In Vivo Writing using Two-Photon-Polymerization

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Two-Photon-Polymerization (2PP) is a fast developing method for the micro- and nanostructuring of three-dimensional parts. The manufacturing of biocompatible structures using this technique is a promising field as it fulfills the demands for parts with high feature resolution. This paper reports the fabrication of scaffolds using photopolymers with embedded living organisms (*Caenorhabditis elegans*). The structuring was performed with a pulsed near-infrared laser with a wavelength of 810nm and adjustable power up to 160mW. Using a 20x magnification microscope objective with a numerical aperture of 0.4, a high resolution scaffold with a base area of 300x300µm and a height of 80µm could be fabricated. Taking advantage of high laser intensities (writing speed: 300µm/s) the structuring process took only 12 minutes.

The required laser intensities do not cause damage at the cellular level for the model organism since biological tissues are very transparent to red and infrared light. The toxicity of the resin increases with its reactivity; therefore there is a trade-off between polymerization time and toxicity in the in-vivo-writing process. To optimize the conditions we tested the toxicity and reactivity of different resins with a focus on water-based, biocompatible and biodegradable hydrogels together with water soluble, near-infrared initiators suitable for 2PP.

Keywords: Additive Manufacturing Technologies, Nanostructuring, High-Resolution Parts, Biocompatible, Biodegradable, Two-Photon-Lithography, Hydrogel

1. Introduction

In contrast to reptiles such as salamanders or lizards, mammals like humans cannot regrow their body parts to the previous anatomical shape. Without any guidance, cells form a two-dimensional monolayer only. Tissue engineering manipulates cells via their extracellular microenvironment to reorganize them in a three-dimensional architecture, a so-called "scaffold". These scaffolds are made of hydrogels, cross-linked polymers swollen in water. The versatility of hydrogels makes them an appealing class of materials for additive manufacturing (AM) applications, since it is possible to fabricate parts with mechanical properties that resemble many biological tissues. The functional and structural properties can be tailored over a large range. In addition, they offer a large potential regarding the rate and type of biodegradation. Together with high-resolution AM technologies these materials can be fabricated to have targeted mechanical and transport properties [4],[1].

Two-photon-polymerization (2PP), a rather new structuring technique, is not restricted to a simple layer-by-layer fabrication. With 2PP, polymerization only occurs in the focal point of a microscope objective. The share of the monomer located between the entry and the focal point is transparent for the near-infrared (NIR) laser beam. An excited energy level rendering radicals can only be achieved near the laser focus. Biological tissue is transparent for the beam, too. Using the 2PP technique it is theoretical possible to structure 3D parts inside a photo-polymerizable resin that is located inside biological tissue.

This feature of 2PP can lead to an improvement of the tissue engineering process. The goal is to inject a mixture of particular cells grown in the laboratory and biocompatible monomers into the patient's body. It should then be possible to polymerize the material from the outside, i.e. to generate the transplants directly in the patient. This new approach has three major advantages.

- No scars would be induced on the patient's skin as there would be no need for a surgical intervention.
- As this treatment is non-invasive it would cause less stress to the patient than an operational intervention. Therefore it would lead to shorter recovery phases and higher chances of curing.
- The shape of the construct can be adapted to the local context in-vivo. Under maturing it can interact with the surrounding structures.

As a step towards a treatment method of this kind, this work presents the manufacturing of parts inside a resin containing living organisms of the species Caenorhabditis

elegans. For a proper in-vivo-writing process it is necessary to find biological degradable and compatible resins with a low toxicity, being structurable with 2PP. These polymers should be on an aqueous basis, similar to biological tissues. Hence, acrylate-based resins, dissolved in different contents of water were tested for their feasibility for 2PP structuring as well as for their toxicity regarding their usability for in-vivo applications. Using a specially adapted two-photon-initiator (TPI), all hydrogels used in this work could be polymerized.

2. Experimental section

2.1. Photopolymerizable hydrogels

We synthesized an already known water-soluble molecule according to Woo et al. [7] 1,4- bis(4-(N,N- bis(6-(N,N,N-trimethylammonium)hexyl)amino)- styryl)-2,5-dimethoxybenzene tetraiodide (WSPI) as a novel TPI for our hydrogel applications (Fig. 1).

