoding the viral structural proteins required for infection; the cationic liposome composition is also shown on the right as reproduced with permission from Geall and coworkers, 2012 [99]. The cationic lipid employed was 1,2-dilinoleoyloxy-N,N-dimethyl-3-aminopropane (DLinDMA) with two double bonds per chain [98]. After immunization, replication and amplification of the RNA molecule occur exclusively in the cytoplasm of the transfected cells, thereby eliminating risks of genomic integration, cell transformation, and safety issues that occur for recombinant DNA, viral vectors, and pDNA vaccines. Furthermore, there is no need for crossing the nuclear membrane, a rate-limiting step for nonviral pDNA delivery.

![Figure 6. Self-amplifying RNA vaccines where this RNA derived from and alphavirus contains a 5′ cap, nonstructural genes (NSP1-4), 26S subgenomic promoter (dark blue arrow), the gene of interest, and a polyadenylated tail. The self-amplifying RNA was protected inside the cationic liposome with the composition shown in the legend on the right. Reprinted from [99].](image-url)

Dendritic cells (DC) process and present antigens to T lymphocytes, inducing potent immune responses when encountered in association with activating signals, such as pathogen-associated
molecular patterns. Monophosphoryl lipid A (MPL) is a ligand of the Toll-like receptor-4 and has been used in several studies on vaccines [100]. Using combined therapy against murine model tumors, both MPL and IL-12 were included in cationic DOTAP liposomes for intratumoral injection [101]. In 4T1 murine model of breast cancer, the injection decreased cellular proliferation and increased serum levels of IL-1β and TNF-α. The addition of recombinant IL-12 further suppressed tumor growth and increased expression of IL-1β, TNF-α, and interferon-γ. IL-12 also increased the percentage of cytolytic T cells, DC, and F4/80(+) macrophages in the tumor. The combination of MPL and IL-12 elevated the levels of nitric oxide synthase 7-fold above basal levels in the tumor and caused cell cycle arrest and apoptosis, also inhibiting the growth of untreated tumor in the same animal and revealing the systemic activity of the formulation [101].

In another very interesting approach, sterically stabilized nanodisks based on high-density lipoproteins (HDL) carried MPL, CpG (ligand of Toll-like receptor-9), and antigen for personalized cancer immunotherapy; synthetic high-density lipoprotein (sHDL) nanodisks were composed of phospholipids and apolipoprotein A1 (ApoA1)-mimetic peptides (the peptides were named 22A because they were synthesized as 22-mer peptides, derived from the repeat α-helix domain of ApoA1) [102]. Thereby the endogenous role of HDL as a nanocarrier for cholesterol was explored in synthetic HDL that carried cholesteryl-CpG, neo-antigens, and tumor Ag peptides (neo-antigens identified via tumor DNA sequencing) to produce homogeneous, stable, and ultrasmall nanodisks in less than two hours at room temperature; nanodisks promoted co-delivery of Ag/CpG to draining lymph nodes; prolonged Ag presentation on antigen-presenting cells (APCs); elicited striking levels of broad-spectrum antitumor T-cell responses; and significantly inhibited tumor growth, also eradicating established tumors [102]. Cationic nanodisks of DODAB, also called DODAB bilayer fragments (BF), have also been used as adjuvants for carrying several antigens, CpG agonist, and oligonucleotides [16] directing excellent Th1 response and also Th2, depending on the administration route [see references 21,25,28]. Figure 7 schematically represents cross-sections of DODAB nanodisks carrying CpG and ovalbumin (OVA). DODAB BF have two major strategic advantages when compared to more sophisticated formulations: (1) DODAB is possibly the less expensive synthetic cationic lipid available nowadays, (2) DODAB dispersion as open bilayers in water solution can be rapidly performed by sonication with a macrotip, and (3) the nanometric size of the DODAB bilayer disks allows direct stimulation of APC in the draining lymph nodes [103].

**Figure 7.** Schematic representation of cross sections for DODAB bilayer fragments (DODAB BF) used for carrying ovalbumin (OVA) and CpG agonist of Toll-like receptor 9. The final assemblies were anionic and directed excellent Th1 response in mice immunized subcutaneously. Curiously, addition of CpG to the assembly did not improve the immune response; DODAB was effective by itself. In addition, the nanosize of the assemblies was more important than the charge. Reprinted from [16] with permission from Elsevier, Copyright 2012.

Whereas DODAB BF harboring CpG did not improve the adjuvanticity of DODAB BF in vivo [16], DOTAP/DC-chol liposomes harboring CpG ODN as a mucosal adjuvant induced both antigen-specific mucosal IgA responses and balanced Th1/Th2 responses so that the combination resolved adverse effects of CpG ODN as mucosal adjuvant by means of dose minimization [104].
Human papillomavirus (HPV) is the most common sexually transmitted biological agent and causes precancer lesions and cancer; three prophylactic HPV vaccines targeting high-risk HPV types are available in many countries worldwide: 2-, 4- and 9-valent vaccines; all three of the vaccines use recombinant DNA technology and are prepared from the purified L1 protein that self-assembles to form HPV type-specific empty shells [105]. There are a few instances of using cationic lipids to formulate vaccines against HPV. DOTAP/oncoprotein E7 of papillomavirus was evaluated for its anti-cancer activity; E7 peptide formulated with DOTAP induced migration of activated dendritic cells (DC) to the draining lymph node (DLN) and efficiently generated functional antigen-specific CD8+ T lymphocyte infiltration and apoptosis at tumor sites; the effect did not change by adding CpG to the same formulation [106]. Efficient eradication of tumors in mice was also achieved using combinations of DOTAP/DOPE cationic liposomes with synthetic long peptides (SLP) derived from OVA alone or combined with different Toll-like receptors ligands including CpG; a single intradermal tailbase vaccination of tumor-bearing mice with a low dose of E7/poly(I:C)-liposomes led to complete clearance of the tumors in 100% of the mice; therapeutic vaccination with SLP could be clinically effective against HPV-induced premalignant lesions; induced antigen-specific CD8+ and CD4+ T cells and in vivo cytotoxicity against target cells after intradermal vaccination; at a low dose (1 nmol) of SLP, our liposomal formulations significantly controlled tumor outgrowth in two independent models (melanoma and HPV-induced tumors) and even cured 75%–100% of mice of their large established tumors; cured mice were fully protected from a second challenge with an otherwise lethal dose of tumor cells, indicating the potential of liposomal SLP in the formulation of powerful vaccines for cancer immunotherapy [107].

SLP-loaded (1,2-dioleoyl-3-(trimethyammonium) propane)-based cationic formulation as a therapeutic cancer vaccine was tested against two independent tumor models. The OVA-derived SLPs containing CTL and Th epitopes were loaded into DOTAP-based cationic liposomes combined with different TLR ligands [poly(I:C), Pam3CysK4, CpG], and the most potent formulations were applied in a foreign antigen (OVA)-expressing melanoma model. In an independent setting, HPV16 E7 SLP was formulated in the same liposomal system and analyzed as a therapeutic vaccine in the TC-1 HPV+ tumor model; both formulations were highly effective in the induction of cellular immunity and tumor control [107].

The humoral and cellular immune responses induced in mice against hepatitis B virus surface antigen (HBsAg) were examined when the antigen was either adsorbed to aluminum hydroxide or administered with DC-Chol. DC-Chol induced cellular immune responses to HbsAg and a balanced Th1/Th2 response, which enabled mice to overcome the inherited unresponsiveness to HBsAg encountered with aluminum-adjuvanted vaccine. Thus, the DC-Chol provided a signal to switch on both Th1 and Th2 responses for vaccination against hepatitis B virus [108].

An early model study on trafficking of cationic-liposome-DNA complexes in the cells attempted to reveal by electron microscopy the intracellular fate of gold-labeled structures. Cells treated with DOTMA liposome-DNA complexes demonstrated endocytosis of the liposome–DNA complexes in coated pits, which were seen in early endosomes, late endosomes, and lysosomes. In isolated alveolar type II cells, the gold-labeled DOTMA lipid apparently mixed with the contents of lamellar bodies. In most cells, gold particles were dispersed throughout the cytoplasmic matrix. In a small proportion of cells, a membrane system resembling the endoplasmic reticulum developed within the nucleus; this novel structure was also present in isolated nuclei from cells and then mixed with DOTMA-containing liposomes [109].

DNA vaccination technologies have been important in several areas despite the difficulties involving DNA transfection efficiency, prevention of DNA degradation, APC targeting, and enhancing DNA escape from endo/lysosomal compartments and attachment of virus-derived nuclear localization sequences facilitating nuclear entry of the DNA [110]. For example, DNA vaccines provide an attractive technology platform against anthrax bioterrorism agents; monovalent and bivalent anthrax plasmid DNA (pDNA) vaccines encoding genetically detoxified protective antigen (PA) and lethal factor (LF)
proteins were formulated in cationic lipids, and immune responses after two or three injections of cationic lipid-formulated PA, PA plus LF, or LF pDNAs were at least equivalent to two doses of anthrax vaccine adsorbed (AVA). High titers of anti-PA, anti-LF, and neutralizing antibody to lethal toxin (Letx) were achieved in all rabbits. All animals receiving PA or PA plus LF pDNA vaccines were protected. In addition, 5 of 9 animals receiving LF pDNA survived, and the time to death was significantly delayed in the others. Groups receiving three immunizations with PA or PA plus LF pDNA showed no increase in anti-PA, anti-LF, or Letx neutralizing antibody titers postchallenge, suggesting little or no spore germination. In contrast, titer increases were seen in AVA animals and in surviving animals vaccinated with LF pDNA alone. Preclinical evaluation of this cationic lipid-formulated bivalent PA and LF vaccine is complete, and the vaccine has received U.S. Food and Drug Administration Investigational New Drug allowance [111].

Early work involving cationic liposomes to carry plasmid encoding antigens revealed that the liposomes indeed protected the liposome-entrapped DNA from degradation in vivo, thereby resulting in greater antibody responses against the encoded antigen when compared with naked DNA, both given via the subcutaneous route; T-cell responses from analysis of interferon-γ and interleukin-4 levels in the spleens of mice treated with liposomes/DNA were also significantly higher than those measured in mice treated similarly with naked DNA [112–114]. More recently, Perrie and coworkers gave an excellent summary of the possible fate of cationic liposomes/DNA (L/DNA) assemblies injected by the subcutaneous route [115]. L/DNA assemblies injected locally are taken up by APC penetrating the site of injection or in the lymph nodes draining the injected site; the clearance of L/DNA from the site of injection depends on L/DNA size; the smaller the size, the more rapid the clearance through the anatomical barriers [116]. The L/DNA become dispersed throughout the lymph node either permeating along the sinuses or being taken up by cells such as macrophages; once within cells, L/DNA are digested by the lysosomes, leaving their contents within the lysosomes or escaping this degradation via fusion with the endosomal membrane (which happens due to the fusogenic lipid DOPE present in the liposomal bilayer) [117–119]. Thereby DNA is displaced from the complex and released into the cytosol for eventual episomal transfection so that cationic lipid and DOPE favor liposome-mediated -DNA immunization [115,117,118]. The possible fates of nanosized adjuvant/antigen assemblies were thoroughly discussed by Smith and coworkers, 2013 [120].

Lecithins are components of cell membranes consisting of combinations of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), plus other substances such as triglycerides and fatty acids; in particular, soy lecithin contains 21% PC, 22% PE, and 19% PI, along with other components. Lecithins are widely used for dispersing, emulsifying, and stabilizing a variety of pharmaceuticals often included in intramuscular and intravenous injectables, parenteral nutrition formulations, and topical products [121]. Hexadecyltrimethyl ammonium bromide (CTAB) surfactant added to lecithin nanoparticles yielded cationic particles with diameters in the range of 100–200 nm where soy lecithin was the matrix material and CTAB, the outer surfactant. The zeta potential of the particles was positive, reached a value of about 40 mV at a CTAB concentration of 2.5 mM, and was used to adsorb plasmid DNA and transfect cells efficiently, representing a potential carrier for DNA vaccines [122].

Cationic liposomes are commonly used as a transfection reagent for DNA, RNA, or proteins and as a co-adjuvant of antigens for vaccination trials. A high density of positive charges close to cell surface is likely to be recognized as a signal of danger by cells or contribute to trigger cascades that are classically activated by endogenous cationic compounds, though carrier/protein or carrier/nucleic acid might have anionic charges and still trigger significant immune responses. There are several cellular pathways, like pro-apoptotic and pro-inflammatory cascades, that can be induced by cationic liposomes, depending on their nature, size, and structural properties (nature of the lipid hydrophilic moieties, hydrocarbon tail, mode of organization) [123]. Their use and design for specific applications such as gene transport or as adjuvants certainly require more knowledge on their structure–function relationship. Excellent reviews are available on this nano-era regarding
applications of nanotechnology in immunology [120,124], progress in prophylactic and therapeutic nanovaccines [124], cancer nano-immunotherapy [125], nanomaterial interactions with the immune system [126], and liposomes formulations for vaccines [127,128].

3. Assemblies Based on Cationic Polymers

Similarly to cationic lipids, cationic polymers constitute another important class of cationic adjuvants despite their often-reported dose-dependent cytotoxicity that requires dose minimization [9, 10,65,129–133]. They easily combine with oppositely charged proteins [40,134]. Biodegradable polymeric particles of poly (lactic-co-glycolic acid) (PLGA) have been combined with cationic surfactants or lipids or polymers (CTAB, DODAB, polyethyleneimine (PEI) or ε-poly-l-lysine (PLL)) for improving antigen adsorption, colloidal stability, and the immune response [135,136]. Nanocomplexes of PEI and antigens (influenza hemagglutinin or herpes simplex virus type-2 glycoprotein D) delivered by the mucosal route activated APC in vivo, promoting dendritic cell trafficking to draining lymph nodes besides eliciting a potent immune response against the viral subunit glycoproteins; a single intranasal administration elicited robust antibody-mediated protection [137]. Systemic administration of the same antigens with PEI induced both Th1/Th2 immune responses and higher titers of both antigen-binding and -neutralizing antibodies than alum [138].

The cationic antimicrobial polymer poly (diallyldimethylammonium chloride) (PDDA) is a polyclation [139–141] able to combine with bovine serum albumin (BSA), yielding NPs with diameters around 50 nm [142,143]. In combination with HIV-1 DNA, nanorods of gold yielded particles of gold/PDDA/DNA, which elicited a Th2 response that was higher than the one obtained using PEI or cetyltrimethylammonium bromide (CTAB). Stimulated cellular and humoral immunity, as well as T cell proliferation, was obtained in comparison with naked HIV-1 Env plasmid DNA treatment in vivo [144]. Recently, NPs of PDDA/ovalbumin were prepared, characterized by their physical properties, and evaluated as stimulators of the OVA-specific immune response [65]. Dynamic light scattering (DLS) showed that these cationic PDDA/OVA NPs at reduced doses of cationic polymer had low size, positive zeta-potential, low polydispersity, good colloidal stability, and low cytotoxicity against mammalian cells in culture eliciting potent Th2 OVA-specific immune response (high OVA-specific IgG1 and low OVA-specific IgG2a production); the OVA-specific antibody production was even higher than the one elicited by Al(OH)₃/OVA [65].

Polycations combine spontaneously with molecules of opposite charge like proteins and nucleic acids [65,137,142]; thus, the main use of polycations in the biomedical field is the delivery of bioactive molecules including DNA, RNA, and protein [145]. Polycations as adjuvants can be used in a variety of assemblies, ranging from the simple complexation of polymer with antigen driven by electrostatic interactions [65,138,146] to the use of the polymers as particle coatings or particle cores [147]. The spontaneous complexation of polycations with negatively charged antigens is the simplest way to use these systems as antigen carriers. Figure 8 shows the chemical structures of some cationic polymers that have been used as antigen carriers.

Polyethyleneimine (PEI) is an organic, hydrophilic, and cationic polymer that displays a strong positive charge density promoting the combination with negatively charged molecules such as DNA, negatively charged antigens, or plasma membranes [146]. An interesting quality of PEI is its ability to leak from endosomes after cell internalization due to its capacity for avoiding the endosomal acidification; the high number of nitrogen atoms in the PEI molecule makes the polymer an excellent buffer also at low pH [148]. This proton sponge effect is due to deprotonated PEI amino groups binding protons as they are pumped into the lysosome, resulting in the influx of Cl⁻ ions and water with lysosome osmotic swelling and stretching of the PEI molecule itself due to repulsion between protonated amino groups; thereby, there is rupture of the lysosomal membrane with release of lysosomal contents into the cytoplasm, the so-called endosomal escape leading to a cellular, Th1 response [149,150]. PEI as an adjuvant protects antigens from enzymatic degradation [151], activates the inflammasomes, up-regulates the transcription factor called interferon regulatory factor 3 (Irf-3) [137] and also other
immunostimulatory genes [152], induces Th1 immune response associated with endosomal escape and cross-presentation [153]; administered by the mucosal route, it improves the uptake of antigen and the activation of APCs [137,154]. PEI promotes dsDNA release by host cells triggering the Irf-3-dependent signaling [137]; Irf-3 is a transcription factor related with the activation of innate response by means of the synthesis of type I interferon [155]. Besides Irf-3, PEI activates Nlrp3 inflammasomes, a critical component of the innate immune system, which directs the immune response toward a Th2-biased type [137,138]. PEI as an adjuvant can induce Th1-, Th2-, or Th1/Th2-biased immune response; although PEI has the proton sponge effect to carry out the lysosomal escape, sometimes the escape does not occur, and antigens are presented via MHC class II, resulting in a Th1/Th2 or Th2 response [137,138,146]. PEI/antigen administered by intranasal route induced Th2 response [137], while PEI/antigen administered by a subcutaneous route yielded a Th1/Th2 mixed response [138].

Figure 8. Chemical structures of some cationic polymers: poly (diallyldimethylammonium chloride) (PDDA), linear polyethylenimine (PEI), branched polyethyleneimine (PEI), poly-γ-arginine (PLAr), poly-γ-lysine (PLL), poly-γ-histidine (PLH), diethylaminoethyl-dextran (DEAE-).

Aiming at improving the mucosal response against major viral pathogens, glycoproteins derived from influenza A virus, herpes simplex virus type-2, or HIV-1 combined with PEI as particles in dispersion, administered as a single intranasal dose induced a robust protection from further lethal viral infection, which was superior to the one elicited by cholera toxin as an experimental mucosal adjuvant; these PEI/antigen nanoparticles were efficiently taken up by APCs in vitro, while in vivo they enhanced the DCs trafficking to draining lymph nodes. The nasal immunization with the recombinant envelope glycoprotein gp140 from HIV-1 carried by PEI induced high titer of antigen-specific IgA in vaginal lavages, demonstrating that nasal immunization can induce a systemic immune response. In Nlrp3-knockout mice, PEI/gp140 complexes elicited a Th1-biased immune response, suggesting
that in normal mice the NPs activated the inflamasome Nlrp3 towards a Th2-type response [137]. In continuation, four different PEI polymers such as linear PEI (40 and 160 kDa) and branched PEI (25 and 750 kDa) were combined with the gp 140 from HIV-1; after immunization, all elicited similar responses characterized by a moderate Th1-biased response. The comparison of PEI with alum showed significantly improved performance of PEI compared to alum. PEI-induced immune response was characterized by an intermediate IgG1/IgG2a endpoint titer ratio, indicating a mixed Th1/Th2 immune response as corroborated from analysis of cytokines profile using antigen-re-stimulated splenocytes from mice immunized with gp140 glycoprotein and PEI. Significant amounts of Th1 cytokines IL-2, TNF-α, and GM-CSF and the Th2-associated cytokine IL-5 were determined [138].

Eliciting a cell-mediated immune response is necessary for achieving effective vaccines against cancer and other major diseases like malaria; thus adjuvants able to enhance the antigen presentation via MHC I are needed. Chen and coworkers reported that PEI/OVA NPs, obtained from mixtures of PEI and OVA, promoted cross-presentation through the major MHC class I pathway. The mouse bone-marrow-derived dendritic cells stimulated in vitro with PEI/OVA NPs resulted in improved and more frequent OVA(257-264)/MHC I complex presentation on dendritic cell surfaces. Besides, DCs pulsed with PEI/OVA NPs but not those pulsed with OVA alone showed significant capacity to stimulate T cells [153]. Using DNA as antigens is an encouraging alternative for designing anticancer therapeutic vaccines. Complexes formed by OVA plasmid and PEI (PEI/DNA) were administered to animals, and the corresponding immune response and antitumor activity were assessed. Animals injected with the PEI/DNA complexes displayed antigen-specific cell lysis, and there was increased antigen presentation via MHC class I and significant lymphocyte infiltration at the intra-tumor inoculation sites; importantly, the vaccine injected either before or after the tumor cell inoculation repressed the tumor growth and increased the survival rate of animals [156].

Poly (diallyldimethylammonium chloride) (PDDA) is a synthetic and linear polycation that combines well with DNA [157,158] or protein [142,143]. Figure 9A,B show scanning electron micrographs of PDDA/OVA nanoparticles (NPs) under low and high magnification, respectively; these NPs elicited OVA-specific Th2 response in immunized mice, which was superior to the one elicited by alum [65].

Figure 9. Scanning electron micrographs of PDDA/OVA NPs assembled at 0.1 mg·mL−1 OVA and 0.01 mg·mL−1 PDDA obtained under low (A) and high magnification (B). At the low PDDA dose employed, PDDA cytotoxicity was not significant against cells in culture. Reprinted from [65].

Biomedical uses for PDDA have mostly focused on the design of biosensors [159], transfection [158], or antimicrobial chemotherapy [139–141,160–163] probably due to its high toxicity [65,129]. PDDA, when combined with OVA, in water, formed cationic, nano-sized, and stable NPs that displayed a dose-dependent cytotoxicity, which could be easily controlled from the use of low doses. Interestingly, PDDA/OVA NPs induced a potent Th-2-type immunity (high ratio IgG1:IgG2a) and elicited an OVA-specific IgG1 antibody production higher than the one induced by OVA or Al(OH)3/OVA; PDDA/OVA NPs displayed low cellular immune response as determined from footpad swelling tests.
for detecting the delayed-type hypersensitivity reaction (DTH) and the low elicited production of IgG2a quantified in serum, both in immunized mice [65].

An important biomolecule that can be carried by cationic polymers is DNA, essential for DNA vaccines despite its relative delivery inefficiency when compared to viral vectors [164]. Cell internalization of the polyplexes (cationic polymer/DNA) and subsequent release of their cargo requires translocation across endosomal and/or nuclear membranes, a determinant factor for therapeutic efficiency, and hence, potential clinical impact. Polyplexes or lipoplexes (cationic lipid/DNA) essentially follow a similar intracellular route once captured by endocytosis [165,166]. Figure 10 shows the intracellular trafficking of a fluorescently labeled oligo-deoxynucleotide (ODN) carried in PEI/fluorescein isothiocyanate-labelled ODN (FITC-ODN) polyplexes; fluorescence is firstly inside the endosome, then in the cytosol after escaping from the endosome due to the endosomal burst, and finally in the cell nucleus as reproduced from ur Rehman et al. 2013 [166]. The limited efficiency of ODN delivery to the nucleus relates to the fact that most endosomes did not burst.

**Figure 10.** PEI-mediated cytosolic delivery of oligonucleotides occurs by endosomal bursting. HeLa cells were incubated with polyplexes carrying fluorescein isothiocyanate labeled oligo-deoxynucleotides (FITC-ODNs) in green and monitored by time-lapse microscopy. Following internalization of the poly (ethylene imine) (PEI) polyplexes in endosomes, endosomal bursting occurs causing the release of ODNs into the cytosol. After an initial appearance throughout the cytosol, the ODNs rapidly accumulate in the nucleus. Reprinted from [165] with permission from 2013 American Chemical Society.