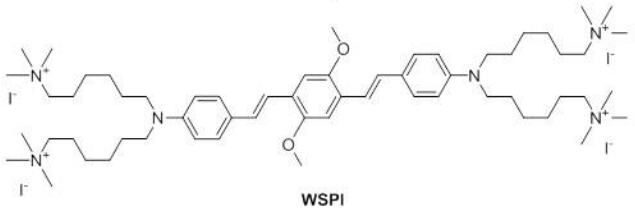

Fig. 1 Water -soluble TPI WSPI

In the literature, this compound and its derivatives were reported to be synthesized for the use in a two-photon-microscope and for the investigation of solvent effects in aqueous media needed for biological imaging [7]. The compound was never tested with 2PP to our knowledge. The solubility in polar media such as water is achieved by additional quaternary ammonium cations in the aliphatic sidegroups bonded to the aniline functionality. The charged end groups do not appear to interact with the electronic structure of the TPA chromophore and do not modify spectral properties but increase the solubility in aqueous media.
The monomers used consisted of a polyethylene-glycol (PEG) spacer with acrylate reactive end groups (Fig. 2). Using an adequate molecular mass of PEG inside the oligomers, the monomers could be easily swollen in water or aqueous solutions like M9 buffer[1].

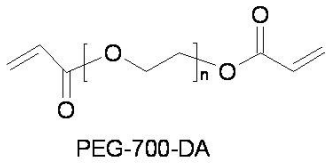

Fig. 2 Monomers applied: polyethyleneglycol-diacrylate (PEG-700-DA) with a molecular mass of 700.

2.2. 2PP setup

The basic setup of the 2PP system used for this work can be seen in Fig. 3. The laser device was an all-diode-pumped Ti:Sapphire laser from HIGH-Q LASER (Model Number IC-800-200fs) with a pulse length of 160fs and a wavelength of 810nm. The pulse repetition rate was 73MHz. The near-gaussian beam profile allowed a line width of 14nm and the output power was 200mW. Its beam passed through an acousto-optic-modulator. This shutter diffracted the laser beam so that first order waves could be used for polymerizing. The intensity was adjusted by a λ/2 waveplate and a polarization depending beam-splitter. Before the beam reached the resin it got focused through a microscope objective. The X- and Y-movements of the laser beam were realized by high precision air bearing axes. The Z-movement was achieved by a similar axis carrying the resin container. The mirror system ensured that the X- and Y-movement was strictly parallel to the direction of the laser beam so that the guidance of the beam was stable throughout the whole structuring process. For the online process observation a camera viewed along the laser beam and got focused on the polymerization spot through the same objective. The axes were mounted on a hard stone frame designed to damp vibrations. The whole setup's base was an optical table with an air friction damping, again to suppress vibrations. For the control of the machine, the axes and the laser intensity power meter were plugged to an electronic device, which processed the commands given by the control computer. The sample was illuminated using a red LED lamp to prevent premature polymerization.

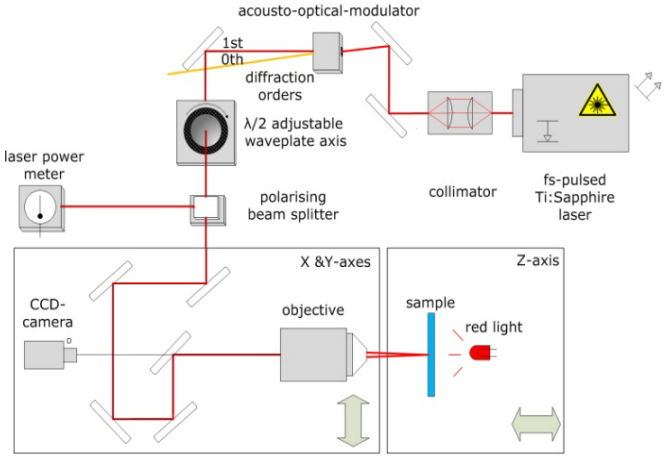

Fig. 3 Experimental setup of a femtosecond micromachining system for in-vivo-writing

[1] M9 buffer is a specially prepared aqueous solution used to maintain C. elegans in aqueous media. It consists of H_20 with 0.3wt% KH_2PO_4, 0.6wt% Na_2HPO_4 and 0.5wt% $MgSO_4$. The animals usually survive up to 4 hours in this aqueous solution [5].