Diethylaminoethyl-dextran (DEAE-) polymer is a quaternary ammonium compound that contains three basic groups with different pKa values [167]. The polymer facilitates the adsorption and penetration of viral particles or bacteria into cells, suggesting that it is adequate for delivering antigens into the APCs [168]. DEAE- has delivered Venezuelan equine encephalomyelitis virus [169] and whole-cells *Vibrio cholerae* Inaba and Ogawa serotypes vaccines [170], although its adjuvant properties have been explored mainly for use in vaccines for veterinarian treatment [171]. DEAE-Dextran mixed with formalin-inactivated Venezuelan equine encephalomyelitis virus exhibited a significant adjuvant effect on the primary immune response in rhesus monkeys [169]. In a whole-cell vaccine, DEAE- combined with *Vibrio cholerae* produced a higher and longer-lasting antibody titer than the one elicited by vaccines without adjuvant; furthermore, there was a greater protection against cholera re-infection [170]. In a breast cancer model, DEAE- induced the production of IFN-beta inhibiting the gene expression of the vascular endothelial growth factor (VEGF) gene and the NOTCH1 gene both related to angiogenesis and tumorigenesis [172].

Poly (2-aminoethyl methacrylate) (PAEM) homopolymers with defined chain length and narrow molecular weight distribution were synthesized using atom transfer radical polymerization (ATRP) so that PAEM of different chain lengths (45, 75, and 150 repeating units) showed varying strength
in condensing plasmid DNA into narrowly dispersed nanoparticles. Longer polymer chain length resulted in higher levels of overall cellular uptake and nuclear uptake of plasmid DNA, but shorter polymer chains favored intracellular and intranuclear release of free plasmid from the polyplexes. Using a model antigen-encoding ovalbumin plasmid, transfected DCs activated naïve CD8(+) T cells to produce high levels of interferon-γ. Efficiency of transfection, DC maturation, and CD8(+) T cell activation showed varying degrees of polymer chain-length dependence, showing the importance of using structurally defined cationic polymers as carriers for DNA vaccines [173]. This model study emphasized the importance of well-defined chain length for cationic polymers in DNA vaccines; this type of cationic polymer poly (2-aminoethyl methacrylate) was also recently reviewed for exploring structure–function relationship while delivering DNA [174]. Excellent reviews are available on micro- and nanoparticles for DNA vaccine delivery [175] and on molecular delivery of plasmids for genetic vaccination [176].

Polyaminoacids are another important class of polycations used for carrying antigens in vaccine formulations [177]. Like other polycations, one major advantage of using them is their ability to combine spontaneously with molecules of negative charge, a phenomenon driven for electrostatic interactions. Within the group of polyaminoacids, poly-l-lysine and poly-l-arginine are among the most studied as adjuvants. For instance, poly-l-arginine on the surface of microcapsules obtained by layer-by-layer or spray-drying techniques supports the particles’ uptake by the APCs [178,179]. Promising research reported the use of poly-l-arginine for carrying tumor antigen-derived peptides for synthesizing a synthetic antitumor vaccine; the work described that the subcutaneous injection of a mixture of poly-l-arginine and peptides induced a large number of antigen-specific T cells detectable in the systemic circulation for more than four months. Another important finding reported by the authors was the presence of numerous APCs loaded with antigen in the draining lymph nodes of vaccinated animals, suggesting that poly-l-arginine is a suitable carrier for targeting antigens into the lymph nodes. An additional relevant result is the absence of antibodies against poly-l-arginine, allowing this compound to be used for numerous booster injections [180].

Employing TLR agonists as adjuvants is an attractive alternative for stimulating a specific type of immunity like a Th1-biased response. Negatively charged TLR agonists could also be easily attached to polycations by electrostatic interactions [138]. The adjuvant activity of combinations between CpG ODN (ligand for Toll-like receptor 9) and different polycations such as poly-l-arginine, poly-l-lysine, poly-l-histidine, or chitosan in an OVA vaccination model, as well as poly-l-arginine and poly-l-histidine, but not poly-l-lysine or chitosan, improved efficiently both the IgG antibody production and the number of splenocytes secreting IFN-gamma after T CD8+ ex vivo stimulation. CpG-ODN/poly-l-arginine is better than complete Freund’s adjuvant and aluminum salt as adjuvants and did not induce local toxicity [181]. The assembly of poly-l-arginine, CpG-ODN, and synthetic peptides induced an improved peptide-specific immune response as compared to the application of peptides with either of the immunomodulators alone. Poly-l-arginine synergized with oligodeoxynucleotides containing Cpg-motifs (CpG-ODN) for enhanced and prolonged immune responses and prevented the CpG-ODN-induced systemic release of pro-inflammatory cytokines; CpG-ODN are known to be potent inducers of predominantly type 1-like immune responses, while polycationic amino acids, facilitate the uptake of antigens into antigen-presenting cells (APCs). The potentially harmful systemic release of pro-inflammatory cytokines induced upon injection of CpG-ODN was inhibited and long-lasting, and high numbers of antigen-specific T cells could be observed from fluorescence-labeled OVA even after only one injection of the vaccine [182].

Cationic polymers are also used for coating or functionalizing different types of NPs, namely, magnetic NPs, metallic NPs, ceramic NPs, hydrophobic NPs, or biocompatible polymer-based NPs. For instance, PEI has been used for coating SPIONs [19,183,184], silica NPs [185], or gold NPs [144,186]. Cationic and nanosized PEI-coated SPIONs in vitro activate macrophages and polarize them to an M1-like phenotype [184]. PEI-coated SPIONs obtained from the sonication of iron oxide suspension and PEI solution was used for carrying the plasmid-malaria DNA vaccine encoding VR1020-PyMSP119;
the intraperitoneal administration of the complex displayed high titers of antigen-specific IgG1 and IgG2a and improved the immunological memory after vaccination [19]. Another example of metallic nanoparticles functionalized with polycations was reported for Xu and coworkers; they coated gold nanorods with the polycations PEI or PDDA or with the surfactant CTAB and attached therapeutic HIV-1 DNA-vaccine. Gold nanorods coated with both polycations show the best capacity for packing DNA, and they were uptaken by DCs in a higher level than the ones coated with CTAB; polycation-coated gold nanorods induced a significant increase of the cellular and humoral immunity as determined by antibody titers (IgG1/IgG2 ratio) and T cell proliferation. It was also described that PDDA enhances a Th2 whereas PEI induces a Th1/Th2 immune response [144].

In situ silicifications and capping treatment produced PEI–silica–PEI coatings on Japanese encephalitis virus vaccine maintaining its immunogenicity better than the vaccines without adjuvant; this fact could be relevant in cases where keeping the vaccine under refrigerated condition is difficult [187]. PEI coating mesoporous silica micro-rods (MSR-PEI) significantly enhanced the antigenicity of a peptide antitumor vaccine. The antigen was coupled to the microstructure by simple adsorption enhancing host DCs activation and T cell response compared to controls; MSR-PEI/E7-peptide eradicated large established TC-1 lung tumors in ~80% of mice and generated immunological memory; MSR-PEI vaccine, using a pool of neoantigens, eliminated established lung metastases and controlled tumor growth [188].

Carboxylated polystyrene particles conjugated covalently with poly-L-lysine carried a DNA vaccine improving the immune Th-1 response; C57BL/6 mice were immunized with the NPs carrying an OVA plasmid yielding a high level of activated CD8 T cells and OVA-specific antibodies. The animals immunized with the poly-L-lysine-coated polystyrene NPs generated a significant antitumor response evidenced by the inhibition of tumor growth after challenging with the OVA expressing EG7 tumor cell line; NPs with diameters of 50 nm yielded the best activity [189].

In summary, polycations can be excellent adjuvants for vaccines, but a quantitative determination of toxicity should always be performed for determining the minimal doses where toxicity is absent and the adjuvant effect still occur; this includes also the equally toxic cationic polyaminoacids. One should notice also that cationic polymers in general are potent antimicrobials as often reviewed [139].

4. Hybrid Cationic Assemblies of Biocompatible Polymer/Cationic Polymer

Hybrid cationic polymer/biocompatible polymer assemblies are useful for improving the characteristics of delivery systems. For example, the use of biocompatible polymers decreases the toxicity of the systems, whereas the cationic polymers improve the colloidal stability, antigen loading capacity, and APCs internalization. Some researchers have used this approach for achieving improved delivery systems [136,190,191].

Biocompatible synthetic or natural polymers can improve body functions without interfering with its normal functioning or triggering side effects [192–194]. Some examples of biocompatible polymers are poly (lactic-co-glycolic acid) [195], poly (ε-caprolactone) (PCL) [196], poly (lactic acid) [197], poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) [198], chitosan [199,200], cellulose [201] and poly (acrylates) including poly (methyl methacrylate) (PMMA) [202,203]. Neutral or anionic biocompatible synthetic or natural polymers as polylactide-co-glycolide microparticles have been cationized with cationic lipid DODAB or cationic surfactant CTAB to assure combination with the oppositely charged DNA antigens to design DNA vaccines [135,204]. In another instance, DODAB cationic bilayer surrounded polystyrene sulfate particles available over a range of diameters [205–207] or silica particles [208,209]; recently optimal coverage with single cationic bilayers on the anionic polymeric or inorganic particles was achieved [207,209]. Synthetic biocompatible polymers such as PMMA were also hybridized with cationic polymers or cationic lipids or surfactants [160–163,210–213]. In summary, a variety of model hybrid and cationic delivery systems are already available to combine with a myriad of oppositely charged antigens for vaccine design [25,163,214,215].
In a brief overview, biocompatible polymers interact with biological systems without exerting significant damage or toxicity, displaying characteristics like biodegradability and/or biosorption. Their broad utility has been reported in different areas of medicine including orthopedics, tissue engineering, and drug and vaccine delivery; eventually, these materials also display intrinsic biological activity [192–194,216]. Medical devices or drug delivery systems based on biocompatible polymers have been approved by the Food and Drug Administration (FDA) [192–194,216]. Biocompatible polymers can be natural (e.g., chitosan, alginate, cellulose) or synthetic (e.g., polylactic acid, poly(lactic-co-glycolic acid)); some excellent reviews on biocompatible polymers are available [217,218]. One of the most promising delivery strategies for activating the immune system is the controlled release of antigens from a biodegradable polymeric matrix, the sustained liberation of antigens may induce strong immune responses [216,217]. Many biocompatible polymers have often been used as vaccine adjuvants such as the biocompatible and biodegradable poly (lactic acid) (PLA), poly (lactic-co-glycolic acid) (PLGA), and chitosan [216,219,220]. Figure 11 shows the chemical structures of some biocompatible polymers used for vaccine delivery.

As a first example of combination of cationic polymer and biocompatible polymer, assemblies of PEI and PLGA have often been used in vaccine delivery systems to carry antigens; they enhanced the antigen-specific antibody titer, lymphocyte proliferation, antigen internalization by phagocytic cells, and Th-1 typical cytokines production [136,221,222]. Biocompatible PLGA NPs were coated with
three different cationic polymers, PEI, chitosan, or ε-Poly-lysine (PLL), aiming at improving the antigen loading capacity of PLGA NPs and evaluating the immune response against OVA as model antigen. PLGA-PEI/OVA NPs displayed the highest OVA loading capacity and colloidal stability, improved lymphocyte proliferation, CD4/CD8 ratio, increased the production of OVA-specific IgG1 and IgG2a antibodies and the secretion of TNF-α, IFN-gamma, IL-4, and IL-6 [136]. Similarly, the cationic PEI-coated PLGA NPs encapsulating the immunopotentiator Angelica sinensis polysaccharide (ASP) significantly activated macrophages, induced the expression of MHC II and CD86 molecules and the production of IL-1β and IL-12p70 cytokines. The adsorption of the porcine circovirus type 2 (PCV2) antigen onto NPs improved the macrophages antigen internalization. Immunized mice significantly improved the production of PCV2-specific IgG and responded with a mixed Th-1/Th-2 biased immune response [221].

Microparticles of PLGA coated with branched or linear PEI were suitable for delivering DNA vaccines demonstrating low cytotoxicity and improvement of APCs internalization [223,224]. In an interesting approach, PEI/DNA complexes were entrapped into PLGA microspheres warranting protection against DNA degradation and efficient uptake by APCs in comparison to the one of naked DNA. The humoral response elicited by PLGA/PEI/DNA against an DNA-HIV-1 antigen was from two to three times higher than one elicited by naked DNA; the cytolytic T lymphocyte at low doses of the antigen was also improved by the complex [224].

Other pairs of polymers used for assembling polymer–polymer hybrid NPs were PLGA/chitosan or PLGA/chitosan-derivatives, these hybrid NPs also improved the uptake rates of antigen by APCs and enhanced the elicited immune response in vivo [191,225,226]. By a modified double emulsion solvent evaporation process, three different NPs based on PLGA were synthesized, chitosan-coated PLGA, glycol-chitosan-coated PLGA, and PLGA NPs, all of them with a diameter smaller than 200 nm and positive zeta potentials; the NPs were used as adjuvants for assessing their capability to induce an enhance immune response. After immunization, glycol-chitosan/PLGA NPs elicited the higher mucosal and systemic response when administered with hepatitis B surface antigen, associated due to in vitro mucoadhesion. Both coated NPs experimented on had less mucosal clearance than PLGA uncoated NPs, resulting in improvement of the immune response [226]; permanence of the vaccine at the mucosa improved the obtained response [226–228]. Slow antigen-releasing N-trimethyl-chitosan-coated-PLGA/OVA NPs with permanent cationic charges yielded high IgA production [229].

Assemblies of PLA with cationic polymer physical properties and immunoadjuvant activity were described [230,231]. Polycation-coated PLA NPs increased the expression of MHC and co-stimulatory molecules, enhanced cytokine production, activated and induced maturation of APC, and improved antibody titer [147,227]. PLA microspheres coated with PEI or chitosan or chitosan chloride significantly increased the loading antigen capacity according to the increment of surface charge; coating PLA microspheres with polycations also augmented the macrophages uptake rates of the adsorbed hepatitis B surface antigen, the expression of CD86, MHC I, MHC II, and the secretion of IL-1β, IL-6, TNF-α, and IL-12. Intraperitoneal administration of cationic microspheres carrying the antigen improved the production of antibodies as compared with aluminum-based adjuvant and free antigen; besides, the cationic microspheres elicited a Th-1 biased showing the assemblies adequacy for performing antiviral, antimalaria, and antitumor vaccines [227].

Chen and coworkers tested chitosan-decorated PLA microspheres carrying hepatitis surface antigen administered via commonly used parenteral administration routes and obtained robust immune responses such as maturation of DCs with overexpression of CD40, CD80, and IL-12 in contrast with the absence of these responses when aluminum-based adjuvant was used; in vivo tests showed increased IgG production induced by both the MPs and the alum-based adjuvants; only the MPs administered by intramuscular route could induce high IgG2a titer, Th-1 cytokines (IL-2, IL-12, and IFN-γ) and Th-2 cytokine IL-4 secretions, demonstrating that PLA-coated NPs improve the cellular and humoral
response [147]. Figure 12 shows a scanning electron micrograph of PLA microparticles coated by chitosan chloride and gives details on mean diameter, zeta-potential, and antigen loading [147].

**Figure 12.** Scanning electron micrographs and dynamic light scattering Zetasizer analysis were used to characterize PLA microparticles (MP) modified by cationic chitosan chloride surface coating. Antigen adsorption was calculated by comparing the amount of antigen in supernatants after centrifugation with total input antigen (protein concentration was analytically determined). The load of HBsAg/particles was 3.2 (µg/mg). Reprinted from [147] with permission from Elsevier, Copyright 2014.

Alginate and chitosan are two versatile biopolymers widely used in drug delivery systems, as they are oppositely charged and it is relatively simple to obtain hybrid systems from them. Taking advantage of the physical–chemical properties of those polymers, one can obtain interesting systems for carrying a myriad of antigens like protein, DNA, or RNA antigen; for instance, it is possible to encapsulate the cargo into the hybrid assembly or to obtain surfaces positively or negatively charged for adsorbing antigens. Regarding the adjuvant properties, one of the main limitations of oral vaccines is that antigens suffer degradation into the gastric cavity losing their native structures and antigenicity; using alginate and chitosan, it is possible to avoid this phenomenon [232–234]. In an interesting work where hybrid NPs from chitosan and alginate were synthesized for vaccine delivery, it was shown that alginate coating antigen/chitosan complex is a good strategy for avoiding antigen degradation from gastric environment. The system showed a pattern of controlled release and low in vitro toxicity; furthermore, alginate/chitosan hybrid NPs elicited systemic and mucosal immune response exhibiting the highest titer of antibody as compared with the controls [234]. However, alginate/chitosan hybrid anionic NPs failed in inducing a Th1 response when the NPs and a hepatitis B virus antigen were administered by the subcutaneous route in a mouse model [235].

Alginate and PLL were assembled as NPs by ionotropic complexation method. The NPs displayed a diameter between 130 and 850 nm, a negative zeta-potential, and a sustained-release behavior regarding the encapsulated BSA. Additionally, the system showed low cytotoxicity and significant increment of BSA internalization in vitro [236].

Hybrid NPs from combinations of biocompatible and cationic polymers have been less studied as antigen carriers. For example, hybrid systems between PEI and alginate are little explored in the context of vaccine delivery, although there is evidence that the two polymers form complexes by electrostatic interactions [237]; combinations of PEI with alginate reduce significantly the toxicity of the polycation favoring the degradation of PEI [238]. Alginate/PEI hybrid systems formed nanogels able to incorporate and deliver antigens enhancing both cellular and humoral response. Nanogels facilitated antigen uptake by mouse bone-marrow DCs; promoted intracellular antigen degradation and cytosolic release, and increased antigen presentation via MHC I and II, vaccine-induced antibody
production, and CD8+ T cell-mediated tumor cell lysis, suggesting that the nanogels are potent immunoadjuvants [239].

PEI-coated PMMA NPs synthesized by emulsion polymerization combined well with DNA and are a suitable alternative for gene delivery, showing low cytotoxicity and high transfection capacity [240,241]; however, these works did not report evidence of the immunoadjuvant activity of those assemblies.

Using emulsion polymerization of the two co-monomers, interesting functionalities were reunited in the same polymeric particle: the cationic moiety represented by the quaternary ammonium nitrogen of comonomer 1 and the hydrophilic poly (ethylene glycol) chains of comonomer 2; thereby poly (methylmethacrylate) (PMMA) was covalently modified to yield core–shell cationic nanoparticles that enhanced cellular responses induced by HIV-1 Tat DNA vaccination. These biocompatible core–shell cationic nanoparticles, composed of an inner hard core of poly (methylmethacrylate) (PMMA) and a hydrophilic tentacular shell bearing positively charged groups and poly (ethylene glycol) chains covalently bound to the core electrostatically and reversibly adsorbed DNA, efficiently delivered it intracellularly and were not toxic in vitro or in mice [242]. Furthermore, two intramuscular (i.m.) immunizations (4 weeks apart) with a very low dose (1 microgram) of the plasmid PCV-tat delivered by these nanoparticles followed by one or two protein boosts induced significant antigen-specific humoral and cellular responses and greatly increased Th1-type T cell responses and CTLs against HIV-1 Tat [242]. Along similar lines, PEG was covalently bound to the cationic polymer poly (2-aminoethyl methacrylate hydrochloride) in order to improve the stability of nanstructures in vivo [243]. Figure 13 shows the core–shell microparticles obtained by Castaldello and coworkers, 2006, incorporating both functionalities, cationic charge and the stability, in aqueous medium imparted by PEG [242].

![Figure 13.](image-url)

**Figure 13.** Scanning electron micrograph of PMMA core–PEG shell microparticles where the hydrophilic non-ionic co-monomer poly (ethylene glycol) methyl ether methacrylate and the cationic co-monomer 2-(dimethylocty) ammonium ethyl methacrylate bromine were used in the emulsion polymerization reaction. Reprinted from [242] with permission from Elsevier, Copyright 2011.

Non-covalent nanoparticle (NP) assemblies of PMMA, a synthetic, non-charged, and biocompatible polymer, and PDDA polycation have been described earlier by our group since the first report on the good compatibility and miscibility of PDDA with PMMA evaluated from microbicidal PMMA/PDDA NPs [160–163,210–214]. The synthesis of PMMA NPs by emulsion polymerization of MMA in the presence of PDDA yielded cationic, homodisperse, and stable NPs [160,212], which are presently being
evaluated in our group for possible applications as immunoadjuvants. In summary, it is possible to take advantage of the biocompatibility of PMMA and the cationic character of PDDA to investigate novel applications for these assemblies obtained in absence of any covalent linkage between the polymers. In addition, our group recently synthesized and characterized hybrid NPs from PMMA, PDDA, and surfactants that could be excellent alternatives for carrying antigen due to the combination of the biocompatibility of PMMA with the adjuvant properties of the cationic polymer PDDA and/or the cationic lipid DODAB [65,161,162]. Figure 14 shows the core–shell PMMA/PDDA NPs and a schematic picture of their nanostructure with PMMA in the core and PDDA non-covalently bound to PMMA, forming a surrounding shell [214]. One should notice that the hydrophobic PMMA is in the core and the cationic and hydrophilic PDDA is in the shell; upon increasing the ionic strength, the shell can collapse due to the screening of the PDDA charges [19–71].

Figure 14. Scanning electron micrograph of PMMA/PDDA NPs and schematic representation of the PMMA/PDDA NPs structure with their core–shell nanostructure that occurs at low ionic strength. Reproduced from reference [160].

Antigen endosomal escape has been observed for protein loaded in cationic and porous maltodextrin NPs [244]. Figure 15 illustrates the endosomal escape or permanence of two types of cationic maltodextrin NPs, those modified with anionic lipids in their cores (named DGNP+) and those with cationic cores (named NP+). The OVA/NPs intracellular traffic was determined from fluorescence-labeled OVA. Both types of NPs efficiently delivered OVA to cells; however, whereas those with the anionic inner core delivered OVA to the cytosol and endoplasmic reticulum, those with the cationic core were less able to release OVA in the cytosol and delivered it to the endosomes. One should notice the punctuated distribution pattern of OVA delivered by NP+ into cells, typical of endosomal localization and the diffused OVA localization throughout the cytoplasm when OVA was delivered with DGNP+ NPs. Both NPs increased intracellular proteolysis of OVA; however, DGNP(+) facilitated OVA escape from endosomes [244].

Cancer vaccines targeting patient-specific tumor neo-antigens have recently emerged as a promising component of the immunotherapeutic treatment against the disease [245,246]. However, neo-antigenic peptides typically elicit weak CD8+ T cell responses, so there is a need for universally applicable vaccine delivery strategies to enhance the immunogenicity of these peptides. Ideally, such vaccines could also be rapidly fabricated using chemically synthesized peptide antigens customized to an individual patient. An interesting approach for implementing the combination between biocompatible polymer and cationic polymer was achieved with the biocompatible propyl-acrylic acid and cationized antigen from covalently binding decalysine peptide to the antigen and then assembling this with the biocompatible polymer via electrostatic assembly; these NPs promoted cytosolic delivery of the antigen and MHC I presentation [245,247]. In addition, poly (acrylic acid) derivatives as mucoadhesive polymers might increase the epithelial permeability for carried antigens or drugs after easily crossing the mucus layer of the mucosae. The attachment to the mucus layer
could be achieved by exclusively non-covalent bonds such as ionic interactions, hydrogen bonds, and van der Waal’s forces, leading also to a certain extent to opening of the tight junctions [248]. Figure 16 shows a schematic representation of the electrostatically stabilized NPs assembled by mixing decalysine-modified antigenic peptides and poly (propyl acrylic acid) (pPAA) poly-anion. NPs increased and prolonged antigen uptake and presentation on MHC-I (major histocompatibility complex class I) molecules expressed by dendritic cells with activation of CD8\(^+\) T cells. The suitable intranasal immunization route inhibited formation of lung metastases in a murine melanoma model; further addition of the adjuvant α-galactosylceramide (α-GalCer) stimulated robust CD8\(^+\) T cell responses, significantly increasing survival time in mice with established melanoma tumors [245]. On Figure 16a, poly (propylacrylic acid)-cationic peptide nanosized assemblies aiming at enhancing antigen endosomal escape and MHC-I antigen presentation are prepared. On Figure 16b, one should notice the simple simple and rapid mixing of decalysine-modified antigenic peptides with poly (propylacrylic acid) (pPAA) forming antigen-loaded nanoplexes, which are electrostatically stabilized nanoparticles. On Figure 16c the scheme shows the nanoplexes promoting cytosolic antigen delivery via endosomal escape, resulting in enhanced levels of antigen presentation on class I major histocompatibility complex (MHC-I).