2.3. Microscope objectives

For the experiments we used two different objectives: one with a 20x magnification and one with 100x with NA 0.4 and 1.4, respectively. Both were manufactured by Carl Zeiss.

It appeared appropriate to use the high-resolution objective to investigate structuring parameters, the other for in-vivo applications for the following reasons: Initially, objectives with a high NA like the 100x facilitate the fabrication of parts corresponding to the desired dimensions. A low NA, in contrast, causes an optical distortion of the volumetric pixel (voxel) shape. Thus, writing parameters leading to optically satisfying objects cannot be determined using the 20x objective. However, the 20x objective has several advantages for in-vivo applications: first, it improves the possibility to increase the objects's dimensions at a comparably high level of structuring speed. The decreased feature size resolution is negligible since it is still substantially higher compared to a common 1PP process. The whole writing process is performed within a reasonably shorter period of time, which implies that the organism is exposed to the harmful resin monomers for a shorter time, leading to a higher survival rate. In addition, the focal point of the objective is substantially larger, leading to decreased laser intensities per area and thus to a lower level of cellular stress within living tissue.

Moreover, the large working distance of this objective offers much more flexibility and three-dimensional versatility of the 2PP system. Especially for the in-vivo-writing process, long working distances are essential as the structuring must occur reasonably deep inside a volume. The latter is especially important as it is the aim, in the long-term, to fabricate parts inside biological tissues.

2.3. Stress response in C. elegans caused by near-infrared (NIR) laser

In general, optical absorption coefficients of biological tissues are determined by the absorption of proteins, DNA, melanin, hemoglobin and water. The variation of their optical activities is strongly dependent on the wavelength [6]. In the red and NIR region, all biological tissues have only little absorption. Nevertheless, when using a laser beam with high light intensity per unit area, the residual absorption is potentially high enough to cause damage. Water is the most important tissue chromophore within these wavelengths. It begins contributing significantly to tissue absorption at $\lambda \geq 900$nm [6]. Thus the heating of the tissue increases with the wavelength. Light below 810nm, however, increasingly causes photo-chemically induced stress inside biological tissues [3]. Thus the damage caused is significantly higher using light with wavelengths below this threshold. Hence, light of 810nm in particular, seems to be most suitable for in-vivo-writing.

In the field of optical tweezers systematics, Leitz *et al.* investigated the influence of an 810nm cw-laser on the expression of the four small 16-kDa heat-shock proteins (hsp16) in the excretory cell of C. elegans, coupled to a heat-shock promoter [3]. The beam triggers a significant reaction in 5% of the laboratory animals only at a power of 240mW for a period of 120s, when utilizing a 100x microscope lens. In our experiments, we use a 20x lens and estimated powers from 20-100 mW. The low magnification lens in combination with lower average power therefore leads to a significantly lower average light intensity compared to the tweezer applications. Furthermore, we use a pulsed NIR-beam. The average laser intensity of the tweezer applications reported might therefore not necessarily be relevant here.

To show that the impact of the beam is neglibly small we performed impact studies similar to Leitz *et al.* Unfortunately, we did not have access to the transgenic C. elegans described by [3]. Thus, we could not use the heat shock promoter as an indicator for stress. Rather, we had to investigate the behavior of the worms compared to reference animals. A number of experimental organisms were exposed to 100mW of laser power utilizing a 20x microscope lens. After this procedure, they were put on NGM[2] petri dishes. There was no significant change in behavior of the worms as the animals' mobility was obviously not affected. Furthermore, the population of the sample grew similarly to that of the reference group, indicating no change in the animals' reproduction.