**Figure 15.** Comparison of ovalbumin (OVA) intracellular delivery by maltodextrin porous nanoparticles with outer and inner cationic charges (NP+) or outer cationic/inner anionic charges (DGNP+) using confocal microscopy to visualize OVA labeled with fluorescent markers (FITC-OVA). NP+ and DGNP+ were loaded with FITC-OVA and incubated for different periods with 16HBE cells. After 30 min incubation, cells were washed with PBS and fixed with 4% PAF. Intracellular FITC-OVA was visualized by confocal microscopy. Scale bar = 10 μm. Adapted from [244] with permission from Elsevier, Copyright 2012.
Figure 16. (a) Poly (propylacrylic acid)-cationic peptide nanosized assemblies for enhancing antigen endosomal escape and MHC-I antigen presentation. (b) Assembly of antigen-loaded nanoplexes via simple and rapid mixing of decalysine-modified antigenic peptides and pPAA, which generates electrostatically stabilized nanoparticles. (c) Schematic representation of nanoplexes promoting cytosolic antigen delivery via endosomal escape, resulting in enhanced levels of antigen presentation on class I major histocompatibility complex (MHC-I). Reprinted from [245] with permission from Elsevier, Copyright 2018.

Inside the endosome, PLGA erosion with release of the glycolic or lactic acids further reduced the pH, improving the antigen degradation and presentation through MHC-II [249,250]. Oral administration of antigens may lead to their uptake by microfold cells (M cells) in Peyer’s patches of intestine to initiate protective immunity against infections, but limitations such as the lack of specificity of proteins toward M cells and degradation of proteins in the harsh environment of gastrointestinal (GI) tract had to be overcome. Mucoadhesive vehicle of thiolated eudragit (TE) microparticles transported an M cell-targeting peptide-fused model protein antigen [251]. Thereby, oral delivery of TE microparticulate antigens exhibited high transcytosis of antigens through M cells resulting in strong protective sIgA as well as systemic IgG antibody responses. The delivery system not only induced CD4+ T cell immune responses but also generated strong CD8+ T cell responses with enhanced production of IFN-γ in spleen [251]. Along similar lines, the mucoadhesive, cationic, and biocompatible chitosan in combination with poly-ε-caprolactone yielded NPs effective in immunization against influenza [252].

In conclusion, hybrid assemblies of polycation and biocompatible polymer have the advantages of reducing the toxicity of the cationic polymer and adding the interesting properties of muco-adhesiveness so important for mucosal vaccines.

5. Cationic Assemblies of Lipid–Polymer and Polymer–Lipid

Assemblies obtained from combining lipids and polymers gather the benefits of lipids’ amphiphilic nature, possibility of high organizational level in the lipid-based structures, and the mechanical and resistance advantages offered by polymers; blending these materials, it is possible to overcome major limitations like degradation of bioactive incorporated principles, short circulation time of drugs and vaccines, and lack of controlled release [253,254]. Diverse types of arrangement between lipids and
polymers have been described; in general, these hybrid assemblies could be classified as lipid–polymer or polymer–lipid, depending on the position of each material in the hybrid structure; either the polymer or the lipid is in the inner part the nanostructure. In addition, polymer and lipid may mix, driven by intermolecular interactions, so that the lipid may become embedded in the polymer matrix displaying good compatibility with the polymer [210,212,213,253,254].

Lipids and polymers can assemble as hybrid materials driven by attractive, non-covalent, and weak but frequent multipoint interactions. For example, lipid deposition from charged bilayers onto oppositely charged spherical solid cores such as polymeric nanoparticles [37,205,255], silica [208,256–258], or hydrophobic drug aggregates [38,259] led to interesting and bioactive hybrid nanoparticles. The so-called biomimetic, lipid–polymer, or polymer–lipid NPs have been finding applications in biomolecular recognition [260], drug delivery [259,261–263], vaccine design [9,10,16,18,21,25,28,46,163], and antimicrobial chemotherapy [131,139–141,160,163,207,211,212,263–271].

The principal forces driving bilayer deposition onto hydrophobic or hydrophilic nanoparticles or surfaces from bilayer vesicles or bilayer fragments (BF) are the electrostatic attraction, van der Waals attraction, and/or the hydrophobic effect [37,205,255,256,258]. Medium composition, pH, and the ratio between the surface areas of the particles (Aparticles) and bilayers (Abilayers) also play a crucial role in achieving the optimal bilayer deposition on the particles: this ratio should be around 1 for complete coverage of all particles with bilayers [38,208,259–263]. The amount of added lipid must be sufficient to surround all particles in the dispersion with one bilayer; otherwise, poor colloidal stability may result with formation of aggregates. Figure 17 illustrates some possible interactions between one bilayer vesicle and two particles, vesicle, and particles with similar sizes [206].

**Figure 17.** The interaction between one bilayer vesicle and two particles. In the first step (step 1), electrostatic and/or van der Waals and/or hydrophobic attraction leads to aggregation of a vesicle and a particle. These same interaction forces may disrupt the vesicle bilayer and promote bilayer adsorption onto the microsphere (step 2) and/or further aggregation with the other microsphere (step 2'). The adsorbed bilayer may attract the second microsphere (step 3). The hydrophobic interaction between an eventually hydrophobic surface and the hydrocarbon chains in the bilayer may completely destroy the bilayer structure, flip-flopping the hydrocarbon chains onto the particle surface and generating a monolayer coverage on each microsphere (step 4). Adapted from [206] with permission from Elsevier, Copyright 1999.

When lipid bilayers are the starting nanostructure, their physical state is an important factor determining lipid–particle interactions; bilayer vesicles in the rigid gel state do not disrupt upon
adhesion onto solid particles, so bilayer coverage does not take place [272]. For avoiding this difficulty, either the lipid has to be changed to a lipid able to form bilayers in the liquid-crystalline, more fluid state at room temperature, or flat and open bilayer fragments of the charged lipid (BF) has to be used. BFs not only can adsorb onto solid particles but also can independently function as scaffolds for deposition of a variety of bioactive molecules such as peptides [207,265,267,268], drugs [131,207,259,263,264,269], antimicrobial polymers [139,141,163,266,273], proteins [21,25,28,40,46,140,141,163,260,264], oligonucleotides [16,46], or nucleic acids [41,42]. Adhesion of a DODAB vesicle layer onto the rough and highly hydrated surface of cells was electrostatically driven; cationic closed vesicles of DODAB at low ionic strength surrounded bacterial cells as a vesicle layer [274]; the absence of DODAB vesicle disruption upon interaction with the bacteria was depicted from absence of (14C)-sucrose leakage from the large vesicles in experiments where this marker was used to label the inner water compartment of the vesicles [275]. Given the quaternary ammonium moiety of the DODAB molecule, its antimicrobial effect was systematically evaluated and its differential cytotoxicity was reviewed [38].

Charged BFs are easily obtained from vesicles by ultrasonic disruption and have been used for the production of a variety of lipid-based biomimetic particles [37–39,41]. Deconstruction of the bilayer by solubilizing cationic lipid and drug or biocompatible polymer in a common solvent has also been a useful approach; this successfully allowed the obtaining of hybrid nanoparticles of hydrophobic drug/cationic lipid/ethanol in water dispersion surrounded or not by the biocompatible carboxy methyl cellulose biopolymer [276]. Another successful strategy was the optimization of single bilayer deposition onto silica nanoparticles from lipid films [209]. In order to ascertain whether bilayer coverage indeed took place, size distribution, polydispersity, and zeta-potential are often determined from dynamic light scattering (DLS) techniques [277] complemented by morphology evaluation from advanced electron microscopy techniques [131,160,212] and quantitative methods for obtaining adsorption isotherms [209]. Figure 18 shows some adsorption isotherms of the cationic lipid DODAB from DODAB films or from pre-formed bilayer fragments (BF) onto silica particles where maximal adsorption values yielded an adsorbed amount consistent with bilayer deposition on silica for depositions from DODAB films [209].

![Figure 18. Adsorption isotherms for DODAB from films or pre-formed bilayer fragments (BF) onto silica (2 mg/mL). The dashed line at 0.288 mM DODAB represents the theoretical concentration corresponding to bilayer adsorption. Reproduced from [209].](image)

If the lipid bilayers and the particles are oppositely charged, poor colloidal stability occurs at a critical lipid concentration where the size is maximum and the zeta-potential is zero [10,18,205,259,263]. Above this critical lipid concentration, the particles exhibit zeta-potentials that are similar to that of the charged bilayer and recover colloidal stability [10,18,205,259]. Aggregates of hydrophobic drugs...
dispersed in water have also been treated as particles and coated with lipids at high drug-to-lipid molar ratios \[140,259,263\]. More recently, protocols have evolved to employ bilayer disassembly and biopolymers for stabilization of very hydrophobic drugs such as indomethacin \[163,276\].

BFs combine well with several antigens and are available as cationic (made of DODAB) or anionic nanostructures (made of the anionic and synthetic lipid sodium dihexadecylphosphate or DHP), allowing combinations with both positively and negatively charged antigens. Transmission electron micrographs of DODAB BF \[278\] and DHP BF \[279\] are on Figure 19A,B, respectively.

![Figure 19. Lipid bilayer fragments of cationic and anionic synthetic lipids. (A) Bilayer fragments of dioctadecyldimethylammonium bromide (DODAB) visualized by cryo-transmission electron micrograph. Adapted from \[278\] with permission from 1995 American Chemical Society. (B) Bilayer fragments of sodium dihexadecylphosphate (DHP) visualized by transmission electron micrograph after negatively staining the sample. Disks were observed edge-on or face-on. Bars denote 100 nm](image1)

DODAB BFs loaded or unloaded with antibiotics were covered consecutively by a carboxymethylcellulose (CMC) and a poly (diallyl dimethyl ammonium chloride) (PDDA) layer \[131,141\] being efficiently captured by macrophages to deliver their antibiotic cargo against difficult intracellular pathogens, as are the mycobacteria \[270\]. The activity of DODAB BF/CMC or DODAB BF/CMC/PDDA in combination with antigens still requires further research.

The differential cytotoxicity of DODAB, its dose-dependent toxicity, and its ability to induce delayed-type hypersensitivity (DTH) in vivo, a marker for cell-mediated immune responses, pointed out the feasibility of using DODAB as an efficient immunoadjuvant mainly for veterinary uses but also in humans in a few instances \[9,13,52,57,280–282\]. Supramolecular assemblies of DODAB BF by themselves or after interaction with supporting particles were also combined with three different model antigens in separate and tested as immunoadjuvants \[9\]. DODAB-based immunoadjuvants carrying antigens at reduced DODAB dose (0.01–0.1 mM) induced superior DTH responses in mice in comparison to alum. Thus, the cationic immunoadjuvant was either reduced to a single-component, nanosized system—DODAB BF—or was a dispersion of cationic nanoparticles with controllable nature and size as obtained after covering silica or polystyrene sulfate latex (PSS) with a cationic DODAB bilayer. DODAB BF interacted with proteins via both the hydrophobic effect and the electrostatic attraction at low ionic strength. DODAB-based adjuvants exhibited good colloid stability while complexed with the antigens, complete absence of toxicity in mice (i.e., local or general reactions), and a remarkable induction of Th1 immune response at reduced doses of cationic and toxic DODAB lipid. DODAB vesicle disruption by probe sonication at low ionic strength (0.1–5.0 mM monovalent salt) produced DODAB BF which remained electrostatically stabilized in dispersion by the electrostatic repulsion in between fragments. DODAB BF also interacted with oppositely charged particles such as silica or polystyrene sulfate (PSS) latex to produce the cationic particulates. Figure 20 shows some
hybrid and cationic immunoadjuvants based on reduced DODAB doses and their compared DTH response [9,10,18].

Figure 20. Schematic representation of nanometric dioctadecyldimethylammonium bromide (DODAB)-based adjuvants inducing delayed-type hypersensitivity (DTH) in mice as compared to alum. The same antigen (Ag) carried by each adjuvant was used for immunization. Ag was carried by DODAB BF at 0.1 mM DODAB (DODAB BF/Ag) or by polystyrene sulfate nanoparticles (PSS)/DODAB or by silica/DODAB particles at 0.01 or 0.05 mM DODAB (PSS/DODAB/Ag or silica/DODAB/Ag), respectively, or by alum (Al(OH)₃/Ag). After immunization, elicitation of the swelling response was done by injecting Ag alone in the mice footpad so that % footpad swelling was measured in comparison to alum [9,10,18]. At the DODAB doses employed, DODAB cytotoxicity was absent against mammalian cells in culture. Adapted from [9,10] with permission from Elsevier, Copyright 2007 and Copyright 2009 and reference [18].
An interesting approach was described in order to apply immunotherapy associated with chemotherapy against *Leishmania* infection: solid lipid NPs using soya phosphatidyl choline and stearic acid were prepared by solvent emulsification-evaporation method followed by ultrasonication. These solid NPs were loaded with amphotericin B before coating with chitosan and displayed diameters lower than 220 nm and positive zeta-potentials showing low hemolytic activity and antileishmanial activity higher than the one for commercial AmBisome or Fungizone. The NPs were taken up by J774A.1 macrophages, which activated the production of TNF-α and IL-12; importantly, cytotoxicity experiments in vitro and acute toxicity experiments in mice evidenced the safety of the formulation in comparison to marketed formulations [283].

Cationic and porous NPs from maltodextrin and the anionic lipid 1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol (DPPG) were synthesized to evaluate their interaction with the nose mucosa; these NPs, combined with OVA displayed low toxicity, were efficiently taken up by airway epithelial cells and significantly improved the OVA delivery to the cells, increasing the permanence time of OVA at the nose mucosa to at least 6 h, in contrast to unformulated OVA, which remained 1.5 h only at the mucosa [284].

DODAB coating the biocompatible PLGA yielded cationic nanoparticles that elicited Th1 and Th17 responses [284,285]. Cationic NPs of PLGA, DODAB, and TDB in a nasal vaccine displayed nanometric sizes and high and positive zeta-potentials while carrying the outer-membrane protein (MOMP) antigen of *Chlamydia trachomatis*, inducing high titers of IgG2a, IFN-γ, and IL-17a [286].

Similarly, PLGA and DODAB were combined to yield cationic NPs for delivering a *Mycobacterium tuberculosis* nasal vaccine; PLGA/DODAB NPs showed uniform size, spherical shape, and smooth surface. PLGA/DODAB NPs loaded with HspX/EsxS antigen (a recombinant fused protein of *M. tuberculosis*) and with monophosphoryl lipid A (MPLA) increased the secretion of IFN-γ and IL-17 and enhanced the antibody titers of IgA, IgG1, and IgG2a [285]. In a different approach, cationic liposomes obtained from DOTAP, hyaluronic acid (HA), and PEG achieved a good immune response after intranasal vaccination with a recombinant antigen from *M. tuberculosis*; there was improved colloidal stability and prolonged antigen release. Besides, the typical cytotoxicity exerted by DOTAP was reduced 20-fold. DOTAP/HA/PEG NPs carrying OVA and MPLA promoted DCs maturation and up-regulation of co-stimulatory markers such as CD40, CD86, and MHC-II. Mice vaccinated with DOTAP/HA/PEG NPs carrying OVA and MPLA via intranasal route generated robust OVA-specific CD8(+) T cell and humoral responses; in addition, the intranasal inoculation of DOTAP/HA/PEG NPs co-loaded with MPLA and F1-V, a fused antigen from *Yersinia pestis*, induced a potent and long-lasting antibody production, evidencing that these hybrid liposome/polymer NPs are appropriate as a mucosal adjuvant [287].

PLGA/DC-Chol core/shell hybrid NPs synthesized by a double emulsion solvent evaporation method were tested as an adjuvant with the peculiarity that the antigen, OVA, was attached to the NPs in three different ways: inside the NPs (OVA in), adsorbed on the NPs (OVA ad), or both ways (OVA in/ad). After the internalization by DCs, FITC-OVA traffic from fluorescence microscopy showed that OVA ad and free OVA remained in the lysosomes, whereas OVA in or in/ad escaped from the endo-lysosome favoring cross-presentation. In vivo experiments showed that OVA in/ad provided not only adequate initial antigen exposure but also long-term antigen persistence at the injection inside. OVA in and OVA in/ad elicited significantly higher antigen-specific immune response than OVA ad [288].

Summarizing this topic, cationic lipids are very versatile and can either carry antigens by themselves via BF or impart positive charges to a variety of polymeric NPs under good control of their usual toxicity. They have the advantage of eliciting a potent cellular immune response in many instances. The adjuvant properties of the hybrid NPs have been often reviewed [21,25,34].
6. Conclusions

Cationic nanostructures such as the lipid-covered particles, the bilayer fragments, the cationic polymers, and the hybrid nanostructures of biocompatible and cationic polymers or lipids are interesting carriers with sizes below 100 nm. Their size directs them to the lymphatic vessels and antigen-presenting cells in the lymph nodes, and their positive charge allows efficient combination with important biomolecules such as peptides, proteins, nucleic acids, epitopes, and enhancers of the immune response.

The composition with lipids allows the inclusion of targeting in the microstructures that cannot directly reach the lymph nodes or that require special targeting to avoid degradation in the endosomes or to reach the cell nucleus.

The cationic nanostructures can protect the carried antigen for vaccine administration by the oral route and can increase the permanence time of the antigen/carrier assembly at the mucosae still enhancing the systemic immunity. The cationic nanostructures are particularly efficient in delivering antigens to APCs, allowing both antigen processing and presentation via MHC-II or processing and cross-presentation via MHC-I. The intracellular traffic depends on administration route and location of the antigen in the complex. The direct connection of the complex antigen/adjuvant to APCs in the lymph nodes avoids permanence at injection sites and local inflammatory reactions since they easily overcome the anatomical barriers due to their nanometric size. Their toxicity is easily controllable by using low concentrations of the cationic lipid or cationic polymer in the nanostructures.

There is a huge variety of cationic nanostructured materials available from nanomaterials science. However, most of them remain untested regarding their properties as immunoadjuvants or their intrinsic toxicity both in vitro and in vivo. This means that a huge area for biomedical research remains still unexplored and it is our hope that this review will be the trigger for further valuable research on vaccine design with novel cationic nanostructures.


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References

2. Coffman, R.L.; Sher, A.; Seder, R.A. Vaccine adjuvants: Putting innate immunity to work. *Immunity* 2010, 33, 492–503. [CrossRef]


32. De Serrano, L.O.; Burkhart, D.J. Liposomal vaccine formulations as prophylactic agents: Design considerations for modern vaccines. *J. Nanobiotechnol.* 2017, 15, 83. [CrossRef]


34. Carmona-Ribeiro, A.M. Biomimetic nanoparticles: Preparation, characterization and biomedical applications. *Int. J. Nanomed.* 2010, 5, 249–259. [CrossRef]


62. Sun, H.X.; Xie, Y.; Ye, Y.P. ISCOMs and ISCOMATRIX. Vaccine 2009, 27, 4388–4401. [CrossRef] [PubMed]


66. Chernikov, I.V.; Vlassov, V.V.; Chernolovskaya, E.L. Current development of siRNA bioconjugates: From research to the clinic. Front. Pharmocol. 2019, 10, 444. [CrossRef]


73. Schweneker, K.; Gorka, O.; Schweneker, M.; Poegg, H.; Tschopp, J.; Peschel, C.; Ruland, J.; Gross, O. The mycobacterial cord factor adjuvant analogue trehalose-6,6′-dibehenate (TDB) activates the Nlrp3 inflammasome. *Immunobiology* **2013**, *218*, 664–673. [CrossRef]


108. Brunel, F.; Darbouret, A.; Ronco, J. Cationic lipid DC-Chol induces an improved and balanced immunity able to overcome the unresponsiveness to the hepatitis B vaccine. *Vaccine* 1999, 17, 2192–2203. [CrossRef]


134. Wang, X.; Zheng, K.; Si, Y.; Guo, X.; Xu, Y. Protein-polyelectrolyte interaction: Thermodynamic analysis based on the titration method (†).*Polymers* 2019, 11, 82. [CrossRef]


162. Ribeiro, R.T.; Galvão, C.N.; Betacourt, Y.P.; Mathiazzi, B.I.; Carmona-Ribeiro, A.M. Microbicidal dispersions and coatings from hybrid nanoparticles of poly (methyl methacrylate), poly (diallyl dimethyl ammonium) chloride, lipids, and surfactants. *Int. J. Mol. Sci.* 2019, 20, 6150. [CrossRef]


170. Joo, I.; Emod, J. Adjuvant effect of DEAE-dextran on cholera vaccines. *Vaccine* 1988, 6, 233–237. [CrossRef]


211. Han, J.; Zhao, D.; Li, D.; Wang, X.; Jin, Z.; Zhao. Polymer-Based Nanomaterials and Applications for Vaccines and Drugs. Polymers 2018, 10, 31. [CrossRef]


225. Han, J.; Zhao, D.; Li, D.; Wang, X.; Jin, Z.; Zhao. Polymer-Based Nanomaterials and Applications for Vaccines and Drugs. Polymers 2018, 10, 31. [CrossRef]


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Abstract: Nanomembranes are the principal building block of basically all living organisms, and without them life as we know it would not be possible. Yet in spite of their ubiquity, for a long time their artificial counterparts have mostly been overlooked in mainstream microsystem and nanosystem technologies, being a niche topic at best, instead of holding their rightful position as one of the basic structures in such systems. Synthetic biomimetic nanomembranes are essential in a vast number of seemingly disparate fields, including separation science and technology, sensing technology, environmental protection, renewable energy, process industry, life sciences and biomedicine. In this study, we review the possibilities for the synthesis of inorganic, organic and hybrid nanomembranes mimicking and in some way surpassing living structures, consider their main properties of interest, give a short overview of possible pathways for their enhancement through multifunctionalization, and summarize some of their numerous applications reported to date, with a focus on recent findings. It is our aim to stress the role of functionalized synthetic biomimetic nanomembranes within the context of modern nanoscience and nanotechnologies. We hope to highlight the importance of the topic, as well as to stress its great applicability potentials in many facets of human life.

Keywords: nanomembrane; bionics; nanoscience; nanotechnologies; ion channels; biosensing; biointerfaces; neural interfaces; nanofluidics

1. Introduction

Biological nanomembranes are ubiquitous and fundamental, and no life as we know it would be possible without them. They ensure the structural integrity of cells and, in eukaryotic cells, of their organelles (including the most complex one, the nucleus) by defining their boundaries and shapes and by shielding their interior, while at the same time providing their defouling. Simultaneously with these protective functions, they enable extremely complex and selective exchange of matter between the cell/organelle and its environment. This includes highly sophisticated and balanced import and export of different matter, from ions to highly complex organic molecules, cell signaling based on receptors and many other functions. While at first glance it could seem that nanomembranes represent a simple envelope around the cell or organelle contents, in reality they are miraculously sophisticated nanomachines ensuring the very existence of life. It is not by accident that biologically functionalized nanomembranes are the most widespread and ubiquitous building block of life.

As far as the composition of biological nanomembranes is concerned, it depends on the type of the cell (prokaryotic or eukaryotic, plant or animal). The basic structure of a vast majority of cell membranes consists of phospholipid layers which are amphipathic, i.e., they simultaneously consist of a hydrophilic (or polar) head and a hydrophobic tail. When a cell membrane is formed, the hydrophobic tails are clustered together, while the hydrophilic head groups face outside of the membrane, thus forming a lipid bilayer [1–3], a two-molecule sheet structure with a thickness of about 4 nm. Biological nanomembranes contradict the widespread misconception that biochemical
processes must occur in water-containing environments only, since very complex biochemical and biological processes take place within the soft and movable yet definitely non-aqueous structure of the cell membrane [4]. Biological nanomembranes contain various functionalizing proteins that ensure exchange of matter with the external world and which can either associate with the polar “heads” of bilayer—the so-called peripheral proteins—or with the membrane matrix—integral proteins. There is a third group of membrane proteins, the non-permanent “ambiguous proteins”. The in-plane dynamics of the membrane system is described by the classic “fluid mosaic” model of Singer and Nicolson from 1972, which has been updated several times since, for instance by Engelmann in 1977 [4,5].

Biological cell membranes are often encased within cell walls that ensure mechanical robustness to the cell and a new level of protection, both from outside agents and inside phenomena like excessive swelling, while at the same time enabling traffic of matter to the cell and from it. They are often composed of polysaccharides. Animal cells do not have a cell wall. In the case of land plants, the wall most often consists of polysaccharide cellulose or pectin, and may incorporate lignin or cutin and some other constituents. In fungi, cell walls are often built of chitin, the same material that is encountered in the exoskeletons of arthropods. Algae also have cell walls made of polysaccharides (e.g., carrageenan and agar). In prokaryotic bacteria, cell walls are built of peptidoglycan. In Archea, the most often encountered constituents are surface layer proteins (S-proteins), glycoproteins, pseudopeptidoglycan and polysaccharides. Some single-cell algae even produce their cell walls from silicon dioxide.

It stands to reason that among the basic goals of biomimetics, especially within the framework of nanoscience, nanotechnologies and micro/nanosystems, one of the important places should belong to the synthesis and use of artificial nanomembranes, and especially those with imparted biomimetic functionalities. We have much to learn and copy from one of the most ubiquitous biological structures and one of the most perfect nanomachines in existence. Whenever possible, this is done using an extended toolbox of materials that includes both the substances that make up biological structures, and those rarely or never encountered in natural nanomembranes. The benefit of such an approach is that it helps us attain biomimetic properties that enhance or completely exceed the natural ones.

A maybe even more important goal is to ensure an artificial pathway to re-creating, enhancing and improving the already extremely rich functionalities of the biological membranes, imparted mostly by the permanently or temporarily associated proteins, including but not limited to the controlled and selective trans-membrane exchange of matter. The possibility of manipulating such fundamental properties of one of the most important building blocks of life in order to make synthetic replicas, enhanced versions, or even versions with completely novel functionalities, is of supreme importance, and the potential benefits for the quality of human life are staggering.

Multifunctionalized biomimetic nanomembranes can be used in a vast number of different fields. For instance, the property of selective transmembrane transport has already shown its value in separation science and technology [6]. Some particular application examples include wastewater treatment [7], desalination [8,9], removal of toxic chemicals [10], of bacterial and viral agents [11] and gathering and concentration of targeted species (e.g., precious metals) from seawater [12]. Another wide field of use is biomedicine and life sciences [13], and covers such diverse areas as biointerfaces including brain–machine interfaces [14], scaffolds for tissue growth [15,16], biosensors [17,18], drug delivery and targeting [19], various labs-on-a-chip [20], DNA sequencers [21], etc. A field where the benefits of biomimetic nanomembranes are already starting to lead to large advancements is renewable energy [22]. For instance, artificial ion channels have proven themselves useful for proton-exchange fuel cells [23]. In addition, there are fields where the biological functionalities are combined in completely novel ways, like in highly sensitive and selective biosensors [24,25], various passive and active optoelectronic devices [26], plasmonic structures and devices [27,28], metasurfaces [29,30], etc.

Some of the research on freestanding synthetic nanomembranes reaches as far back as to the 1930s [31], but the real advent of related investigations occurred with the discovery of graphene [32,33] and fabrication of other quasi-2D materials like silicene [34], molybdenum disulfide [35], borophene [36],
phosphorene (black phosphorus), germanene [37], stanene [38], plumbene [39], MXenes [40–44] and other 2D materials synthesized to date [45].

The first freestanding artificial nanomembranes had only modest aspect ratios, but since 2004 the experimental designs have started to evolve rapidly and the range of available materials has been expanding ever since. Historically, the development of freestanding nanomembranes, including their fabrication, functionalization and, in recent times, application, proceeded in the direction of larger and more durable membranes, in spreading the toolbox of the available materials and the number of types of the freestanding structures and in introducing them to various field of human life. Today there is a vast number of different types of synthetic nanomembranes and mechanisms of their biomimetic functionalization. This development is continually expanding to encompass more and more fields, and the focus is gradually shifting to targeted structures that are fit for specific applications. The aim of this paper is to review this exciting and ever-expanding field, concentrating on the more recent achievements. Some topics and even whole fields are surely skipped, as well as some important teams who made immeasurable contributions to the subject of biomimetic nanomembranes. The authors are only left to say in their defense that such things unavoidably happen with basically all reviews of this kind. Of course, we did our best to avoid such occurrences. We apologize to all of those who significantly contributed to this vast field and whose work was nevertheless omitted or presented in insufficient detail or at an inadequate level.

In this review paper, we first systematically outline various types of the synthetic nanomembranes described so far, then we present a short overview of the methods for their fabrication. This is followed by a review of different approaches to nanomembrane biomimetic functionalization, together with a consideration of the main synthetic functionalizing structures drawn from biology, including, among others, artificial ion channels, ion pumps and nuclear pore complexes. The rest of the paper describes in some detail different applications of multifunctionalized biomimetic synthetic nanomembranes. We continue with a description of some possible future research directions and finish with a conclusion.

In this article, we directed our maximum efforts to render the text as exhaustive as possible and to cover as many diverse fields connected with biomimetic nanomembranes as we were able. One of the highlights is the inclusion of the most recent developments in the field. About two thirds of the references we cited were published within the last five years, while a number of them were published in 2020. Another contribution is that many topics appearing throughout this review, to the best of the authors’ knowledge, have never before been reviewed in dedicated reviews. Among the key points is the comprehensive review of synthetic structures for biomimetic functionalization.

2. Principles

2.1. Definitions and Terminology

Here we define the synthetic nanomembrane as an organic, inorganic or hybrid quasi-2D artificial freestanding or free-floating structure with a thickness below 100 nm, with the bottom limit being a monatomic/monomolecular thickness, and simultaneously having a large thickness-to-lateral size aspect ratio. “Large” here means at least 2 to 3 orders of magnitude, but much higher aspect ratios are readily achieved. If the obtained aspect ratio is one million or higher, such nanomembranes are denoted as “giant” [46]. One may notice that this definition excludes nanocomposite membranous structures with a thickness exceeding 100 nm, although there are some instances in the literature where the term “nanomembranes” is reserved for the above described structures with a thickness of up to 300 nm–400 nm. Additionally, the designation “nanomembranes” is sometimes used for much thicker structures with high aspect ratios, even those in excess of hundreds of micrometers, if they are nanostructured. A typical example would be nanoporous membranes for separation, which are also sometimes denoted in the literature as nanomembranes.

Biomimetic nanomembranes can be defined as a subclass of synthetic nanomembranes with some of their functionalities partially or fully mimicking those of natural biological membranes. This means
that we can use any material or structure that in itself is not biomimetic for their production and functionalization, for instance metals or other inorganic materials and structures such as carbon nanotubes or metalorganic frameworks, as long as they perform a function within the nanomembrane that mimics a biological one. It is not the composition, but the function that makes something biomimetic. Our definition excludes the use of natural biological structures as parts or building blocks—the biomimetic membranes and their functional parts we consider here are purely synthetic.

Freestanding nanomembranes have been variably called “interfaces without bulk” [47], “quasi-2D objects”, “freestanding films”, “self-supported films”, “unbacked films”, “suspended films”, and, depending on the type of a nanomembrane, “free-floating films”. The word “films” in all of these expressions may be replaced with “nanofilms”, “ultrathin structures”, “2D objects”, “2D structures”, or simply “membranes”.

On the other hand, the term “nanomembranes” has been used in the literature to refer to quasi-2D structures backed with some solid support or substrate. For instance, in separation science a porous nanomembrane to be used as a nanofilter or a nanosieve will often be backed with a macroporous substrate whose function is to ensure a robust support without hindering fluid flow. Despite being denoted as “nanomembranes”, such structures may have a thickness of the order of hundreds of micrometers, even a few millimeters. The term has been even sometimes been applied to fully macroscopic membranes, simply because they are nanostructured (for instance, they may have nanometer-sized pores).

2.2. Properties of Non-Functionalized Synthetic Nanomembranes

A biomimetic nanomembrane may be self-supported in air/vacuum or it may be positioned between two different fluids (including gas/liquid, a very frequently encountered combination). As a rule, we do not consider nanomembranes transferred and fixed to any kind of solid support. We chose the upper thickness limit of 100 nm because simple scaling laws valid from the macroscopic world to the microsystems cease to be applicable at approximately that point. Physics and chemistry of nanomembranes often simply do not obey the scaling laws. Quantum effects become necessary for the description of charge carrier transport, heat transfer and even optical effects (the appearance of the evanescent fields and localization effects) [48]. Additionally, nanofluidics is governed by a different set of rules in comparison to either conventional fluid mechanics or even microfluidics [49]. This “nanofluidic book of rules” must be applied when describing fluid transport through nanometric pores in nanomembranes. Even mechanical properties do not remain the same—for instance, Young’s modulus is modified and becomes thickness-dependent [50]. Many surprising and counter-intuitive phenomena arise, of which we list some in the remainder of this subsection.

Counterintuitively, synthetic nanomembranes may be very robust and tough, especially bearing in mind their minuscule thickness. It has been recorded that even monatomic/monomolecular membranes are able to withstand single-sided pressures in excess of a few bar [47]. It has also been noted that 35 nm thick composite giant nanomembranes made of polymer host containing zirconium oxide are sufficiently strong to hold quantities of liquid 70,000 times heavier than the nanomembranes themselves [51].

The property of toughness is at the same time combined with extreme foldability, i.e., the flexural rigidity of freestanding nanomembranes is extremely low. This is valid even for those membranes consisting of relatively brittle materials that are easily damaged or broken if their thickness is macroscopic. For instance, Vandamme et al. [51] showed that a nanomembrane can reversibly, repeatedly and without any damage pass through a syringe with an internal diameter 30,000 times smaller than the membrane area and unfurl itself after being ejected into fluid, completely restoring its previous shape (Figure 1).
We propose one such classification in Table 1. In the further text, we proceed by elaborating further details on some of the main classes of artificial freestanding nanomembranes.

From the point of the mechanics and the theory of elasticity, external pressure is most often balanced in nanomembranes exclusively by in-plane membrane stresses, i.e., without a bending rigidity component. This stems from the theory of plates and shells [53,54], to which thick plates, diaphragms, membranes and nanomembranes all belong and conform. However, there are exceptions, which form the basis of stress engineering in nanomembranes.

Nanomembranes exhibit extremely large surface-to-volume ratios (“interfaces without volume”) [47]. This proves useful for those chemical or biomedical applications that are proportional to the active surface (e.g., catalytic or enzymatic function, drugs delivery) or require soft and pliable materials (e.g., implantable structures). A consequence of their enormous aspect ratio is that nanomembranes represent one of the rare nano-objects that can be seen by the naked eye and manually handled with only a modest degree of precautions [55].

The nanomembrane stiffness decreases with their thickness and the critical bending radius also decreases. As an example, we quote the case of silicon nanomembranes [56]. It has been shown that a brittle silicon wafer becomes foldable and stretchable at low values of thickness. A silicon membrane 300 nm thick has a critical bending radius of over 5 µm. At the same time, a silicon nanomembrane 10 nm thick has a bending radius of 500 nm [57]. Thus, biomimetic nanomembranes are highly stretchable and easily assume the shape of the surface to which they are transferred (mechanical conformability). Such low flexural rigidity makes them convenient for stretchable and foldable electronics [56].

Nanomembranes generally, not only the biomimetic ones, exhibit self-healing (self-repair) properties [58,59]. This means that small damage is spontaneously restructured and repaired. A possible explanation for this effect is that in such a low-dimensional structure, the in-plane dynamics of its constituents are very different and much higher than that in bulk.

Biomimetic nanomembranes offer the possibility of integrating bio-inspired functionalities with those seldom or never found in nature. Examples include plasmonic biomimetic nanomembranes and nanomembranous metasurfaces [27,60].

2.3. Types of Synthetic Nanomembranes

Synthetic membranes can be classified based on the types of material used for their fabrication. We propose one such classification in Table 1. In the further text, we proceed by elaborating further details on some of the main classes of artificial freestanding nanomembranes.

Figure 1. Successive steps after local administration of metal-composite nanomembrane by syringe; 1. membrane being ejected into liquid or tissue, but still partly within the needle; 2. membrane completely ejected into the fluid and beginning to unfurl; 3. fully unfurled membrane. The authors' own work from [52].
Table 1. Classes and types of synthetic nanomembranes based on membrane material.

<table>
<thead>
<tr>
<th>Classes of Synthetic Nanomembranes</th>
<th>Types Belonging to the Class</th>
</tr>
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<tbody>
<tr>
<td>Inorganic nanomembranes</td>
<td>Metal nanomembranes</td>
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<tr>
<td></td>
<td>Metal composite and alloy nanomembranes</td>
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<tr>
<td></td>
<td>Diamond nanomembranes</td>
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<tr>
<td></td>
<td>Diamondoids</td>
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<tr>
<td></td>
<td>Diamond-like carbon (DLC) nanomembranes</td>
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<tr>
<td></td>
<td>Semiconductor nanomembranes—single element</td>
</tr>
<tr>
<td></td>
<td>Semiconductor nanomembranes—compounds</td>
</tr>
<tr>
<td></td>
<td>Freestanding monatomic sheets (incl. graphene)</td>
</tr>
<tr>
<td></td>
<td>Freestanding inorganic monomolecular sheets</td>
</tr>
<tr>
<td>Hybrid organic/Inorganic</td>
<td>Interpenetrated structures</td>
</tr>
<tr>
<td></td>
<td>Metal–organic frameworks</td>
</tr>
<tr>
<td>Organic nanomembranes</td>
<td>Single-polymer (pure)</td>
</tr>
<tr>
<td></td>
<td>Copolymer (blended)</td>
</tr>
<tr>
<td></td>
<td>Carbon nanomembranes (CNM)</td>
</tr>
<tr>
<td>Synthetic biological nanomembranes</td>
<td>Model lipid bilayers (black lipid membranes, painted bilayers, synthetic lipid bilayers)</td>
</tr>
</tbody>
</table>

2.3.1. Inorganic Nanomembranes

Metal Nanomembranes

The first freestanding pure metal ultrathin membranes were produced as early as 1931 (unbacked gold) [31]. Their thickness was about 200 nm. The lateral dimensions of their filament structures, however, were quite small, about 25 µm × 3 mm. The production technologies for pure metal freestanding nanomembranes evolved over years and Jia et al. reported in 2019 a routine production of large-area (up to 75 cm²) freestanding gold nanomembranes with arrays of nanoholes and with thickness values as low as 50 nm [61].

Metal Nanocomposite (Mixed Matrix) and Alloy Nanomembranes

These include constituents mixed at the atomic/molecular level or consisting of intermixed nanocrystallites. An example would be nanomembranes in which metallic alloys are their basic material [62]. Among other examples are nanomembranes that represent nanocomposites of metals with non-metallic components; e.g., in [55] chromium-based nanomembranes with giant aspect ratios (>1,000,000) containing silicon and oxygen atoms were reported.

Diamond

Freestanding sub-micrometer diamond membranes were reported in [63]; however, their smallest achieved thickness was 210 nm. To produce these diamond membranes, the authors used a double-implant process, followed by annealing, and finally wet etching. For many years, the reported thickness was a de facto standard for ultrathin diamond membranes [64–66]. However, Yoshikawa et al. succeeded in producing nanocrystalline diamond freestanding nanomembranes with the thickness values between 10 nm and 50 nm [67]. Many freestanding diamond membranes were used to fabricate 3D self-assembled forms using the methods of rolled-up nanotechnology [65]. The obtained forms included, among many others, tubes, nested tubes, rings, nested rings, nanoribbons, jagged nanoribbons, and various helical structures. Diamond nanomembranes were used for helical biomimetic structures with controllable properties in [66].

Diamondoids

Diamondoids [68] are diamond crystal molecules formed in the shape of cage, fused to each other, terminated by a hydrogen atom and superimposed upon diamond (bulk or thin film). The simplest
diamondoid is adamantane, consisting of only one diamond cage; a higher diamondoid is tetramantane, which contains of four cages, etc. Self-assembled monolayers of tetramantane with large areas were reported in [69]. The stability of diamondoids can be enhanced by graphene.

Diamond-Like Carbon

Diamond-like carbon (DLC) or hard carbon is an amorphous allotrope of carbon, containing mostly sp\(^3\)-hybridized carbon atoms. It is characterized by high hardness akin to that of diamond. Out of seven different existing forms of DLC that differ in the types of crystal lattices of their crystallites, the hardest is tetrahedral amorphous carbon. Young’s modulus of DLC may reach over 500 GPa, similar to that of natural diamond. The discovery of DLC was announced by Aisenberg and Chabot in 1971 [70]. The material has been since intensively used for hard coating protection of steel machine tools and has excellent tribological properties.

Freestanding DLC nanomembranes were used as ultrafast nanofilters of viscous liquids in [71]. They showed high hardness and at the same time resistance to organic solvents, similar to thin DLC films deposited on metal substrates.

Semiconductor Nanomembranes

Among the most often used semiconductor nanomembranes are those made of silicon [56]. Due to maturity of silicon microelectronic and microsystem fabrication technologies, processes are available that enable production of relatively low-cost and high-quality freestanding Si nanomembranes with arbitrary thickness and giant aspect ratios. Such membranes are used in a plethora of applications due to their favorable properties, which include high flexibility (convenient for foldable and stretchable electronics), good thermal properties (due to phonon confinement in structures with nanometric thickness), desirable electrical and optical performance due to electron confinement in the quasi-2D structure (convenient for microelectronic and optoelectronic devices), good dissolution and degradability over time in bodily fluids (convenient for implantable structures intended for biomedicine), etc.

The most frequently used technique for fabrication of high-quality freestanding single crystalline silicon nanomembranes is etching of commercially available silicon-on-insulator (SOI) wafers, first that of silicon in order to obtain the desired thickness of the thin top layer, then the sacrificial etching of the buried silica layer in order to completely remove the nanomembrane from the substrate. Layers with thickness as low as 2 nm have been thus fabricated, although typical values of SOI-obtained Si nanomembrane thickness fall in the range between 20 nm and 50 nm.

Other semiconductor materials can be formed into freestanding nanomembranes as well. Examples include molybdenum disulfide (MoS\(_2\)) whose bandgap from indirect in bulk materials transforms to a direct one in nanomembrane form, germanium sulfide (GeS), germanium selenide (GeSe), perovskite Sr\(_2\)Nb\(_3\)O\(_{10}\), among others [72]. An often-used method to produce such nanomembranes is either chemical etching exfoliation (for instance, in aluminum arsenide/gallium arsenide sandwiches the AlAs layer is sacrificially etched in the course of exfoliation or, as another example, in aluminum gallium arsenide (AlGaAs)/gallium arsenide (GaAs) multilayers the AlGaAs layers are sacrificial.)

Freestanding Monatomic Sheets

The prototype monatomic material is graphene [73–75]. It is a crystalline allotrope of carbon in the form 2D hexagonal lattice consisting of a single layer of carbon atoms. Its mechanical strength is two orders of magnitude greater than that of steel. Graphene represents a zero-bandgap semiconductor, its valence band touching its conduction band, and shows plasmonic behavior. Atomically thin freestanding graphene membranes have been made, and they showed high mechanical robustness and chemical stability [76]. They can be made porous or non-porous. Their proposed applications include DNA sequencing, water filtering and purification and various sensing applications.

Besides carbon, other chemical elements that form 2D allotropes include 2D silicon (silicene), 2D boron (borophene), 2D phosphorus (phosphorene or black phosphorus, also blue phosphorus) 2D
germanium (germanene), 2D tin (stanene), 2D lead (plumbene) and 2D bismuth [45]. All of these 2D sheets have been experimentally produced by exfoliation technique, by physical vapor deposition, PVD or chemical vapor deposition, and CVD (including atomic layer deposition, ALD).

Freestanding Inorganic Monomolecular Sheets

The prototype monomolecular materials are MXenes [40], defined as compounds consisting of carbides, nitrides or carbonitrides of early transition metals (Ti, Cr, Sc, V, Nb, Zr, Hf, No or Ta). Their atoms form 2D monolayer sheets that are interconnected into laminar structures such that the manner of obtaining the sheets is to exfoliate them by chemical etching. They can be described as electrically conductive clays. There is a large number of 2D materials that belong to MXenes, with a wide range of different properties, with the most frequently encountered one among them being titanium carbide [77]. Other 2D compound materials include gallium arsenide, transition-metal dichalcogenides, sulfides, selenides and tellurides of tungsten, niobium, molybdenum or tantalum, aluminum carbide, cadmium selenide, and many more.

2.3.2. Organic/Inorganic Hybrids

Interpenetrated Structures

Usually this term denotes structures made of polymers and reinforced or otherwise functionalized by inorganic nanofillers. As an example, in 2006 Vendamme et al. produced hybrid organic/inorganic nanomembranes using spin-coating. They fabricated free-standing, 35-nm-thick membranes with lateral dimensions of several centimeters consisting of polyacrylate interpenetrated with zirconia (ZrO$_2$) [51]. In 2007 the same research team fabricated another type of large interpenetrated organic/inorganic membranes, this time a few tens of nanometers thick, of elastomeric polyacrylate networks interpenetrated with silicon dioxide [78]. Polymer membranes to which reinforcing nano-building blocks were added could also be classified to this group, a prime example being nanomembranes reinforced with single- or multiple-walled carbon nanotubes (CNT.)

Metal-Organic Frameworks

Metal-organic frameworks (MOF) represent 1D, 2D or 3D nanocrystalline compounds, usually with a highly porous structure, which consist of metal ions or ion clusters and organic molecules [79,80]. Their tailorability, combined with adsorbability, makes MOF nanomembranes convenient for different highly selective sensing applications, as well as for their use as efficient molecular sieves [81].

2.3.3. Organic Nanomembranes

Completely organic freestanding nanomembranes that are in entirety composed of one or more organic materials represent a huge class of the existing freestanding nanomembranes. There is a vast number of organic compounds and their combinations that could be used.

Organic materials are defined as carbon compounds. That means that they do not include pure carbon in any of its allotrope forms. However, this classification also excludes the simplest compounds of carbon, like oxides of carbon, carbonates, carbides, and cyanides. All other carbon compounds belong to organic substances. This does not mean that any organic compound can be used to make nanomembranes—some of these compounds are gaseous or liquid, while others simply cannot be formed into membranous structures. It appears that the best suited organic compounds for production of freestanding nanomembranes are many of those with macromolecular/polymeric structure. They are characterized by large molecules, sometimes very large. These include polysaccharides [19], synthetic lipids [82], synthetic polymers [83], proteins [84], DNA [85] and RNA [86]-based membranes.

Most macromolecular nanomembranes are characterized by some common traits. They are usually highly sensitive to elevated temperatures and actually retain their useful properties in a narrow temperature range, are attacked and often dissolved by organic solvents and sometimes are even
endangered by increased humidity. Their mechanical properties tend to be impaired with a decrease of membrane thickness. Most of them have a low Young’s modulus. They usually start to creep and are permanently plastically deformed under permanent stress. Probably the first produced organic nanomembrane was made in 1907 by Bechhold from pyroxylin (nitrocellulose, collodion) [87].

Here we consider some exemplary types of macromolecular nanomembranes, those consisting of synthetic polymers. Depending on their composition, they may be classified into single-compound (pure) [88], and blended polymer (copolymer) [89] nanomembranes.

Single-Compound (Pure) Organic Nanomembranes

As an example, here we quote a small number of polymers used to date to fabricate macromolecular nanomembranes. They include epoxy resins [88], polysulfone [90], polyethersulfone [7], polyacrylate [91], polycarbonate [90], polystyrene [92], nylon [93], cellulose [94], nitrocellulose [87], generally polysaccharides [19], polyamide [90], polyimide [95], polydioepamine [96], polypolyamine [90], polyurethane [19], poly(methyl methacrylate) (PMMA) [97], polyvinylchloride (PVC) [90], polyester [98], polytetrafluoroethylene (PTFE, Teflon) [90], poly(vinylidene fluoride) [90], poly(lactic acid) [99], polyacrylonitrile (PAN) [90], polydimethylsiloxane (PDMS) [90] and many more. There is a host of convenient macromolecules besides those listed here and their systematic classification falls by far outside the scope of this work.