2.4. Investigating process parameters for 2PP

Due to the suitable voxel shape, we investigated feasible structuring speeds and respective laser powers using the 100x microscope objective. We fabricated an array containing default scaffolds produced using different writing speed and power settings (dv/dP). The power ranged from 2 mW to 32 mW, changing by 2 mW for each scaffold in the X-plane. The speed ranged from 10 µm/s to 200 µm/s. The distance between the single lines was adjusted to 5µm. In total, 20 layers were manufactured on top of each other. Depending on the resin, different combinations of parameters led to different qualities of the structures. To determine the optimal resin-specific process window, structures were classified according to their arrangement, their regularity and the thickness of their hatches.**2.4. Toxicity analysis**

The determinant for the in-vivo-writing process is the toxicity of the resin. As a measure of the biocompatibility of different hydrogels, the survival time of the animals in these substances were determined. In each case, 50 animals were transferred to a drop of the respective substance. An equal number of reference animals were kept in a drop of M9 buffer. As an indicator for the life-death analysis of the worms, we investigated their pharyngeal pumping. In case of stopping, the worm was considered dead. This is a

[2] Nematode growth medium: In the laboratory, C. elegans are maintained on NGM petri dish. A dish has a lawn consisting of 3g NaCl, 2.5g Peptone and 17g agar per liter M9 medium (described above). This lawn is spread with E. coli as a food source. On such a petri dish, the animals can live and reproduce for several days [5].

standard method used for toxicity studies at the Vienna Biocenter [2].

3. Results and discussion

3.1. Structuring Parameters

First structuring attempts were performed using a mixture of 50wt% PEG-700-DA and 50wt% M9 buffer together with 2wt% WSPI. The most appropriate structure is displayed in the left picture of Figure 4. Despite the high water content, the single lines of the small scaffold are fully detailed. The picture shows their regularity. Except for the side walls, the structure fits perfectly to the desired CAD dimensions. These promising results made us further increase the water content of the resin.

In the centre of Figure 4, a structure manufactured from a resin with 40wt% PEG-700-DA, 60wt% M9 buffer and 2wt% WSPI is displayed. It is fabricated at 20 mW of power with 190 µm/s of writing speed, again using the 100x objective. In the middle of the structure, the lines appear regular. Yet the walls do not fit the desired dimensions. An increase in the dimensions would presumably render a more regular structure. Using an objective with lower magnification would probably lead to reasonably satisfying structures.

A scaffold fabricated from a hydrogel with 70wt% M9 buffer, 30wt% PEG-700-DA and 2wt% WSPI content is displayed in the left picture of Figure 4. It is fabricated at a speed of 190 µm/s with a power of 28 mW. Although the structure does not have the slightest similarity to the CAD file, this scaffold renders the proof that it is possible to create structures even at very high water contents. This is remarkable regarding the already quite high molecular mass (mw 700) of the PEG spacer inside the monomer PEG-700-DA. This results show that WSPI has the potential to render hydrogels suitale for 2PP.