Polymer-Composite (Copolymer) Organic Nanomembranes

Probably the most often used polymer-composite blend is block copolymers [89], simultaneously made from two or more monomers and consisting of homogeneous blocks of pure polymers whose number is identical to that of the monomers used for polymerization. Numerous combinations of the above-listed polymers can be also used to fabricate copolymers, as well as many macromolecules not even mentioned here. The number of possible combinations is literally endless. More details on block copolymers and their ordered self-assembly can be found later in this text, in Section 3.2.

Carbon Nanomembranes

Carbon nanomembranes (CNM) [100] actually represent freestanding monomolecular sheets of cross-linked carbon precursors, fabricated by self-assembly. Thus, their name is somewhat misleading, since they are not composed solely of carbon atoms as one may assume, but are based on carbon instead. Thus, they are a separate group within the wider class of organic nanomembranes. They can, however, be converted to freestanding graphene sheets, for instance using pyrolysis. CNM are about 1 nm thick (a single molecular layer), and despite this they show remarkable mechanical strength. They can be produced as continuous surfaces or they can be built with a system of pores. Their precursors can be a variety of organic molecules, including but not limited to, polyaromatic thiols—oligophenyls and condensed polycyclic hydrocarbons. Recently, terphenylthiol monomolecular CNM nanomembranes with subnanometer pores acting as water channels were used for ion exclusion filtering and separation [101].

The production of CNM includes self-assembly of organic precursors into ordered monolayers of molecules on a solid surface, their subsequent cross-linking by forming covalent bonds between neighboring molecules using electrons, electromagnetic radiation or ions, and finally their release from the surface as free-standing or free-floating monolayer nanomembranes. The CNM unite extremely low thickness of graphene with simple production and easy and versatile surface functionalization, some examples of which include linking with fluorescent materials, dies, target-specific ligands or biomolecules. There are some excellent review papers of CNM, including [47] and [100]. They cover the fabrication of carbon nanomembranes, their functionalization and applications.
2.3.4. Model Lipid Bilayers

Model lipid bilayers are actually synthetic lipid bilayer nanomembranes. Strictly speaking, they could be classified into the previously described wide group of organic nanomembranes. However, since they represent replicas (and in some cases even upgrades) of the living nanomembranes, we decided that they merit a class of their own, belonging more to synthetic biology than to general organic chemistry.

Historically, the first model lipid bilayers were synthesized in 1962 [102], and were at first known as the so-called “black lipid membranes” and also as “painted bilayers”. They were intended as scaffolds to study the membrane processes in vitro, to facilitate the analysis of the transmembrane mechanisms and the function of ion channels.

Many different model lipid bilayers were subsequently fabricated, and they are being developed at this very moment. A review of methods and approaches to synthesis of artificial lipid bilayer membranes has been written by Siontorou et al. [103].

Today functionalized model lipid bilayers are built, among other purposes, for experiments with artificial cells in synthetic biology (the ultimate research subject in biomimetics—the fabrication of biochemical “artificial life” i.e., “synthetic biogenesis.”)

3. Fabrication

3.1. General Strategies

In this subsection we present the three main generic approaches to the fabrication of freestanding nanosheets/nanomembranes. This classification is based on the prevailing strategies used in micro- and nanomanufacturing. Besides the well-known top-down approach, used in the semiconductor industry and in microsystem fabrication, and the bottom-up approach, used for chemical synthesis and most often applying various self-assembly techniques, here we quote the third method, exfoliation of nanometer-thick sheets from starting macrostructures, which may be obtained in a variety of ways. As far as the starting substrates go, they may be solid or liquid. A summative table is given below (Table 2).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Fabrication Strategies</th>
<th>Particular Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid</td>
<td>Sacrificial structure</td>
<td>Top-down (thin film technologies)</td>
</tr>
<tr>
<td></td>
<td>Exfoliation</td>
<td>Mechanical or chemical delamination</td>
</tr>
<tr>
<td>Liquid</td>
<td>Gas–liquid interface</td>
<td>Bottom-up (self-assembly) Thin film technologies</td>
</tr>
<tr>
<td></td>
<td>Liquid–liquid interface</td>
<td>Self-assembly</td>
</tr>
</tbody>
</table>

In most situations, after the fabrication procedure, nanomembranes will have to be either completely freed from the surrounding liquid solution by direct removal from it, or in some cases by replacing the original liquid with another one. Liquid interchange represents an intermediate step before extracting the membrane. In that case, one replaces a liquid with a higher surface tension that could damage or destroy the membrane during extraction with one having a lower surface tension.

3.1.1. Solid Substrates and Etching of Sacrificial Structures

This is a method with its roots in microelectromechanical system (MEMS) technologies, i.e., it belongs to the top-down procedures. In short, one first chooses some kind of material (often silicon) as a substrate. A layer to be selectively etched and thus removed (“sacrificed”) at a later stage may then be deposited on the substrate. An ultrathin (thickness below 100 nm) layer of material to become a nanomembrane is deposited over the sacrificial layer. Some means of etching that selectively removes
the sacrificial layer material is then applied. After this process, the nanomembrane remains free where the sacrificial layer was. If the etchant was a liquid, the nanomembrane remains freely floating in the etchant solution or, alternatively, suspended on its edges connected to the solid substrate. If a non-liquid etching procedure was applied, then the latter part of the procedure is still valid and one is again left with a freely suspended ultrathin structure.

A variant of this approach is that the substrate, made of single crystalline silicon, is directly etched (for instance, through an opening in a previously deposited photolithographic mask) using anisotropic bulk micromachining. Etching is applied through the mask opening and finished when the nanomembrane layer is reached, since etchant does not react with it. The remaining part of the substrate serves as the edge support for the suspended freestanding nanomembrane. In this manner, the substrate performs the dual role of being simultaneously the support and the sacrificial layer.

3.1.2. Fabrication on a Liquid-Air Interface

Among the generic methods for nanomembrane fabrication, an important position belongs to structures fabricated without a solid substrate. Most of the self-assembly (bottom-up) methods and generally chemical synthesis of ultrathin free-floating films may occur at liquid-air interfaces (the surface of liquids). Basically, the method consists of synthesizing or depositing a free-floating film on the surface of a liquid. Many ultrathin film deposition methods used with solid substrates (top-down) are applicable here as well. The difference is that here one does not have to produce a sacrificial layer, and instead finishes with the nanomembrane already floating on the surface of the solution. The approach is especially useful for soft organic membranes. The method has even proven itself convenient for high-quality nanocrystalline nanomembranes [104].

3.1.3. Exfoliation

The third generic procedure for nanomembrane fabrication is direct detachment of monatomic/monomolecular or multilayer films from a bulk material. It is used if the starting material already has a laminar structure (i.e., consists of nanosheets of material to become nanomembranes or “nanoflakes”. Such is the case with many materials used to produce monolayers, e.g., graphene, various types of MXenes, etc. Single or multiple layers may be detached from the structure using the “Scotch tape” method, i.e., by literally sticking a piece of adhesive tape to the laminar structure with weak interlayer bonds and simply pulling it off, after which flakes of monatomic/monomolecular nanomaterial remain attached to the tape [75,105]. In other words, this kind of exfoliation is done in a purely mechanical way. This method has been successfully applied to obtain even relatively large graphene monolayers (size on the order of a millimeter). It was the chosen method when fabricating the first graphene monolayers.

Another approach to exfoliation is applied if the bond between layers is strong enough to render mechanical cleavage useless. In such situations, one uses a liquid etchant to remove the bonds between the neighboring monolayers, leaving them freely floating in the etchant solution. This is the approach used to obtain MXenes [106]. Other exfoliation methods include the electrochemical approach [107], sonication using ultrasound in some kind of fluid (e.g., ionic liquid, mixture of two immiscible liquids) [108], cleaving by a sharp edge, shearing by mixers, etc.

3.2. Nanomembrane Production Methods

At the beginning of this subsection we would like to describe a distinction between additive/subtractive manufacturing and top-down/bottom-up approach, which denote completely different processes. The additive/subtractive fabrication regards what is done with the membrane material (additive—new material is added to the membrane; subtractive—material is removed from it, typically to obtain a pore). The top-down/bottom-up approach regards how addition/subtraction is done (top-down refers to standard planar, microelectronic, MEMS and NEMS batch processing,
while bottom-up is actually self-assembly). Both top-down and bottom-up approaches can be used to add or remove material.

3.2.1. Top-Down Approach: Thin Film Technologies

A majority of ultrathin film deposition techniques used in microelectronics and microsystem (MEMS) technologies can be used in the production of biomimetic nanomembranes as well, combined with the sacrificial layer etching technique. An excellent source of information on different microsystem techniques of deposition in general can be found in the seminal work of Madou [109]. Previously, we reviewed different techniques applicable for nanomembrane fabrication in [110], so in this work we cover the field only briefly, accentuating the new developments that have become prominent in recent years. For a more in-depth approach, although it is necessarily an older report, we direct the readers to the mentioned review.

The top-down deposition techniques can be divided into two main groups: physical methods and chemical methods. We only list here the main procedures. Some procedures are present in both lists, because they may include chemical processes, but do not have to.

Physical methods for depositing nanomembranes in a controlled way include radiofrequency sputtering, evaporation (e.g., thermal evaporation and laser-assisted evaporation), physical vapor deposition (PVD), epitaxial growth (homoepitaxy or heteroepitaxy), spin coating (aka spinning or spin casting—only if no chemical changes and no self-organization occur when depositing thin films in this manner), drop-coating, dip-coating, electrospray deposition, molecular beam epitaxy, ion beam deposition, electron beam (e-beam) deposition, atomic layer deposition or molecular layer deposition, cathodic arc deposition, pulsed laser deposition, etc.

Chemical methods encompass chemical vapor deposition (CVD), plasma enhanced CVD, again atomic layer deposition (ALD), plating (including electroplating), sol–gel method (chemical bath deposition), spin coating (if a chemical process like polymerization occurs during the process), again molecular beam epitaxy, etc. For exhaustive details on the mentioned methods, the reader is directed to the above-referenced work of Marc Madou.

3.2.2. Bottom-Up Approach: Self-Assembly Methods

Self-assembly is a natural process of structures organizing themselves on their own into larger units, as defined by the properties of their smallest constituents (their geometry, chemical and physical properties). It is an ubiquitous process, and its examples can be encountered in a vast range of dimensions and systems, from atoms and molecules through viruses to galaxies and metagalaxies [111]. Thus, it is truly omnipresent and not only multiscale, but rather an “all-scales” process.

Self-assembly can be defined as a process in which the constitutive parts of a system reach an ordered spatial distribution within the boundaries of the system through self-organization. A review of self-assembly methods and approaches used for freestanding nanomembranes can be found in [112]. Here we mention some of the most prominent techniques used in self-assembly.

Langmuir-Blodget Method

The Langmuir-Blodget (LB) technique [113] is a biomimetic self-assembly procedure. It uses molecules possessing a lipophilic (hydrophobic) “tail” and a hydrophilic “head” (amphiphiles or surfactants—surface-acting agents); examples include fatty acids, phospholipids, and glycolipids, but also various polymers like polyimides, as well as some inorganic nanoparticles. When placed at an air–water interface, a monomolecular film is formed (a Langmuir monolayer), since the amphiphilic molecules orient themselves to minimize free energy. Thus, the formed monomolecular surface layers are insoluble in water.
Layer-by-layer (LbL) Self-Assembly

Layer-by-layer deposition also belongs to self-assembly methods [13]. It uses adsorption of alternating macromolecular layers, each with an opposite electric charge with respect to the previous. It proceeds in the following manner: a dilute solution of a cationic (+ charge) material is brought to a substrate and is there adsorbed in a single monomolecular layer whose exact thickness will be determined by the particular molecules used for the monolayer adsorption, but as a rule of thumb it will be on the order of a nanometer. In the next step, after rinsing and drying, the substrate covered with cation is placed into a dilute solution of anions (− charge), and thus a new monolayer is adsorbed on the previous one, and the wafer is again rinsed and dried. Now the process may be repeated a desired number of times. When designing a multilayer, arbitrary chosen materials can be used under the condition that their charges are alternating. The method gives multilayers with a thickness from about 5 nm to over 500 nm. Due to its simplicity, the method has been called the “molecular beaker epitaxy”. This is a popular method, often used to produce high-quality giant aspect ratio nanomembranes.

Block Copolymer Self-Assembly

A copolymer is a polymer produced by simultaneous polymerization of two or more starting monomers. A block copolymer is a kind of copolymer in which there are two or more distinct kinds of blocks that consist of a single pure copolymer (homopolymer) produced from one monomer and are chemically different and even immiscible. These blocks are covalently bonded among themselves. Block copolymers in solution naturally tend to self-organize in various shapes. To date, more than 20 aggregate shapes have been recognized, including spherical micelles, lamellae, rods, tubes, onion-shaped forms, egg-shaped forms, tubules, etc. An excellent tutorial on block copolymer self-assembly has been published by Mai and Eisenberg [114]. Self-assembly of block copolymers has gained distinct popularity for fabricating complex dielectric nanostructures for optical, photonic crystal and metamaterial applications [29]. Its application in the fabrication of various types of freestanding nanomembrane has been extensive. One of the useful traits of block copolymer self-assembly is that it generates ordered patterns that can be useful for membrane functionalization, while at the same time producing the membrane and performing its functionalization. Examples include self-supported perforated polymer nanomembranes intended for protein separation [83] and UV-polymerized nanomembranes of phospholipid and copolymer fabricated by Langmuir-Blodgett method at the air–water interface [115].

Sol-Gel Process

Sol-gel technology is a deposition procedure in which sol (a colloidal solution) deposited on a substrate gradually sets into a gel (semi-solid colloidal suspension) consisting of a continuous integrated solid network of nanoparticles or/and polymer in liquid. The excess liquid is removed e.g., by centrifugation. This step is followed by drying and possibly heat treatment (firing). Under some conditions, nanoparticles will self-assemble while setting. This technology has been very popular for the fabrication of different types of freestanding nanomembranes, including interpenetrated polymer-zirconium oxide membranes with giant aspect ratio [51]. The technique has been used for the fabrication of sol–gel on-polymer processed indium zinc oxide intended for wearable soft electronics [116].

Dip-Coating

Dip-coating is a method of depositing thin films where an object to be coated is dipped (immersed) in the solution containing the material to be deposited or, alternatively, a suspension of its nanoparticles. The object is left for some time in the liquid and then it is withdrawn from it. During the emersion, a layer of liquid remains attached to its surface. The object is held for some time for the excess liquid to flow and drop from it. After drying or evaporation, the nanoparticles remain attached to
the surface in the form of a film, whose thickness can be tailored by the amount of liquid and the filler material dissolved or suspended in it. Care must be taken that the liquid uniformly covers the surface. The procedure is convenient for sol–gel and hydrogel deposition, as well as for deposition of self-assembled monolayers (although self-assembly may be or may be not present, depending on the process materials and parameters). Dip-coating is convenient for large-scale industrial production, since the object may be continuously movable (e.g., a tape). Figure 2a illustrates the dip-coating process.

![Figure 2a](image)

**Figure 2.** Illustration of dip-coating and drop-coating techniques: (a) Dip-coating; left: before drying; right: after self-assembly of nanoparticles and drying. (b) Drop-coating; top: before drying; bottom: after self-assembly of nanoparticles and drying. (c) An example of functionalization of synthetic nanomembrane by lamination. A larger number of layers can be used, as well as different materials for any of them. Authors’ own work from [52]. (d) Some different shapes of nanoparticles that can be used as nanofillers (a small and arbitrarily chosen part of what became known as the “nanotechnological zoo”).

**Drop-Coating**

Drop-coating is a method of depositing thin films where drops of the solution or suspension containing the material to be deposited are sprinkled on the surface of the object to be coated in accurately controlled quantity. The object is left until all of the liquid dries through drying or evaporation, leaving a solid film or nanoparticles attached to the surface. In this case, like in dip-coating, the thickness of the film can be tailored by the amount of liquid and the filler material dissolved or suspended in it. Once again, care must be taken that the liquid uniformly covers the surface. This procedure is convenient for sol–gel and hydrogel deposition, as well as for deposition of self-assembled monolayers. Figure 3b illustrates the drop-coating process.
Any of the fabrication methods listed in Section 2.2 can be used. The number of layers can be arbitrary, performed by biological nanomembranes are due to built-in protein structures that ensure additional functionalities. These external layers can consist for instance from a biocompatible material, thus ensuring a possibility to use the structure for biomedical and life science application.

Figure 3c. It shows a metal composite nanomembrane in the middle, with two external layers enveloping it from both sides. These external layers can consist for instance from a biocompatible material, thus ensuring a possibility to use the structure for biomedical and life science application.

4. Functionalization

Similarly to their biological counterparts, the role of synthetic nanomembranes would be more or less purely mechanical and protective if there were no additional functionalities. Most of the roles performed by biological nanomembranes are due to built-in protein structures that ensure additional functionalities. A very similar situation is encountered in synthetic structures. The difference is that structures enabling biological functionalities are much more sophisticated than the artificial ones, but for the price that they resort to a much smaller toolbox, in the sense of chemical composition and the available choice of materials, operating temperature and humidity ranges and available functionalities. With artificial structures the methods are much more crude and imperfect, but the number of possible ways to perform functionalization is much larger, and the material choice is wider, as is the pure range of functionalities. The latter include many options and paths not encountered in nature. One is free to avoid the limitations of protein chemistry and reach for a much larger toolbox to incorporate in a biomimetic way many novel non-biological properties, including plasmonic, magnetic, electric and optical ones. In 2010, the authors published a review of possible approaches to multifunctionalization of nanomembranes [117]. Here we give just a brief overview of the methods and approaches, concentrating to the more recent findings published in the meantime and to the methods that were skipped in [117].

4.1. Five Basic Methods of Functionalization

4.1.1. Lamination/Multilayering

Lamination is a method of functionalization by making multilayered structures, each layer contributing its own properties and functions. It is the method often used by nature in biological membranes. It is also the simplest method of functionalization, but is very versatile nevertheless. Any of the fabrication methods listed in Section 2.2 can be used. The number of layers can be arbitrary, as can be the added functionalities.

An illustration of the use of lamination to functionalize biomimetic membranes is shown in Figure 3c. It shows a metal composite nanomembrane in the middle, with two external layers enveloping it from both sides. These external layers can consist for instance from a biocompatible material, thus ensuring a possibility to use the structure for biomedical and life science application.
4.1.2. Nanofillers

One method to functionalize a nanomembrane at the fabrication stage is to incorporate nanoparticles, nanofibers, etc., into its body (the host) [110,117]. One must take care during design not to compromise the existing desirable properties of the nanomembrane.

For instance, a nanomembrane structure may be mechanically reinforced by incorporating carbon nanotubes. One could incorporate one type of nanofillers only or several types simultaneously, each with its own functionality, thus obtaining multifunctionality. Examples include incorporation of nanofillers with biological or chemical functions, catalytic (including photocatalytic), magnetic, light-emitting, plasmonic, piezoelectric, etc. A nanotechnological “zoo” showing some possible nanofiller geometries is presented in Figure 3d. Naturally, the number, the kinds, types and materials of available nanofillers vastly surpasses what is shown in Figure 3d for illustrative purposes only.

4.1.3. Nanopatterning

Functionalization of a nanomembrane may be done by defining a pattern on it by adding material (additive approach) or by removing it (subtractive approach)—the fabrication of nano-topographies. Bearing in mind their minute thickness, subtractive patterning of nanomembranes is usually equal to pore making. The patterns may be ordered (arrayed) or disordered. The same or different pattern motifs may periodically repeat in one or both directions across the membrane surface. Additive patterning may be done in a single layer or in multiple layers.

Top-Down Approach to Additive/Subtractive Patterning

While still on the surface of the sacrificial layer, the nanomembrane may be processed using any of the microelectronic/microsystem (MEMS) or nanoelectronic/nanosystem techniques [118]. The standard photolithographic procedures may be used to define patterns for both additive and subtractive patterning, but only if the features are larger than 130 nm. The smallest details are determined by Abbe diffraction limit, according to which the resolution achievable by light is the operating wavelength divided by about 2.8 (depending on the optical equipment used). Since the standard photolithographic light sources operate at 365 nm, this gives us the detail size given above. The same is valid for direct laser writing techniques that may be used to delineate motifs in the photore sist mask, or even to directly process the membrane material. For the definition of details of the order of a nanometer, one has to resort to more recently developed nanolithography methods [118–120], like electron beam lithography, focused ion beam lithography, proton beam lithography, neutral particle lithography, extreme UV lithography, X-ray lithography, synchrotron radiation lithography, magnetolithography, nanoplasmonic lithography, liquid immersion lithography, quantum optical lithography, stencil lithography, nanosphere lithography, etc.

One of the favorite tools for fabrication of nanometric details is nanoimprint lithography [121]. Near-field optical methods can be used to obtain nanometric details, like superlenses based on metamaterials. Another method is scanning probe nanolithography (which includes the dip-pen method, direct writing by scanning microcantilever, thermal and thermochemical scanning probe nanolithography, scanning probe lithography, local oxidation nanolithography, etc.).

Most of the nanolithographic methods listed can be also used for subtractive patterning, i.e., pore forming. Some of them have been successfully used directly on freestanding nanomembranes, for instance, focused ion beam [122].

Material for additive patterning may be deposited through the mask openings, or removed by selective etching when forming pores. Most of the standard deposition techniques can be used for additive patterning, even at nanodimensions. These include (in no specific order) sputtering, electrochemical deposition, chemical deposition, CVD, ALD, PVD, local epitaxy (including molecular beam epitaxy), thermal evaporation, laser evaporation, vacuum arc deposition, electric arc deposition, etc.
Bottom-Up Approach to Additive/Subtractive Patterning

Nanometric and even subnanometric features may be defined by self-assembly techniques [123] using various supramolecular chemistry approaches and starting from various precursor materials [124], including both additive and subtractive patterns. Regarding fabrication of nanopores, a very convenient pathway to it are those processes and materials for fabrication of nanomembranes which intrinsically result in fabrication of nanopores in ultrathin sheets, for instance by using supramolecular self-assembly. In this way, regular nanoporous networks were fabricated, with the nanopore diameters ranging from 1 nm to over 10 nm [125]. Various supramolecular pore shapes can be produced, including circular, hexagonal and square, as well as various dimensions in the nanometric range. The Langmuir-Blodgett method can be useful for additive patterning, and layer-by-layer for both additive and subtractive. Due to the quality and a large variety of available geometries, a prominent place among bottom-up nanomembrane patterning methods belongs to block-copolymer self-assembly.

4.1.4. 3D Sculpting

Nanomembranes are characterized by high tailorability and customizability. They can adjust their shape to whatever body they are transferred to. Despite their minuscule thickness, even when freestanding, they can generally be sculpted into various 3D shapes that are surprisingly robust. Such surface formations include waves [78], pyramids [126], rolling into tubules [123,127], nano-origami [128,129] and nano-kirigami [130,131], spirals, curved sheets [132], as well as other structures [123].

One can use built-in stresses in nanomembranes to fabricate various 3D shapes (tubules, spirals, Swiss rolls, helicoids, etc. [65,66], or, alternatively, the sacrificial layer may be sculpted before depositing nanomembrane layer; after sacrificial etching, the shapes remain permanently sculpted in the freestanding structure [126].

A technique of choice to induce folding of nanomembranes into 3D shapes is strain engineering. To this purpose, one induces spatially distributed strain into a nanomembrane or a nanoribbon, which may be done by built-in structuring or externally in a mechanical way. Many physical properties are changed in strain-deformed nanomembranes, and they sometimes effectively behave as if they were fabricated in a completely new material. Examples of strain engineering in the fabrication of nanomembranes include, e.g., [133,134].

4.1.5. Surface Activation

Surface activation or surface modification represents the application of some external means to a part of or the whole surface of the nanomembrane to modify the behavior of its interfacial parts that interact with the environment [135]. The implemented changes may be located strictly at the interface with the environment or may spread throughout the volume of the nanomembrane.

The activation procedures may include chemical, electrochemical, photochemical, etc., treatment [136]; they may involve irradiation with ionizing electromagnetic waves, including extreme UV, X-ray and gamma ray [137]; or bombardment with nuclear particles (alpha and beta particles, neutrons and protons, accelerator particles). Illumination by non-ionizing electromagnetic waves may include radiofrequent or microwave radiation, infrared, visible or near-UV rays. Last but not least, plasma processing is a widespread method to activate membrane surfaces [138].