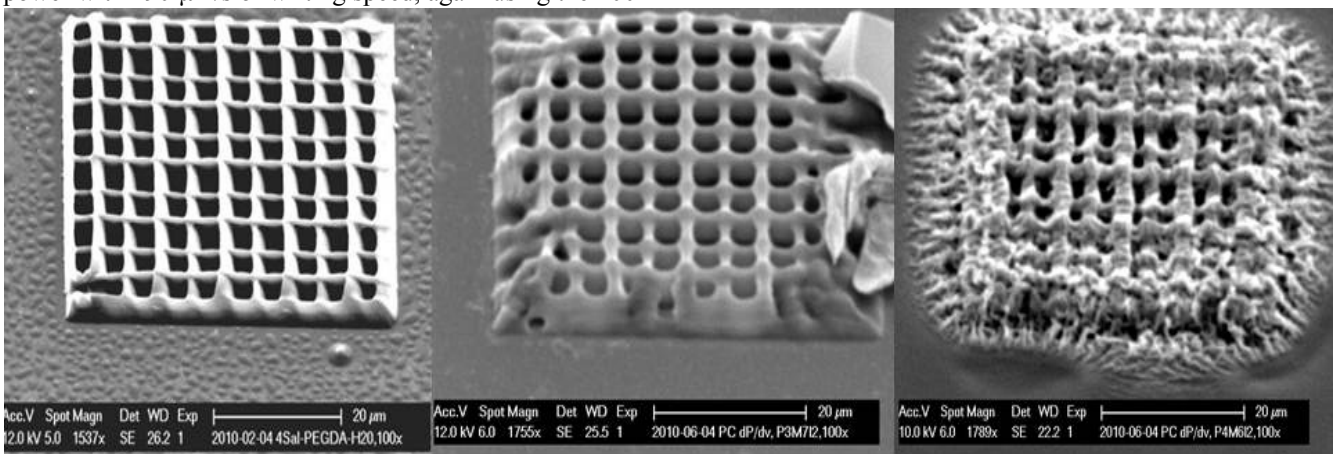

Fig. 4, Left picture: SEM images of **s**caffolds structured in different concentrations of PED-700-DA in M9 buffer using 2wt% of WSPI, respectively (left image: 50wt% PEG-700-DA, 50wt% M9 buffer; center image: 40wt% PEG-700-DA, 60wt% M9 buffer; righ imaget: 30% PEG-700-DA, 70% M9 buffer), magnification 1600x, Tilt 45°

3.2. Toxicity of the resin

Regarding the results of the previous chapter only, the hydrogel consisting of the oligomer PEG-700-DA seems to offer a high potential for in-vivo applications. Toxicity studies, however, show a different picture. Fig. 5 indicates the averaged survival-rate of the worms in different concentrations of PEG-700-DA and WSPI in water. The animals were exposed to the substances for 10 minutes in total.

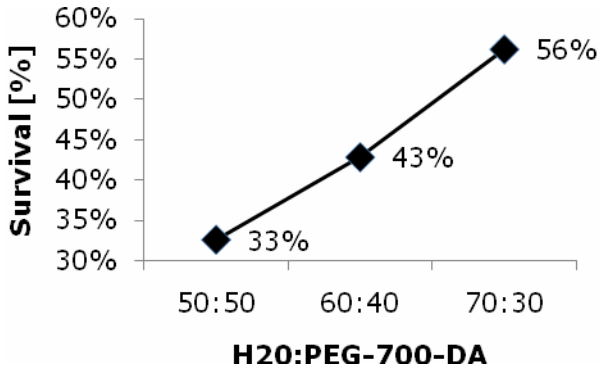

Fig. 5 Survival-rate of C. elegans exposed to three different concentrations of PEG-700-DA and WSPI in H_2O for 10 minutes.

The comparatively high toxicity is a result of the reactive acrylate-groups of the oligomer. Despite the considerable high reactivity of acrylates, they show a significant tendency towards Michael addition side reactions with amino groups of proteins or DNA. This results in hydrolytically non-cleavable aliphatic adducts. The toxic effects of acrylate have already been described by Heller *et al.* [1]. Methacrylates, carbonates and carbamates as well as vinyl esters, in contrast, do not show any such tendency [1]. Hydrogel formulations using these substances will definitely play a role in further research in this field.

3.3. In-vivo-writing

A single scaffold with a base area of 300x300 µm and a height of 80µm was manufactured into a water-insoluble methacrylate-based resin with an embedded living specimen of C. elegans. For the benefit of the resin's high reactivity, we accepted its considerable toxicity. In addition, we chose a high viscous resin (3100 to 3900 mPas at 25°C) to limit the movement of the animal. Together with high laser intensities available (35 mW, measured past the objective), we managed to reduce the polymerization time to 12 minutes. Fig. 6 shows online pictures of the fabrication process. The distance between the single lines was set to 30 µm, resulting in 10 lines per layer. In total, 10 layers were fabricated with a distance of 8µm between them. As polymerization time had to be kept short, a comparable high writing speed of 300µm/s was paramount for a successful in-vivo-writing process.

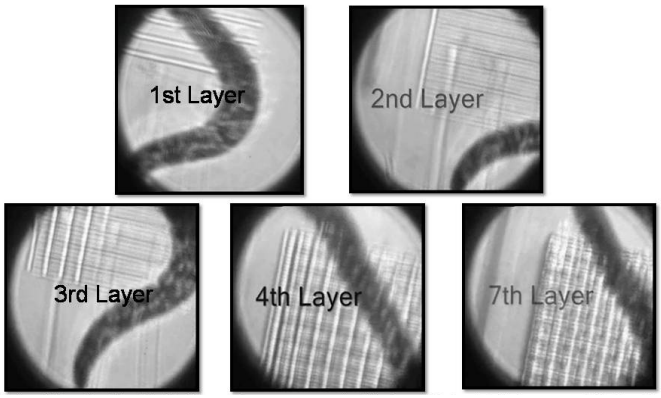

Fig. 6 In-vivo-writing: C. elegans in methacrylate-based resin whilst the fabrication of a scaffold, CCD-camera images, Magnification 20x

During the structuring of a single line, the laser was not turned off. Thus the focal point definitely hit inner organs and cells of the organism. As the animal showed no response to the exposure, we can agree to the propositions made by Leitz *et al.* [2].