Surface activation may involve breaking surface bonds or generating new ones, removal or altering of the boundary atoms/molecules, creation or destruction of polar groups, etc. This may introduce dramatic changes in the nanomembrane parameters and modify them even for several orders of magnitude.

Activation processing may affect practically all of the membrane parameters. For instance, the diffusive properties of a biomimetic nanomembrane may be modified by adding a specific chemical agent. An example is the work of Sharma et al., who applied capsaicin to the membrane surface in
order to change its interaction with certain molecules [139]. Chemical activation of diamond and silicon surfaces for biosensing of proteins was investigated in [140].

As a visual summary, Figure 3 shows the five main methods of nanomembrane functionalization. These include nanopatterning (subtractive, a-1, and additive, a-2), nanofillers (b), 3D-sculpted nanomembrane (rolled-up) (c), lamination (d), and activated surface (e).

4.2. Structures for Nanomembrane Multifunctionalization

4.2.1. Synthetic Ion Channels

Ion channels in biological cell membranes are ionophoric transmembrane protein receptors that selectively facilitate the transport of ions in the direction of lower concentration and charge, i.e., down the electrochemical gradient. An ion itself could not pass the hydrophobic lipid bilayer wall since it requires the presence of an aqueous environment to retain its ionic structure and is destabilized without it. An ion channel represents a gateable pore (i.e., a structure that can be opened/closed by some of the gate mechanisms) that replaces interaction between the ion and water with an equivalent interaction between the ion and the channel, thus ensuring a decrease in the barrier energy for its transport.

There are many kinds of synthetic ion channels [141,142]. Probably the oldest is gramicidin, a semi-synthetic ion channel that acts as an ionophoric substance, first obtained in 1939 by René Dubos and still used nowadays as an antibiotic. It has a helical peptide structure, with a pore inside. When built into a lipid bilayer, it ensures a high-permittivity path through it, acting as a channel for light metal ions like sodium (Na⁺) or potassium (K⁺). In 1977 Kennedy et al. synthesized four different peptides and reported their properties as synthetic ion channels in vitro after incorporating them in their black lipid membranes (model lipid bilayers) [143]. The first fully synthetically produced ion channel was tetra-substituted β-cyclodextrin [144], which was reported as early as in 1982.

Figure 4 shows a simplified depiction of a freestanding lipid bilayer nanomembrane with built-in synthetic ion channels (shown in a simplified manner as spirals).

![Figure 4](image-url)

**Figure 4.** Simplified depiction of a freestanding lipid bilayer nanomembrane with built-in synthetic ion channels; blue spheres are particles to be separated, while brown helices represent synthetic ion channels.

Artificial ion channels may be based on cyclodextrin (the first synthesized ion channel), calixarenes, macrocycles (including peptide macrocycles), G-quadruplex, p7 viroporin, etc.

Their opening or closing (gating) may be controlled by different mechanisms [142]. The gating mechanisms include

- Voltage gating (ionic switching) [145]
Chemical gating (ligand gating) [146]
- Light (optical) gating [147]
- Mechano-sensitive gating [148]

4.2.2. Synthetic Ion Pumps

Similarly to ion channels, the ion pumps are ionophoric transmembrane protein receptors that enable selective and controlled ion transport through lipid bilayer, but, contrary to ion channels, they ensure transport against the electrochemical gradient, i.e., in the counter-direction relative to diffusion. Their function is perpetrated by the fluctuations of the external field.

The synthesis of ion pumps is much more complex than that of the ion channels. Synthetic ion pumps were reported in [149]. They fabricated an asymmetric nanostructure with a narrowing channel (a “nanopump”), so that it functions as a kind of electrochemical ratchet. In this manner, they ensured a net flow of potassium ions against the direction of the electrochemical potential, rectifying the ion current. The rectification amount is dependent on the angle of the conical nanopore, as well as on its side length. The concept actually represented a scaled down version of a previously proposed microsystem that performed the same function on micrometer-sized particles [150]. The principle of conical pore-based synthetic nanopumps proposed in 2002 is still used today [151], and the production of nanocone ion rectifier pumps remains a daunting task.

4.2.3. Artificial Water Channels

Aquaporins or water channels are integral channel proteins forming pores in cellular membranes. Their main role is to facilitate selective transmembrane transport of water, which proceeds in addition to the osmotic transport directly through the lipid bilayer. They also enable, in a smaller amount, the transport of some smaller neutral solutes (urea for instance), as well as gases (carbon dioxide, ammonia). They consist of six alpha helices having amino and carboxylic terminals within the cytoplasmic part, while the inner wall is padded by asparagine-proline-alanine complex. They have an hourglass structure with the diameter at the narrowest part of 0.3 nm. Water molecules in it form a “water wire”, i.e., a single file molecule array. A cluster of amino acids called the aromatic/arginine filter selectively binds to water molecules and ensures their passage while blocking other molecules that do not bind to it. The efficiency of their transport is up to $10^9$ molecules per second per a single aquaporin channel. There is several various types of aquaporins in both plant and animal cell membranes. Peter Agre was awarded the 2003 Nobel Prize in Chemistry for the discovery of aquaporins. Natural biological aquaporins are built into separating membranes and used in water separation at an industrial scale. However, they are expensive, have low stability and pose constraints for both fabrication of separators and the range of their operating conditions. This was the reason synthetic alternatives were sought for. The synthetic structures serving a function analogous to natural aquaporins are called artificial water channels [152,153].

Carbon nanotubes (CNT) with an inner diameter of about 0.9 nm, which allows the formation of water wires, have been proposed as artificial water channels for incorporation into nanomembranes [154]. For this purpose, short CNT (5–20 nm) with diameters 0.9–20 nm are used. They easily self-incorporate and self-align in lipid bilayer membranes.

Other artificial water channels can be based on single molecules or supramolecular assemblies. The first synthetic water channel was reported in [155], and was based on imidazole quartets.

Single molecular channels include the PAP1 pillar[5]arenes, as proposed by Hu et al. [156]. PAP1 molecules are impermeable to protons, but show no selectivity against cations. The next solution included PAP2 pillar[5]arenes incorporating peptidic poly phenylalanine arms. Their water permeability is comparable to that of aquaporins. In addition to that, they are permeable to amino-acids, but show a poor ionic selectivity.

Self-assembled supramolecular channels include tubular imidazole I-quartets as described in [155,157]. Other molecular assemblies used for artificial water channels include peptide-appended
hybrid[4]arene (PAH[4]) [153], aquafoldamers, reverse osmosis (RO) membranes and double helical water T-channels [157].

Monolayer carbon nanomembranes based on terphenylthiol with subnanometric pores have been used as simple artificial water channels in [101]. A virtue of this solution is its simplicity, while such structures ensure extremely rapid passing of water (due to the shortness of the path the molecules have to pass) and at the same time show an outstanding rejection of undesired ions.

4.2.4. Artificial Nuclear Pore Complexes

Nuclear pores are protein-based pores built into the nanomembranous envelope of the nucleus of a biological cell [158]. A nuclear pore is a part of the cell’s massive and highly complex nuclear pore complex (NPC), which serves as a scaffold for the translocation passageway. The role of an NPC is to mediate transport of macromolecules between the cell cytoplasm and the nucleus, but also to take part in genome organization and transcription processes. A nuclear pore complex consists of protein-based structures called nucleoporins (nups). There are 34 different nucleoporin proteins in each nuclear pore complex, containing about a thousand different proteins as their building blocks.

Biological nuclear pores are the largest pores in the cell. In vertebrate animals, the whole nuclear pore complex has a diameter of about 120 nm, while in other organisms it is smaller. The diameter of the nuclear pore channel is about 40 nm and its depth is about 45 nm.

The transport through the core of the NPC consists of export from the nucleus into the cytoplasm (this includes ribosomal proteins and RNA) and import from the cytoplasm to the nucleus (this includes proteins such as lamins and DNA polymerase, signaling molecules, lipids and carbohydrates) against the concentration gradient. Smaller molecules are transported through a nuclear pore by diffusion, while the larger ones are translocated only with the help of specific signal sequences based on karyopherin transport factors.

Jovanović-Talisman et al. succeeded in synthesizing artificial nuclear pores [159] in polycarbonate nanomembranes, consisting only of a passageway and its lining composed of scaffold-anchor phenylalanine-glycine nucleoporins (FG nups), which represent a binding structure for transport factors. Their artificial NPCs demonstrated nanoselective filtering and allowed passing of the transport factors and their cargo complexes that bind FG nups, while they simultaneously inhibited the transport of the proteins that did not bind these nucleoporins.

A large progress in understanding nuclear pore complexes has been achieved in recent times [160]. Still, many factors governing the behavior of biological NPCs and the role of several its sophisticated structural parts are yet to be understood, while the synthetic NPCs produced to date remain necessarily simplified.

4.2.5. Artificial Organic Nanotubes

There are numerous synthetic structures, both organic and inorganic, in the form of tubes with nanometric dimensions that could be convenient for the use as ion channels. Generally, nanotubes can be made from basically the same toolbox as nanomembranes. Thus, inorganic nanotubes can be made of pure chemical elements (carbon, boron, silicon), inorganic compounds (tungsten sulfide, titania—titanium dioxide, gallium nitride, boron nitride, etc.) or their mixtures (including BCN, the composite of boron, carbon and nitrogen atoms in approximately same amounts). One can introduce a classification of organic nanotubes in a similar fashion as we did for the inorganic ones. Organic nanotubes can be made from different macromolecules (examples being peptides, polysaccharides, block copolymers, macrocycles, artificial amphiphiles, oligophenylacetylenes, metal-organic polymers, etc.) or their mixtures (e.g., cyclic peptides containing beta-amino acids), but also from DNA molecules. A comprehensive review on self-assembled organic nanotubes can be found in [161]. Another approach is to produce hybrid organic-inorganic nanotubes, for instance by combining the above-mentioned materials with inorganic nanofiller as structural reinforcing agents.
Artificial inorganic nanotubes were, for instance, reported by Li et al. in [162]. They applied pyrolysis to lamellar mesostructures made of mixture of tungsten disulfide with surfactant. Thus, the layered sheets rolled and formed tungsten disulfide nanotubes 0.2 to 0.5 μm long and with diameters in the range from 5 nm to 37.5 nm.

Artificial self-assembled organic nanotubes were described by Ghadiri et al. They described the design, production and characterization of self-assembled nanotubes based on cyclic polypeptides [163]. They protonated these compounds, after which they crystallized into tubular nanostructures. The structures were very uniform, their length being several hundreds of nanometers, and the diameter 0.7 nm to 0.8 nm. The same team later presented an approach to the design of artificial membrane ion channels using the obtained self-assembled polypeptide nanotubes [164].

4.2.6. Carbon Nanotubes

Carbon nanotubes (CNT) [165,166] belong to the wider class of artificial nanotubes (Section 4.2.5 of this text), but merit a class of their own because of their peculiarities, their importance and their widespread use in nanomembrane functionalization, as well in many other fields.

The discovery of CNT is often credited to Sumio Iijima in 1991 [167], despite the fact that almost 40 years earlier, in 1952, carbon nanotubes were observed and reported by Soviet researchers L.V. Radushkevich and V.M. Lukyanovich in the Soviet Journal of Physical Chemistry [168].

CNT are an allotrope of carbon and represent a hollow tube consisting of carbon atoms forming a hexagonal 2D crystal lattice (Figure 5). They may be single-walled or multiple-walled, their walls being monatomic. The thinnest freestanding single-walled CNT had a diameter of 0.43 nm, and a typical value is 1–2 nm. They have extreme aspect ratio, and actually CNTs with lengths in excess of 50 cm have been observed. Theoretically, the CNT length could be infinite. Typically, the single-walled CNTs are 5–30 nm long. CNTs may behave as metals with extremely high electrical and thermal conductivity or as semiconductors. CNTs exhibit an exceptional tensile strength.

![Figure 5. Schematic presentation of single-walled carbon nanotubes.](image)

Important from the point of view of biomimetic nanomembrane functionalization is that the inner side of CNTs is hydrophilic. This makes them useful for incorporation into nanomembranes as artificial transmembrane channels. For that purpose, they have to be relatively short and vertically aligned relative to the nanomembrane. They are used as porins in membranes for water filtration and removal of undesired ions, for instance in desalination [154].

4.2.7. Antifouling Structures

The membranes of all biological cells are exposed to various kinds of external fouling based on different mechanisms. Fouling agents belong to one of the following three classes [169]: (1) passive, non-self-migratory particles and substances (for instance various external proteins, small particles of organic or inorganic matter, polysaccharides, etc.; also prions and viruses, which do not proliferate...
while outside the cell walls and become active and multiply only upon entering the cell); (2) passive spreadable substances (external liquids, for instance droplets of oil); and (3) active and proliferating foulants (bacteria, protozoans).

Antifouling structures and layers are essential parts of all living cells. The biological antifouling mechanisms can be roughly divided into the following groups: (1) physical structures or chemicals repelling fouling agents; (2) self-cleaning physical structures or chemicals shedding (passively removing) already present fouling agents; and (3) aggressive chemicals that actively destroy fouling agents [169,170]. Biomimetic antifouling structures can and should follow these strategies, since biomimetic membranes, especially those intended for separation applications, will be exposed to similar kinds of fouling.

**Fouling Agent Repellents**

These may include layers of superhydrophilic chemicals (e.g., hydrophilic polymers) on the membrane surface that bind a thin sheet of water (hydration layer) and thus behave as a kind of fish scales not leaving the fouling agents a place where to adhere. Another approach is the grafting of polymer brushes (nanobrushes) on the membrane surface which represents a physical method to repel a would-be fouling agent. Finally, the surface topology may be modified by making structures reject fouling agents and prevent their adhesion in the same manner a lotus leaf repels water.

**Self-Cleaning**

Self-cleaning layers and structures are intended to remove fouling agents that already found a way to adhere to the membrane. One approach is to use a layer of chemical with low surface energy (an example being perfluoro/silicon-containing polymers/macromolecules).

Active chemicals that attack fouling agents. These include metal coatings, graphene coatings, sulfides or oxides of transition metals, titanium dioxide photocatalytic nanoparticle layers and other catalytic/ enzymatic agents that actively take part in destroying the foulants.

A review dedicated to imparting antifouling properties to artificial membrane surfaces was published in [169].

4.2.8. DNA Transmembrane Channels

Channel structures in lipid bilayers may be formed of DNA double helix strands. It may consist of one or more DNA strands. If there are more, their incorporation into the nanomembrane is done by using the DNA origami technique [171,172]. This customizable method allows one to organize several DNA molecules in a funnel-shaped structure with a polygonal cross-section. This structure is built into the membrane orthogonally and stands upright within the lipid bilayer. The cavity within the thus-woven DNA channel represents an artificial transmembrane pore. To incorporate a DNA channel into a lipid bilayer nanomembrane, one has first to functionalize DNA. This is done by chemical modification, and in this way hydrophobic “anchors” (porphyrin-tags or cholesterol-tags) are added to DNA. After that, the DNA funnel spontaneously aligns itself orthogonally into the lipid bilayer.

The largest transmembrane channels to date were built using a DNA origami structures and their area was ten times larger than any previously man-made synthetic ion channel, comparable in size to nuclear pore complexes [173]. Its electrical conductance was an order of magnitude higher than any reported previously. The smallest DNA-based artificial transmembrane channels, consisting of only one DNA double helix, were reported in [174].

4.2.9. Summary of the Artificial Structures for Nanomembrane Multifunctionalization

A summarizing illustration is shown in Figure 6. It shows a choice of artificial structures for nanomembrane multifunctionalization, including spiral synthetic nanochannels, functionalization by DNA and CNT and two antifouling methods, 3D sculpting of membrane surface and grafted nanowires.
5. Applications

This section gives an overview of some proposed applications of biomimetic nanomembranes. The text only highlights the main fields of use, as a definitive list would be extremely long. For one, novel types of nanomembranes with many (multi)functionalities are continually being invented and developed, so it is likely that they will bring about new applications as well. As an example, if somebody makes a bionic replica of a nuclear pore complex with more functionalities than today, it could find its way into labs on a chip, and the implications for the whole genomics and proteomics would be enormous. Additionally, a bionic nanomembrane is a fundamental building block, in fact so fundamental that nature built it into basically all organisms. Thus, whole fields of science and industry could be reinvented looking through such a prism. An example would be micro- and nanosystems, where it appears as a new building block, in addition to only a few existing ones (micro/nanocantilever, microbridge, diaphragm, etc.). So, the list is necessarily open, and new applications will be appearing as you read this text. A summative table reviewing some applications of biomimetic nanomembranes classified by their fields of use can be found below (Table 3).

5.1. Active Nanofluidic Devices Based in Ion Transport

Nanofluidics [49] investigates the transport of fluids and ions at the nanometer scale [175]. In this way it is directly connected with basically all nanopore-based mechanisms of biomimetic multifunctionalization. In this moment, one can say that the development of nanofluidics is in full swing.

Various active devices have been developed, akin to their micro/nanoelectronic counterparts, but based on the transport of ions instead of electrons and holes. These start with ion pumps, which incorporate some nanofluidic-based diodic behavior [151], continue with nanofluidic diodes [176,177], bipolar ionic transistors [176], and nanofluidic field effect transistors (FET), which were theoretically conceptualized in [178] and practically implemented in [179]. More recent implementations include [180,181]. The operation of active nanofluidic devices is based on the fact that in nanofluidic
channels, the surface charges usually have larger effects on the flow of ions than to the flow of liquids, in contrast to the phenomena observed in microfluidics. This is a consequence of the Debye length, the distance scale for ion screening. Thus, the nanofluidic ionic current can be tuned by modifying the surface charge density when the electrical double layers in the channel do not overlap [176].

**Table 3.** Some select applications of biomimetic nanomembranes classified by fields of use.

<table>
<thead>
<tr>
<th>Application Field</th>
<th>Application Type</th>
</tr>
</thead>
</table>
| Environmental Protection | (1) Air pollution control—removal of pollutant particles and volatile compounds from airstreams  
(2) Wastewater treatment—removal of pollutants and recycling  
(3) Remediation |
| Biosensing and chemical sensing | (1) Ultrasensitive chemical, biochemical and biological sensors  
(2) Simultaneous sensing of multiple analytes by a single sensor |
| Toxicology, forensics and homeland defense | (1) Recognition of toxic inorganic, organic and biological agents  
(2) Removal of toxic agents |
| Renewable energy & power industry | (1) Fuel cells  
(2) Solar cells  
(3) Water splitting  
(4) Micro-power sources and microbattery arrays  
(5) Nuclear fuel production, purification and enrichment  
(6) Hydrocarbon fractionation  
(7) Environment-friendly fuel purification (e.g., desulfurization) |
| MEMS/NEMS | (1) Active nanofluidic devices based on ion transport  
(2) Self-healing micro and nanostructures  
(3) Stretchable and foldable devices  
(4) Very high frequency microoscillators and microresonators  
(5) Catalytic membrane microreactors  
(6) High temperature microreactors  
(7) Smart Labs on a chip |
| Molecular sieves and Separators | (1) Water, oil, gas separation from undesired ingredients  
(2) Removal of heavy metals  
(3) Reclaiming of precious materials including noble metals  
(4) Desalination |
| Biomedical applications | (1) Two-dimensional scaffolds for tissue regrowth  
(2) Biointerfaces including neural interfaces  
(3) Wearable and implantable artificial kidneys  
(4) Portable artificial lungs  
(5) Drugs delivery and disease control  
(6) Pathogenic bacteria, viruses, prions recognition and deactivation |
| Bioengineering and genomics | (1) DNA analysis/separation/replication  
(2) Gene sequencing, genomics  
(3) Cell biotechnology  
(4) Biomarker detection |
| Food and drinks industry | (1) Food and beverages purification  
(2) Potable water production through purification |
| Chemical Engineering | (1) Multicomponent gas mixtures separation  
(2) Gas dehydration  
(3) Separation of liquid chemicals  
(4) Chemicals analysis  
(5) Microfiltration, ultrafiltration and nanofiltration  
(6) Environmentally friendly petrochemistry |

One might argue that the speed of contemporary active nanofluidic devices is much lower than that of their electronic counterparts. On the other hand, a counterclaim may be found in the fact that all our neural processes are based on nanofluidic ion transport. Hence, we are talking about a process that brought to this development the most sophisticated micro- and nanostructured biological machines that ever existed, namely our brains. This proves the point that there is much room for further biomimetic development. In 2020, Bocquet proposed [175] the development of ionic machines inspired by the functioning of our neuronal systems, including building synthetic neuromorphic biomimetic computer elements.
5.2. Two-Dimensional Nanofluidics

Koltonow and Huang proposed the idea of fluid flow confined to 2D space [182] as a parallel to 2D charge carrier transport in quasi-2D sheets (graphene, MXenes, generally nanomembranes). For this purpose, they proposed making sandwiches of nanomembranes/monolayers (e.g., graphene) at a distance smaller than the Debye lengths of the surrounding solid nanosheets. The interspace between the sheets is filled with fluid, and there are pores in the sheets that enable transport between different “levels”. In a situation similar to semiconducting 2D systems, the confinement of fluid into 2D nanochannels should ensure novel physical effects in such 2D fluid sheets, mainly vastly increased conductivity of cations.

5.3. Biosensors

The field of nanomembrane-based biosensors is very wide, and it opens pathways to extensive and numerous practical uses. Many different types have been proposed to date, of which we give an overview of a few.

A combination of biomimetic nanomembranes with plasmonics and metamaterials has found use in ultrasensitive chemical or biological sensors [27,183,184]. These sensors are refractometric and affinity-based (using the effect of selective adsorption and desorption). They typically consist of metal-dielectric nanocomposites, ordered in a form of 2D arrays. The presence of adsorbed analyte at the metal-environment interface modifies the effective refractive index near the surface of the nanoplasmonic structure or metamaterial (which may be a freestanding metasurface [60] or a bulk structure, the so-called Catalyst Plus sensor [185]). In this way, the propagation of surface electromagnetic waves (evanescent waves) is tuned proportionally to the amount and species of the analyte. Such sensors exhibit extreme sensitivities, even reaching a single-molecule accuracy [17], dependent on the nanostructure used. Biosensors that use ion channel switches were described by Cornell et al. [24].

More recently, Medina-Sánchez et al. described a high-performance DNA detection biosensor that recognized DNA strands of avian influenza virus H1N1 [186]. Their sensor was based on rolled 3D nanomembranes. The authors proposed the use of their sensor for detection of proteins and enzymes, as well as for monitoring of in vivo cell behavior.

El Afandy et al. described a nanomembrane-based sensor of thermal diffusivity and conductivity [18]. Their technique is based on the increase of phonon-boundary-scattering rate of nanomembranes. They showed it on a single cell. The low flexural rigidity and softness of nanomembranes ensure a close contact with the cells, independently on the irregularity of their shapes. The method enabled a novel biomedical diagnostic technique, since it can detect differences between, e.g., different types of cancer cells.

5.4. Renewable Energy

5.4.1. Fuel Cells Based on Proton Exchange Membranes

Conventional proton exchange membranes (PEM)-based fuel cells [23] (also denoted as polymer electrolyte membrane fuel cells) customarily use 50–150 µm-thick Nafion® (a copolymer of tetrafluoroethylene with sulfonic acid-terminated perfluoro vinyl ether) membranes to ensure proton conduction. Membranes made of other materials are also used sometimes, for instance Gore-Select™mechanically reinforced membranes which are much thinner (about 10 µm). PEM fuel cells operate at low temperatures (below 100 °C), are easy to scale up, offer high power densities and high efficiencies, while at the same time emitting zero amount of greenhouse gases (their only exhaust is pure water). Today they are already being used as safe power sources for hydrogen-fueled cars (Toyota Mirai, Hyundai Tucson, etc.). Other practical uses include portable devices like laptops, smartphones, tablets, etc. Extensive research efforts are currently directed toward the development of improved
components for PEM fuel cells, with a large portion of these efforts being spent on researching novel types of membranes.

Their operation is based on two mechanisms: vehicular transport where hydrogen ions (H\(^+\)) diffuse in the form of hydronium ions (H\(_3\)O\(^+\)); and the Grotthuss mechanism (“proton jumping”) in water wires, where H\(^+\) ions hop between neighboring water molecules in a string within nanochannels spanning the membrane. For a proper operation, a PEM fuel cell must be kept hydrated at a percentage within required limits.

In this text, we briefly describe approaches to replace the conventional Nafion® membranes with much thinner biomimetic nanomembranes functionalized by nanopores acting as water channels. Their advantage, apart from vastly increased packaging density and thus available power per unit volume, would be a much faster proton transport; instead of labyrinthine ways for water channels as in the conventional membranes, they require only short and straight biomimetic nanopores, which should furnish proton transport rates comparable to those in biological cell walls and much higher than in Nafion®. The mechanism is here also a combination of the vehicular one and Grotthuss effect [187,188].

As far as the artificial pores are concerned, a convenient choice would be short and vertically aligned carbon nanotubes whose interior is hydrophilic, so that water wires are spontaneously formed within them, ensuring proton transport. Many polymer hosts are available for nanomembranes for PEM, including but not limited to amorphous fluoropolymers and polybenzimidazole. The membranes would need to be reinforced, e.g., by lamination with an inorganic layer.

5.4.2. Solar Cells

Biomimetic nanomembranes have been used in stretchable and curved electronics. Silicon single-crystalline nanomembranes have been proposed for the use in solar cells [57], due to the excellent performance of silicon single crystal compared to amorphous silicon or polysilicon. Single crystals are very expensive in comparison, but this sole advantage of the other types of silicon solar cells becomes unimportant when minute amounts of material are used, as is the case with nanomembranes. The use of silicon microcells on foldable substrates has been proposed. The performance of these solar cells can be further enhanced by e.g., lenticular optical concentrators, antireflection structures and other optical enhancement techniques [189].

Sculpted nanomembranes in a rolled-up geometry have been proposed for use in both infrared detectors and solar cells [190], due to their improved light trapping properties enabled by the rolls.

5.4.3. Hydrogen Economy—Water Splitting

Water splitting to hydrogen and oxygen represents a crucial step towards a hydrogen-based clean economy. Nanomembranes can be used to make ultra-compact catalytic microreactor cells to perform water splitting using solar energy (photocatalysis) [191]. For this purpose, titanium dioxide, the well-known material for catalytic water splitting, was used. Another approach to solar-induced photocatalytic water splitting is to use composite organic-inorganic nanomembranes laminated with photocatalyst or, alternatively, filled with its nanoparticles.

Silicon diphosphide (SiP\(_2\)) and silicon diarsenide (SiAs\(_2\)) nanomembranes have also been proposed for highly efficient photocatalytic water splitting [192]. Both materials promise high carrier mobilities and good mechanical, thermal and dynamic stabilities. Another pair of alternative materials for the same purpose are beryllium nitride (BeN\(_2\)) and magnesium nitride (MgN\(_2\)). Monolayers of these materials as water-splitting photocatalysts were considered in [193]. Janus monolayers (i.e., with different front and back side, “two-faced”) of metal chalcogenides Ga\(_2\)XY and In\(_2\)XY (X = S, Se, Te; Y = S, Se, Te) have also been described as promising materials for the same purpose [194].

Instead of using a photocatalytic structure, an alternative approach is to use electrocatalyst nanomembranes [195]. Wang et al. used nanomembranes with reduced graphene oxide combined with NiO/Ni. Alternatively, metallic Ni\(_2\)S\(_2\) ultrathin sheets fabricated by atomic layer deposition proved themselves efficient and stable electrocatalysts for this purpose [196].
5.5. Nanomembrane Separation

Porous nanomembranes are often used as separators or molecular sieves, filtering various kinds of fluids (both liquid and gaseous) in a great many applications. Their pores may belong to some of the previously described classes (ion channels, ion pumps, nuclear pore complexes), but do not have to. Simple nanometric openings are convenient for many of applications in separation. The necessary requirement, however, is that the size of pores across the membrane is uniform. The nanomembranes do not even have to be porous—structures based on diffusion through non-porous synthetic nanomembranes are also used.

First, we classify some mechanisms used in filtration that are applicable to biomimetic nanomembranes. A possible classification is presented in Table 4.

It may be useful to quote here the definitions of pores according to IUPAC: micropores have diameters below 2 nm. Mesoporous materials have pores 2–50 nm, while macroporous materials have pores with diameters above 50 nm [197]. Unofficially, all pores with nanometric dimensions are denoted as nanopores; their diameters may range from the order of 0.3 nm to about 20 nm.

Membrane separation science is an enormous field, a science in itself, with numerous related technologies, processes and methods [198,199]. Basically, in a lot of fields where conventional membranes are used, their place could be filled by biomimetic nanomembranes. The questions of mechanical strength can be solved, instead of using porous substrates to mechanically support the active part of the separation membrane, by applying porous nanomembranes functionalized with nanofillers (e.g., woven CNT, zirconia fillers, etc.) that may drastically increase their strength and avoid the need for porous substrates altogether. What we are left with are structures that basically perform the same function, but with at least several orders of magnitude decreased space requirements. Additionally, the throughput of nanomembranes would be vastly higher than that of their micro or macro counterparts, because instead of having to pass through drastically longer and meandering separation channels, the filtrates would have to pass only a nanometer-long and straight pathway that actively rejects the undesired agents and species. Additionally, nanomembranes can be functionalized with target-oriented biomimetic pathways like ion channels, ion pumps and even simplified nuclear pore complexes. In Table 5 we present some applications of biomimetic nanomembrane separation.

Without any claims for exhaustiveness, we describe in the next subsections a choice of some areas where the use of biomimetic nanomembranes has been notable. This is only intended as an illustration of the much wider field of applications of biomimetic nanomembrane in separation science and technology.

5.5.1. Remediation and Environmental Protection

Environmental uses of nanomembranes include waste water treatment, removal of inorganic and organic pollutants, especially the persistent ones (e.g., dioxin, radioactive nuclides, pesticides, etc.). Nanotechnological methods for water remediation include three approaches: the use of nanomembranes, nano-adsorbents and nanocatalysts. The application of functionalized nanomembranes for environmental protection and remediation was reported in [200]. It appears that the optimum results are obtained if nanomembranes are multifunctionalized by photocatalytic nanoparticles and thus perform a dual role of simultaneously filtering some pollutants while actively destroying others. An essential functionality of membranes for the use in remediation and wastewater treatment is imparting the antifouling properties at the “dirty” side.
### Table 4. Classes and types of filtration by biomimetic nanomembranes.

<table>
<thead>
<tr>
<th>Class</th>
<th>Type</th>
<th>Mechanism and Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pore filtration</strong></td>
<td>Particle filtration</td>
<td>Pressure-driven filtration through &gt;1 µm pores and above, removes large suspended particles (^1)</td>
</tr>
<tr>
<td></td>
<td>Microfiltration</td>
<td>Pressure-driven filtration through 0.1 µm to 1 µm pores, removes bacteria and suspended organic and inorganic particles</td>
</tr>
<tr>
<td></td>
<td>Ultrafiltration</td>
<td>Pressure-driven filtration through 1–100 nm pores, removes all above plus viruses</td>
</tr>
<tr>
<td></td>
<td>Nanofiltration</td>
<td>Pressure-driven filtration through ~0.3–1 nm pores, removes all above plus large (polyvalent) ions</td>
</tr>
<tr>
<td><strong>Filtration by diffusion</strong></td>
<td>Reverse Osmosis</td>
<td>An applied pressure is used to overcome osmotic pressure for separation through a semi-permeable membrane, removes all above plus small (monovalent) ions</td>
</tr>
<tr>
<td></td>
<td>Forward Osmosis</td>
<td>Separation driven by osmotic pressure gradient through a semi-permeable membrane</td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td>Solute separation is induced by the difference in solute diffusion transport through the membrane</td>
</tr>
<tr>
<td><strong>Filtration assisted by liquid-gas phase transition</strong></td>
<td>Pervaporation</td>
<td>Separation of mixtures of liquids by permeation through a membrane, followed by vaporization</td>
</tr>
<tr>
<td></td>
<td>Membrane Distillation</td>
<td>Thermally driven separation of liquids where only vapor molecules move through a microporous hydrophobic membrane</td>
</tr>
<tr>
<td></td>
<td>Gas Permeation</td>
<td>Separation of gas mixtures permeating a membrane based on the fact that the flux of each gas is different</td>
</tr>
<tr>
<td></td>
<td>Evapomeation</td>
<td>Separation of mixtures of liquids by full vaporization through a non-porous or porous membrane</td>
</tr>
</tbody>
</table>

\(^1\) Only applicable if the biomimetic nanomembrane is supported by a porous substrate.

### Table 5. Some applications of biomimetic nanomembrane separation.

<table>
<thead>
<tr>
<th>Class</th>
<th>Type</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water treatment</strong></td>
<td>potable water</td>
<td>removal of organic pollutants, arsenic, ammonium, manganese, iron, etc.</td>
</tr>
<tr>
<td></td>
<td>process water</td>
<td>removal of all process pollutants, especially heavy metals incl. lead, mercury, also dyes, organic by-products, aromatic hydrocarbons …</td>
</tr>
<tr>
<td></td>
<td>waste water</td>
<td>removal of contaminants, including bacteria, viruses, cyanobacteria, protozoans, organic and inorganic pollutants</td>
</tr>
<tr>
<td></td>
<td>remediation</td>
<td>removal of contaminants, including toxins, heavy metals and radioactive nuclides</td>
</tr>
<tr>
<td></td>
<td>chemical spillage</td>
<td>removal of contaminants</td>
</tr>
<tr>
<td></td>
<td>concentration of trace ingredients</td>
<td>harvesting of e.g., noble metals from seawater, present in it in trace amounts</td>
</tr>
<tr>
<td><strong>Food and beverages treatment</strong></td>
<td>food ingredients</td>
<td>removal of biological and other pollutants, sterilization</td>
</tr>
<tr>
<td></td>
<td>sugar, oil production</td>
<td>organic and inorganic material removal</td>
</tr>
<tr>
<td></td>
<td>dairy products</td>
<td>biological contaminants removal</td>
</tr>
<tr>
<td></td>
<td>wine production</td>
<td>filtering, contaminants removal</td>
</tr>
<tr>
<td><strong>Process industry</strong></td>
<td>chemical processing</td>
<td>undesired species removal, refinement</td>
</tr>
<tr>
<td></td>
<td>petrochemistry</td>
<td>separation of undesired compounds from liquids, gases</td>
</tr>
<tr>
<td></td>
<td>fertilizer production</td>
<td>removal of organic and inorganic contaminants</td>
</tr>
<tr>
<td></td>
<td>pulp and paper mills</td>
<td>refinement</td>
</tr>
<tr>
<td></td>
<td>pharmaceutical industry</td>
<td>removal of organic and inorganic contaminants, refinement</td>
</tr>
<tr>
<td></td>
<td>refining of biofuels</td>
<td>refinement</td>
</tr>
<tr>
<td><strong>Power industry</strong></td>
<td>refining and fractioning of natural oil</td>
<td>hydrocarbons separation, desulfurization, refinement</td>
</tr>
<tr>
<td></td>
<td>refining of natural gas</td>
<td>hydrocarbons separation, removal of N(_2) and CO(_2), refinement</td>
</tr>
<tr>
<td></td>
<td>fuel cell membranes</td>
<td>water wires in PEM structures (incorporation as an integral part)</td>
</tr>
<tr>
<td></td>
<td>batteries production</td>
<td>removal of organic and inorganic contaminants, refinement (incorporation as an integral part)</td>
</tr>
</tbody>
</table>
5.5.2. Food and Beverages

Nanomembranes in the food industry find applications in removal or photocatalytic/enzymatic destruction of viruses, bacteria, aflatoxin, cyanotoxins and other toxins, pesticides, bisphenol and other undesired contaminants, also for filtering inorganic substances including heavy metals, etc. Among examples are bioconversion and removal by separation of lactose in milk and dairy products, obtaining cheese and other fermented products from milk, filtering beer from mother solution, etc. [201]. Other uses of biomimetic nanomembranes in the food and beverage industry include nanomembrane-based sensors for in situ monitoring of various critical ingredients that contribute to the overall quality, also the detection of the above mentioned dangerous, cancerogenic and even potentially lethal contaminants.

5.5.3. Desalination and Potable Water Production

One of the often-encountered approaches to water treatment to remove sodium chloride and other minerals from seawater and to make it potable are nanoporous nanomembranes functionalized by aquaporins channels [9,202]. Usually structures with a porous support are used to ensure mechanical integrity. Nowadays, the use of aquaporins for desalination is becoming a profitable industry. Another approach is to use carbon nanotube-based water channels for the same purpose [203,204]. It has been shown that water moves through carbon nanotubes exceptionally fast (“hyperlubricity”). Other approaches include nanoporous graphene layers [205].

5.6. Biomedical Applications

5.6.1. Two-Dimensional Scaffolds for Tissue Regrowth

The main goal of tissue engineering is to repair, regenerate and restore tissue damaged or degenerated due to, e.g., disease or injury including mechanical or toxic trauma to its original form as fast and safely as possible. A very important role in this task belongs to artificial scaffolds whose role is to stimulate and sustain the regrowth and proliferation of new cells, which is a very complex process by itself. To facilitate regrowth of cells, cell systems, tissues and even whole organs, one typically uses a 3D or 2D structure (the scaffold) to serve as a template for cell growth, to which one seeds the desired cells. The follow-up may be that one adds the necessary growth factors, applies some kind of external biophysical stimulus, or performs both of these procedures [16]. The whole regrowth process may occur in vitro, in vivo, or combined.

While designing a scaffold to facilitate the initial steps of regeneration and repopulation of the differentiated cells, including mandatory revascularization, one of course must take care of biocompatibility (a scaffold must not cause an inflammatory process or tissue rejection and must not release any toxic components either directly or indirectly (through the process of its intermediate or final decomposition or resorption by the organism). Ideally, it will remain in the organism as long as necessary and then it will spontaneously break apart and be dissolved (biodegradability).

Various tissues can be engineered in a biomimetic manner, from bones [206,207] and cartilage [208], over muscle cells and skin (including hair follicles) [209], cardiovascular tissues including heart muscle tissue [210,211], internal organs (e.g., liver, pancreas, intestines, kidneys), [212] airways in lungs, to highly complex neural tissues [14,16,213]. The implemented scaffolds must be mechanically compatible with the surrounding tissue and at the same time ensure easy handling. Their structure must be porous and ensure easy adherence of the cells to be regrown. The pores should be interconnected to allow a further cell growth and spreading in a manner that maximally corresponds to the natural one.

Two main kinds of materials are usually used for tissue engineering scaffolds, ceramics and polymers (either natural or artificial ones) [16]. In addition to these, one sometimes uses a combination or composite of both. Ceramics are often used for bone regeneration and include materials like hydroxyapatite or tri-calcium phosphate. The most popular natural polymers are chitosan and
collagen, while there is a host of synthetic polymeric scaffolds like, e.g., hydrogels, polystyrene, copolymers, acrylates, polyglycolic acid, etc.

Among various scaffolds for tissue regrowth used to date, there is a group of 2D scaffolds including synthetic nanomembranes that ensure 3D cell growth. The materials for these nanomembranes include the above-mentioned ones [214], but also novel structures like carbon nanomembranes and graphene, polycaprolactone and many more [215–217].

Wu et al. [215] investigated the biocompatibility and biotoxicity of graphene as well as its potential uses in tissue regeneration and regrowth, as well as its role in cell reorganization and regrowth. Jakšić et al. considered a possible use of metal-composite nanomembranes as 2D scaffolds for nerve regrowth [52]. An analysis of the use of biomimetic nanomembranes as a ultrathin extracellular scaffold for free-standing cell sheets is given in [15]. Figure 7a shows a simplified presentation of a metal-composite nanomembrane sieve as a nerve cuff bypassing a nerve damage.

![Figure 7a](image_url)

**Figure 7.** Summary of biomedical applications of biomimetic nanomembranes. (a) Simplified depiction of metal-composite nanomembrane sieve bypassing a nerve damage, with partially re-grown neural tissue. Authors' own work from [52]. (b) Comparison of conventional and nanomembrane-based in vivo neural interfacing. Left: Conventional implanted electrodes. Right: non-invasive injected soft plasmonic nanomembrane-based electrodes, interrogated by modulated infrared beam. Authors' own work from [52]. (c) Structure of a nanomembrane-based neural interface with coupling based on long range surface plasmons polaritons. Authors' own work from [52]. (d) Schematic presentation of the operation of the artificial kidney. (e)
5.6.2. Neural Interfaces/Neural Cuffs

We considered the feasibility of using functionalized nanomembranes as a minimally invasive link connecting external optical signals and neural tissue, using long-range surface plasmon-polaritons (SPP) [218,219]. Neural activity was already recorded in vitro, using coupling of propagating free-space modes with neural impulses by way of long-range SPP, with excellent sensitivity.

We continue by describing a potential use of a metal composite nanomembrane as an implantable “nerve cuff” electrode that may be positioned around the epineurium as a kind of “shrink-wrap”, with tens of nanometers thickness, which ensures intimate contact with the neural tissue [52,220]. When conventional microelectrodes are inserted (Figure 7b, left), they damage capillaries, extracellular matrix and glial and neural cells. The results are inflammations, swelling, bleeding, necrosis, etc. In the long run, glial cells try to phagocytose the foreign body, damaging it and isolating it by a few hundreds of µm of scar tissue.

The bulk metal of a conventional microelectrode is replaced here by a metal nanomembrane with a thickness three orders of magnitude smaller than a bacteria size, making the latter outstandingly soft and flexible at macroscopic level and thus minimally invasive; such “soft” biomimetic electrodes will cause minimal mechanical damages (Figure 7b right). An illustration of surface-plasmon polariton soft nerve cuff for the readout of neural activity is shown in Figure 7c.

A previously described method of nanomembrane insertion [51] (Section 2.2 of this text) is the injection by a simple syringe, which makes use of their surprising robustness and toughness, as well as of their extreme foldability.

Excitation and readout could be done by modulated infrared laser beam, since it propagates well through living tissue. The surface relief of the semi-crumpled membrane ensures coupling of propagating with evanescent modes. Additionally, it has previously been shown in this text (4.1.1) that nanomembranes can be made fully biocompatible through proper material choice and functionalization through both-sided lamination.

5.6.3. Wearable Artificial Kidneys

The development of wearable artificial kidneys [221,222] is of great interest for patients with kidney failure (chronic kidney disease and end-stage renal disease). The need to keep the patients connected to large systems for dialysis for prolonged periods of time is disabling, drastically altering their life, the procedure is slow and painful, and abrupt changes in blood composition may cause a shock to the patient (the so-called dialysis washout), requiring at least hours to recover. On the other hand, prolonged periods between dialysis sessions may leave the patient with increased levels of toxins in blood. A wearable device for dialysis would ideally allow for continuous detoxification and at the same time permit the patient to be mobile while under treatment, thus drastically improving his/her quality of life. Most contemporary portable devices weigh somewhere between 3 and 30 kg [223]. Because of this, one of the key targets of contemporary microfluidics and microelectromechanical systems (MEMS) technology is the development of wearable or even implantable kidneys through a decrease of their dimensions and an increase in efficiency [224].

A simplified principle of operation of an artificial kidney (renal replacement device) is shown in Figure 7d. Its main component is a system of polymer or copolymer hollow microfibers with nano-sized pores. The patient’s blood flows through the microfibers, which are surrounded by dialysate, is filtered through the system of pores, and waste components such as urea and creatinine are removed and washed into dialysate.

There are several experimental systems that mimic the toxin removal function of natural kidneys and have been scaled down for possible use in wearable systems. Such systems are based on the use of inorganic porous nanomembranes and have been reported by Hill et al. [225]. They used silicon nitride membranes with a thickness below 100 nm and with nanometer-sized pores. Such nanomembranes showed high filtration performance while at the same time exhibiting excellent robustness. They reduced the necessary membrane area for hemodialysis compared to conventional,
floor-standing equipment by 100 times or even more. This may enable the production of miniaturized systems that could lead to hemodialysis at home, with longer duration and with improved removal of toxic components from blood. Thus, nanomembranes represent an important enabling step towards wearable artificial kidneys, potentially ensuring continuous blood purifying through ultrafiltration hemodialysis [226].

5.6.4. Portable Artificial Lungs

State-of-the-art artificial lungs aim to enhance the level of oxygen exchange in the blood of patients with lung failure [227,228]. To this end, the devices use large bundles of hollow fibers made of rolled porous membranes with typical radii of 100 to 200 µm. Oxygen fills the space inside of each microfiber, while blood flows around the fibers. Diffusion ensures flow of oxygen through pores into the blood, while simultaneously the same mechanism ensures that carbon dioxide exiting the blood enters the microfluidic fiber. A simplified principle of operation of the artificial lungs is shown in Figure 7e.

The critical goals in the fabrication of the artificial lungs are to minimize the dimensions of the hollow fiber bundle for gas exchange (since it represents the most part of the bulk of the artificial lungs), and at the same time to maximize the gas throughput. Both of these ensure a decrease in the dimensions of the structure and thus contribute to improved mobility of the patient while on artificial lungs. Typically, biocompatible polymer materials are currently used for the hollow fibers, for instance polypropylene [227].

Bearing in mind the dimensions of nanomembranes, they seem ideal for both of the main improvement goals—since gas has to cross a much shorter path through the pores than in the conventional thick membranes, throughput will be enhanced. Simultaneously, the fiber walls will be much thinner, thus contributing to the improved portability of the whole system.

5.6.5. Selective Drug Delivery and Therapy

The pharmacokinetic behaviors of drugs is closely related to cell membranes. A majority of therapeutic drugs target either receptors within membranes or organelles and their parts like chromosomes/DNA within cells, i.e., they interact with biological membranes either by acting upon their built-in blocks or passing through them using some of their existing pathways for matter exchange (for instance, membrane proteins that assume the role of transport pumps, or ion channels) to proceed to targets inside the cell or even inside organelles.

Nanoparticles can be used for medical treatment in several manners [229]. The oldest approach among them is their use as vehicles to carry drugs and therapeutic agents (diagnostic tools for marking the affected tissue, mediator agents for photodynamic therapy, chemotherapy, gene therapy, etc.) to their target [230], which is often cancerous tissue [231]. A variant of this approach is to “hide” therapeutic agents in freely movable cells, thus using them as carriers [232]. Another method is direct introduction of, e.g., metallic or titanium dioxide nanoparticles into the affected tissue as mediator agents for hyperthermia treatment. For this purpose, one causes heating of nanoparticles by remote tissue-penetrating electromagnetic radiation, e.g., laser [233] or microwaves [234], and the hot nanoparticles cause thermal death of the heat-sensitive cancer cells [235]. Plasmonic effects are used for this purpose too [232]. Finally, one of the approaches to drugs delivery (e.g., antidiabetic, anti hypercholesterolemic, etc., drugs) is the direct injection of their nanoparticles, without a carrier [236].

Problems with all of the quoted approaches are the poor targeting of the affected tissue due to bodily fluid circulation in vivo, a short life of nanodrugs in the living tissue, and poor penetration depths. This is the reason why only a few carrier-based nanoparticle drugs have been approved by Food and Drugs Administration [232].

Biomimetic nanomembranes could be used as carriers for nanodrugs and other therapeutic agents [19]. Whether the drugs are in the form of nanoparticles or not, their uncontrolled release and spreading through the body could potentially be harmful and toxic. However, if they are embedded in a biomimetic nanomembrane functionalized to ensure biocompatibility, then the whole ensemble
functions as a soft macroscopic carrier with nano-volume and with very good targeting. It is necessary to ensure the adherence of the unfurled biomimetic nanomembrane to the treated tissue in the position where the nanomembrane was injected. In this case, the drugs will be concentrated in exactly the position where the soft nanomembrane is located. In an ideal case no free nanoparticles will roam the organism. Furthermore, the active area of nanomembranes is close to the theoretical maximum ("the interface without volume"); thus, they offer high exposure of the tissue to embedded nanoparticles and maximized efficiency combined with localization (Figure 8). The critical steps here represent possible decomposition and resorption of the nanomembrane under the influence of body fluids and biological processes and subsequent potential cytotoxicity [237].

![Figure 8. Drug nanoparticles (violet spheres) embedded in nanomembrane (golden) as a drug delivery system.](image)

6. Future Outlook

Here we briefly present a few of possible directions for future research. There is a vast number of such directions that we do not mention here, and the area keeps expanding on a daily basis. The topic of biomimetic nanomembranes opens literally endless horizons for both research and applications.

6.1. Novel Types and Architectures of Artificial Pore Complexes

One field that leaves much to be desired is the fabrication of artificial ion channels, ion pumps and nuclear pore complexes. Structurally, all types of ion-transporting pores are currently far less complex than their living counterparts. Functionally, many artificial ion channels are close to the biological structures. Ion pumps require much more research, and it will require a lot of effort to achieve both the functionality and complexity of the natural structures. A similar claim, only on an even larger scale, can be made for pores located in the membrane around the cell nucleus. Huge advances have been made in recent years. Yet larger ones remain to be achieved.

6.2. “Living” Plasmonics and Metamaterials

One promising direction of research is the integration of plasmonics with functionalized biomimetic membranes. The use of nanomembranes for plasmonics and metamaterials was considered in [27]. A further logical direction would be research into biomimetic plasmonics. Publications in this promising area are almost nonexistent. One of the rare papers in the field investigates sensors of tumor cells using surface plasmon resonance functionalized by artificial lipid bilayer membranes [238]. Even more scarce are publications on biomimetic metamaterials.

In this subsection we consider the possibility of researching nanomembranes with plasmonic and/or metasurface properties functionalized by some of the biomimetic structures previously
considered in this text. Functionalization of nanomembranes made of metal or some other plasmonic material using synthetic ion channels, ion pumps, water channels or artificial nuclear pore complexes seem to be especially promising for the field of biological sensing. Additionally, all plasmonic nanomembrane-based sensors could benefit from antifouling functionalization which could improve their applicability in the real field conditions.

6.3. Quantum Functionalities

Nanomembranes represent quasi-2D objects, which means that charge carrier transport in them will obey the rules of low-dimensional systems and thus will show marked quantum mechanical effects. This in itself is a large field of investigation, since each low-dimensional material will have its own set of properties and peculiarities, including effects rarely or never observed in nature. It may be said that the topic of combining biomimetic nanoobjects and quantum physics is in its very infancy, although the results to date are encouraging and have already brought us to a host of novel effects and even novel and improved electronic devices [48].

6.4. Meta-Bilayers Adding Functionalities beyond Natural to Biomimetic Nanomembranes

The possibility of using an extended toolbox of materials compared to biological structures opens the path to imparting new functionalities. For instance, one can make lipid bilayers in which proteins are not the main functionalizing agents. Various alternative functions could be imparted to them, including incorporation of particles with magnetic, optical, plasmonic, catalytic including photocatalytic, pharmaceutical and other properties. This would make “meta” lipid bilayers out of them—those with properties that go beyond the natural ones.

6.5. Shape-Shifting Nanomembrane Bulges with Active External Control

The curvature and the overall profile of spatially sculpted nanomembranes could be externally controlled. It is known that several macroscopic properties of micro and nanosurfaces are influenced or even defined by their surface shapes—examples include self-cleaning and superhydrophobic behavior, actually a drop-repellent property (the lotus leaf effect) [239,240], and drop-pinning on hydrophobic surfaces (the rose petal effect) [241]. An active control of the surface microstructure could switch between these two modes of superhydrophobic behavior. Another property dictated by the shape of the surface details is adhesion, observed in some organisms ranging from simple bacteria to such sophisticated organisms as some frog species (e.g., Litoria caerulea), where the active control over the adhesive properties is obtained through the change of the surface shapes. A practical approach to control over the adhesion properties of membranes was considered in [242] for the case of thick elastic membranes. The actuation needed for the surface relief change was obtained through pneumatic mechanism.

In this work we extrapolate the active approach to shape shifting to the field of nanomembranes, which, being much thinner than the conventional membranes, could ensure a more energy-efficient and precise switching between different modes of hydrophobicity and hydrophilicity, as well as dynamical control over adhesive properties. The actuation mechanisms that could be used for such shape shifting include applied electric field, single-sided pressure, mechanical change of the membrane support through stress/stretching or bending, the external optical field, etc.

7. Conclusions

In this paper, we considered the main properties of biomimetic synthetic nanomembranes, and their fabrication and functionalization. We also considered the main biological functional parts of cell nanomembranes, the ones ensuring basic complex functionalities that make up life processes, including membrane ion channels and ion pumps, as well as parts of the cell nucleus envelope, the nuclear pore complexes. After that we proceeded to consider their biomimetic counterparts. One of the important functionalities that we considered is imparting the biomimetic antifouling properties to the membranes.
In the next part we gave an overview of selected applications of biomimetic nanomembranes, either theoretically proposed or actively used. At the end we gave some extrapolations and possible directions for future research.

It was our aim in this review to stress the importance of functionalized synthetic biomimetic nanomembranes within the context of modern nanoscience and nanotechnologies. We hoped to highlight the importance of the topic, as well as its profound applicability potential in many facets of human life.

Biological nanomembranes are the most ubiquitous nanomachine in nature, which says a lot about their usefulness and versatility. Mimicking their functions and complexities offers a cornucopia of rewards. It is only up to us how deep we will be able to reach.

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**References**


41. Huang, K.; Li, Z.; Lin, J.; Han, G.; Huang, P. Two-dimensional transition metal carbides and nitrides (MXenes) for biomedical applications. Chem. Soc. Rev. 2018, 47, 5109–5124. [CrossRef]
54. Altenbach, H.; Mikhasev, G.I. Shell and Membrane Theories in Mechanics and Biology; Springer: Berlin/Heidelberg, Germany, 2016. [CrossRef]


87. Nosheen, S. Nanomembrane Applications in Environmental Engineering. In Nanotechnology Applications in Environmental Engineering; Nazir, R., Ed.; IGI Global: Hershey, PA, USA, 2019; pp. 103–120. [CrossRef]
88. Watanabe, H.; Kunitake, T. A large, freestanding, 20 nm thick nanomembrane based on an epoxy resin. Adv. Mat. 2007, 19, 909–912. [CrossRef]
89. Salvatore, S. Optical Metamaterials by Block Copolymer Self-assembly; Springer: Cham, Switzerland, 2014. [CrossRef]
116. Matovic, J.; Kettle, J.; Brousseau, E.; Adamovic, N. Patterning of nanomembranes with a focused-ion-beam. *Nano Today* 2015, 12, 41–45. [CrossRef]


141. Sakai, N.; Matile, S. Synthetic ion channels. Langmuir 2013, 29, 9031–9040. [CrossRef]


144. Tabushi, I.; Kuroda, Y.; Yokota, K. A, B, D, F-tetrasubstituted


152. Kocsis, I.; Sun, Z.; Legrand, Y.M.; Barboiu, M. Artificial water channels—deconvolution of natural Aquaporins through synthetic design. npj Clean Water 2018, 1, 1–11. [CrossRef]


154. Tunuguntla, R.H.; Henley, R.Y.; Yao, Y.-C.; Pham, T.A.; Wanunu, M.; Noy, A. Enhanced water permeability and tunable ion selectivity in subnanometer carbon nanotube porins. Science 2017, 357, 792–796. [CrossRef]


170. Shen, Y.-X.; Saboe, P.O.; Sines, I.T.; Erbakan, M.; Kumar, M. Biomimetic membranes: A review. *J. Membr. Sci.* **2014**, *454*, 359–381. [CrossRef]


192. Shojaei, F.; Mortazavi, B.; Zhuang, X.; Azizi, M. Silicon diphosphide (SiP2) and silicon diarsenide (SiAs2): Novel sTable 2D semiconductors with high carrier mobilities, promising for water splitting photocatalysts. *Mater. Today Energy* 2020, 16, 100377. [CrossRef]


201. Kumar, L.Y. Role and adverse effects of nanomaterials in food technology. *J. Toxicol. Health* 2015, 2, art. 2. [CrossRef]


211. Xue, Y.; Sant, V.; Phillippi, J.; Sant, S. Biodegradable and biomimetic elastomeric scaffolds for tissue-engineered heart valves. *Acta Biomater.* 2017, 48, 2–19. [CrossRef]


Biomimetic Nanocarrier Targeting Drug(s) to Upstream-Receptor Mechanisms in Dementia: Focusing on Linking Pathogenic Cascades

Joseph S. D’Arrigo † ⁉️

Cavitation-Control Technology Inc., Farmington, CT 06032, USA; cavcon@ntplx.net
† Present address: Cav-Con Inc., Bellevue, WA 98007, USA.

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Abstract: Past published studies have already documented that, subsequent to the intravenous injection of colloidal lipid nanocarriers, apolipoprotein (apo)A-I is adsorbed from the blood onto the nanoparticle surface. The adsorbed apoA-I mediates the interaction of the nanoparticle with scavenger receptors on the blood–brain barrier (BBB), followed by receptor-mediated endocytosis and subsequent transcytosis across the BBB. By incorporating the appropriate drug(s) into biomimetic (lipid cubic phase) nanocarriers, one obtains a multitasking combination therapeutic which targets certain cell-surface scavenger receptors, mainly class B type I (i.e., SR-BI), and crosses the BBB. Documented similarities in lipid composition between naturally occurring high-density lipoproteins (HDL) and the artificial biomimetic (nanoemulsion) nanocarrier particles can partially simulate or mimic the known heterogeneity (i.e., subpopulations or subspecies) of HDL particles. Such biomedical application of colloidal drug-nanocarriers can potentially be extended to the treatment of complex medical disorders like dementia. The risk factors for dementia trigger widespread inflammation and oxidative stress; these two processes involve pathophysiological cascades which lead to neuronal Ca\(^{2+}\) increase, neurodegeneration, gradual cognitive/memory decline, and eventually (late-onset) dementia. In particular, more recent research indicates that chronic inflammatory stimulus in the gut may induce (e.g., via serum amyloid A (SAA)) the release of proinflammatory cytokines. Hence, an effective preventive and therapeutic strategy could be based upon drug targeting toward a major SAA receptor responsible for the SAA-mediated cell signaling events leading to cognitive decline and eventually Alzheimer’s disease or (late-onset) dementia.

Keywords: Alzheimer’s disease; calcium dyshomeostasis; dementia; drug targeting; endothelial dysfunction; inflammation; nanocarrier; oxidative stress

1. Introduction

Microvascular endothelial dysfunction, due to cerebrovascular risk factors, precedes cognitive decline in Alzheimer’s disease and contributes to its pathogenesis (see [1,2] for reviews). These risk factors (e.g., hypertension, diabetes, obesity, atherosclerosis, smoking, aging) trigger widespread inflammation and oxidative stress; these two processes involve pathophysiological cascades which lead to neuronal Ca\(^{2+}\) increase, neurodegeneration, gradual cognitive/memory decline, and eventually (late-onset) dementia [3].

2. Endothelial Dysfunction, and Targeted Treatment for Early Dementia

It has been reported repeatedly that endothelial modulation and repair is feasible by pharmacological targeting [1,2,4–10] of the SR-BI receptors (i.e., “scavenger receptor class B, type I”) [10]. As the detailed review by Mahringer et al. [11] points out, the blood-brain barrier (BBB) is equipped with several
endocytic receptors at the luminal surface (i.e., the capillary endothelial membrane), including SR-BI. Recently, Fung et al. [12] specifically found that SR-BI mediates the uptake and transcytosis of high-density lipoproteins (HDL) across brain microvascular endothelial cells (i.e., across the BBB). Since SR-BI has already been identified as a major receptor for HDL (with their major apolipoprotein (apo)A-I), as well as for the recently reviewed [1,2] “lipid-coated microbubble/nanoparticle-derived” (LCM/ND) nanoemulsion (see below), this multitasking lipid nanoemulsion can arguably serve as a targeted, apoA-I-based, (SR-BI mediated) therapeutic agent for common (late-onset) dementias [13–15].

This targeted-delivery-approach, using the proposed LCM/ND lipid nanoemulsion for treating the more common (late-onset) dementias, receives added impetus from continual findings of cerebrovascular pathology [1,16–26] and an apparent endothelium dysfunction [2,15,19,22,27–33] in both Alzheimer’s disease and its major risk factors [1,2,26–38]. By incorporating drug molecules into the LCM/ND lipid nanoemulsion type (yielding particle sizes mostly < 0.1 µm in diameter—see Figure 1), known to be a successful drug carrier [39,40], one is likely to obtain a multitasking combination therapeutic capable of targeting cell-surface SR-BI. This (intravenous) combination therapeutic would make it possible for various cell types, all potentially implicated in Alzheimer’s disease [1,2], to be simultaneously sought out and better reached for the localized drug treatment of brain tissue in vivo [39,40].

![Figure 1. Lipid-coated microbubble/nanoparticle-derived (LCM/ND) nanoemulsion stability over time [2].](image)

3. Colloidal Nanocarrier Formation, and Targeting via Lipid Cubic Phases

In this particular targeted-delivery approach, the self-assembled colloidal “nanocarrier” structure itself (upon the intravenous injection of LCM/ND lipid nanoemulsion) is apparently successfully utilized as the “active” targeting ligand—which is directed via (adsorption of) plasma lipoproteins (including notably apoA-I) toward the appropriate endocytic receptors on the target-cell surface [39].

Previous reports concerning colloidal nanocarriers (e.g., [41,42]) do not fully explain how various (biobased) lipids, and their mixtures, are able to reliably form self-assembled non-lamellar nanostructures (i.e., lipid cubic phases)—which, in turn, have been observed to serve as colloiddally stable nanocarriers for drug(s) in excess water (e.g., in blood plasma). The answer to this fundamental
question resides in the physicochemical tendency of these biobased lipids to adopt a non-lamellar inverse topology [43]. This special tendency of these surface-active lipids is itself a function of lipid head-group hydration, acyl chain length, and cholesterol content (cf. below). In general, by increasing the average negative curvature of the lipid/water interface (e.g., by means of the water concentration or temperature), inverse-topology liquid–crystalline lipid phases (viz. different from lamellar) can be obtained—namely, inverse bicontinuous cubic phases, inverse hexagonal phases, or inverse (discontinuous) micellar cubic phases [44]. Moreover, Pouzot et al. have asserted that there is actual consensus on the fact that the formation of an (Fd3m) micellar cubic phase is promoted in systems where lipids have a negative preferred curvature, which is practically realized when long alkyl hydrophobic tails are associated with weakly hydrated, hydrophilic head groups ([44]; cf. [45]).

Notice that this actual consensus that amphiphilic lipids with weakly hydrated, hydrophilic head groups serve to promote the formation of an Fd3m cubic phase (also known as phase Q227) is particularly relevant to the earlier-described [42] LCM/ND nanoemulsion formulation(s): Specifically, the saturated glycerides and cholesterol (and its ester derivatives), which together compose the basic Filmix® (LCM/ND) nanoemulsion formulation [39], are all non-ionic and therefore each amphiphilic lipid in such a lipid mixture would only have a weakly hydrated, hydrophilic head group. Consequently, the above facts considered together support the earlier provisional conclusion that the dispersed Fd3m micellar cubic phase represents the most probable or preferred lipid polymorphic form adopted by the particles in the LCM/ND nanoemulsions [42,46].

In summary, the dispersed lipid particles of LCM/ND nanoemulsions very likely represent liquid–crystalline inverse-topology nanocarriers, i.e., dispersed lipid cubic phases (cf. [39]).

4. Cardiovascular Risk Factors, Inflammation, Oxidative Stress, Calcium Dyshomeostasis, and SR-BI

The cardiovascular risk factors for dementia induce brain tissue hypoxia, leading to endothelial cell activation. The result is the production/release of reactive oxygen species (ROS) and proinflammatory proteins, which together trigger widespread inflammation and oxidative stress—both of which can lead to BBB disruption [47]. (Note that inflammation is intimately associated with oxidative stress in Alzheimer’s disease. The redox status modulates inflammatory factors involvement in signaling processes, which are critical mediators of oxidative stress and neuroinflammation, causing neurodegeneration. The resultant cellular damage promotes further neuroinflammation in the Alzheimer’s-disease brain [48].) These pathological cascades lead to a neuronal Ca\(^{2+}\) increase, neurodegeneration, gradual cognitive/memory decline, and eventually Alzheimer’s disease [3].

It is believed by many researchers that enhanced calcium load may be brought about by extracellular accumulation of amyloid-\(\beta\) (A\(\beta\)) in the brain. Such studies have laid the foundation for the popular idea that A\(\beta\) peptides (39–42 amino acid molecules) are, in part, toxic to brain tissue because they form aberrant ion channels in cellular membranes and thereby disrupt Ca\(^{2+}\) homeostasis in brain tissue and increase intracellular Ca\(^{2+}\) [49,50]. Historical support for the above amyloid-\(\beta\) ion channel hypothesis, or so-called “calcium hypothesis”, has also been observed at the clinical level [51]. A good correlation exists between early cognitive impairment and levels of soluble forms of A\(\beta\) in the brain (but not the (insoluble) amyloid deposits or plaques at autopsy) [52]. Moreover, a recent biochemical study [53] of the two major A\(\beta\) variants, A\(\beta\)(1–40) and A\(\beta\)(1–42), has shown that: 1) A\(\beta\)(1–40) aggregated into amyloid fibrils; 2) contrariwise, A\(\beta\)(1–42) assembled into oligomers that inserted into membranes (i.e., artificial bilayers and/or biological membranes excised from cells of neuronal origin) as well-defined pores. (These amyloid pores adopted characteristics of a \(\beta\)-barrel arrangement.) Because A\(\beta\)(1–42), relative to A\(\beta\)(1–40), has a more prominent role in Alzheimer’s disease, the higher propensity of A\(\beta\)(1–42) to form \(\beta\)-barrel pore-forming oligomers is an indication of their importance in Alzheimer’s disease [53]. Furthermore, ion channel conductance results suggested that A\(\beta\)(1–42) oligomers, but not monomers and fibrils, formed pore structures. The authors concluded that their findings demonstrate that only A\(\beta\)(1–42) contains unique structural features that facilitate membrane insertion.
and channel formation, now aligning ion channel formation with the neurotoxic effect of Aβ(1–42) compared to Aβ(1–40) in Alzheimer’s disease [53]. (In addition, tea polyphenols have been repeatedly reported (e.g., [54]) to protect cells from Aβ-mediated neurotoxicity, by dose-dependently inhibiting the formation of Aβ aggregates (e.g., from fresh Aβ(1–42) peptides), through the destabilization of preformed Aβ aggregates. These green tea polyphenols (regularly ingested worldwide via tea beverage consumption) are considered to be valuable, for the prevention and therapeutic treatment of Alzheimer’s disease, via the combined effect of inhibiting Aβ aggregate formation and protecting neurons from the toxicity (e.g., oxidative stress) induced by Aβ [54].)

Note too that, while this Section 4 began with an acknowledgement that the risk factors for dementia trigger widespread inflammation and oxidative stress (e.g., [3]), it is also true that these two processes can result in more biological effects than enhanced calcium load in brain tissue and neurodegeneration (cf. [55]). In fact, oxidative stress and inflammation each involve pathophysiological cascades associated with a wide range of pathologies and especially aging. However, these two processes/cascades are not always associated with biological damage. (For example, oxidative stress constitutes an important mechanism in many physiological processes, such as adaptations to physical exercise and cell signaling.) Yet, when oxidative stress and/or inflammation are dysregulated, their action is harmful [55]. (In this situation, one corresponding example [of many] occurs in Alzheimer’s disease, where growing evidence links the ROS-mediated damages with molecular targets including mitochondrial dynamics/function, autophagic pathways, and proteostasis balance [56].) Accordingly, Khalil et al. [57] found that Alzheimer’s disease impaired the interaction of HDL (and ApoA-I) with the SR-BI receptor, and their experimental results indicated that such patients had higher levels of oxidative stress [57]. The authors concluded that their clinical study provides evidence for the first time that the functionality of HDL is impaired in Alzheimer’s disease, and that this alteration may be caused by Alzheimer’s disease-associated oxidative stress and inflammation [57]. This conclusion is consistent with earlier work where SR-BI was identified on astrocytes and vascular smooth muscle cells in Alzheimer’s disease brain, and has been demonstrated to mediate the adhesion of microglia to aggregated Aβ (cf. [58]). Moreover, these authors further report that SR-BI mediates perivascular macrophage response, and regulates Aβ-related pathology and cerebral amyloid angiopathy, in an Alzheimer’s-disease mouse model [58].

5. Gut-Brain Axis, Serum Amyloid A (SAA) versus SR-BI Targeting, and Alzheimer’s Disease or (late-onset) Dementia

Particularly noteworthy is more recent research [59,60] indicating that chronic inflammatory stimulus in the gut may induce (e.g., via serum amyloid A (SAA)) the release of proinflammatory cytokines. At the same time, increased BBB permeability due to aging (or dysfunction), in turn, allows these proinflammatory cytokines to enter the brain, inducing glia reactivity [59,60]. These recent findings and various past studies indicate that inflammation plays an important role in the process of Aβ deposition and, therefore, the inhibition of inflammatory cascades may attenuate amyloidogenic processes—such as Alzheimer’s disease [61] (cf. [57,62]). Hence, an effective preventive and therapeutic strategy could be based upon targeting drug(s) toward a major SAA receptor responsible for the SAA-mediated cell signaling events leading to cognitive decline and eventually Alzheimer’s disease or (late-onset) dementia.

Specifically, earlier research [63] has already confirmed that SR-BI receptors (or its human ortholog CLA-1) function as cell-surface SAA receptors—which bind, internalize, and mediate SAA-induced proinflammatory effects (cf. [64]). However, Baranova et al. additionally report that (in cell culture) CLA-1/SR-BI ligands “efficiently compete” with SAA for CLA-1/SR-BI binding [63]. (For example, it has already been documented in the literature that both apoA-I and SAA are substrates for SR-BI, which indicates that SR-BI could mediate the transport of both proteins across the BBB (e.g., [65])). Not surprisingly, therefore, Robert et al. have recently asserted that many lines of evidence suggest a protective role for HDL and its major apolipoprotein (apo)A-I in Alzheimer’s disease [14]. Accordingly,
a similar benefit (of “competitive binding” to SR-BI receptors) may well accompany the clinical intravenous use of the LCM/ND lipid nanoemulsion vehicle—which has already been repeatedly described in the peer-reviewed literature (based upon numerous in vivo animal studies) as a targeted, apoA-I-based, (SR-BI mediated) drug-delivery agent (see Section 2). Moreover, by incorporating drug molecules into the LCM/ND lipid nanoemulsion type, one is likely to obtain a multitasking “combination therapeutic” capable of targeting cell-surface SR-BI. This (intravenous) colloidal-nanocarrier therapeutic would make it possible for various cell types, all potentially implicated in Alzheimer’s disease [1,2] and/or (late-onset) dementia, to be simultaneously sought out and better reached for localized drug treatment of brain tissue in vivo [39,40].

6. Conclusions

Cerebrovascular risk factors trigger widespread inflammation and oxidative stress, both of which can lead to BBB disruption. These pathological cascades lead to neuronal (intracellular) Ca\(^{2+}\) increase, neurodegeneration, gradual cognitive/memory decline, and eventually Alzheimer’s disease. In particular, more recent research indicates that chronic inflammatory stimulus in the gut may induce (e.g., via serum amyloid A (SAA)) the release of proinflammatory cytokines. At the same time, increased BBB permeability due to aging and/or dysfunction, in turn, allows these proinflammatory cytokines to enter the brain, inducing glia reactivity. An effective preventive and therapeutic strategy could be based upon early (or even proactive) targeting of drug(s) toward a major SAA receptor responsible for the SAA-mediated cell signaling events leading to cognitive decline, and eventually Alzheimer’s disease or (late-onset) dementia.

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**References**


46. Gamba, P.; Testa, G.; Gargiulo, S.; Staurenghi, E.; Poli, G.; Leonarduzzi, G. Oxidized cholesterol as the driving force behind the development of Alzheimer’s disease. *Front. Aging Neurosci.* 2015, 7, 119. [CrossRef]