Fig. 7 shows pictures taken after the successful fabrication of the scaffold. The worm was fully covered by the structure and could not flex inside the structure. However, it seemed as if the animal could move forward and backward suggesting that the lines of the fabricated part did not actually stick to the cuticle (the animal's exoskeleton). As a result, we assume that resins having a high viscosity – as that one used – might lead to a better chance for the animal surviving. In contrast, resins with lower viscosities could favour the attachment of 2PP parts to living biological tissue. This will be subject to further research in this field.

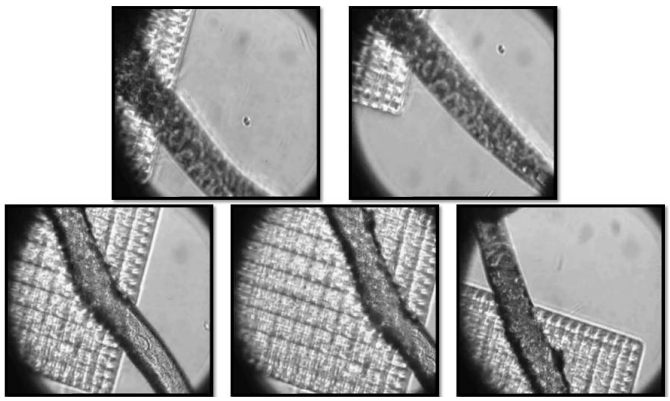

Fig. 7 C. elegans caught in a scaffold after 12 minutes of polymerization, CCD-camera images, Magnification 20x

Finally Fig. 8 provides the proof that the model organism survived the procedure. The sequence of pictures show that the tail of the worm actually moved up and down.

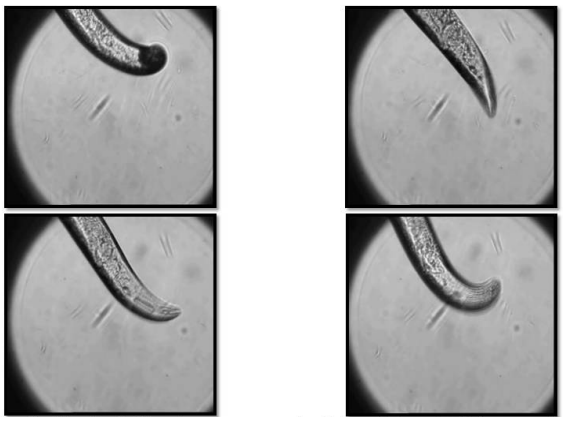

Fig. 8 Prove of live after the structuring process, CCD-camera images, Magnification 20x

The necessary irritant solvents (isopropanol or isobuthylmethylether) for the development of the structures finally killed the animal. In the future, a resin soluble in water based solvents is therefore absolutely necessary. Not until then, a complete in-vivo-writing process, starting from the animal's exposure to the resin and ending in a proper development, doing away with all monomer residues while safeguarding the survival of the model organism will be possible.

4. Summary

A method to fabricate three dimensional parts inside resins with embedded living organisms is presented. As we know from previous research, the NIR laser-beam does not harm experimental animals of the species C. elegans at the intensities used for structuring proper two-photon-lithography (2PP) parts. The main limitation of this process is therefore the toxicity of the resin.

Acrylate-based hydrogels suitable for 2PP applications are presented. They could be polymerized using the radical initiator WSPI described by Wu *et al.* [3] and used as 2PP initiator for the first time. The toxicity and the reactivity of hydrogels could be tailored over a wide range including different water contents. Yet a trade-off has to be made between these two parameters.

A scaffold of 300x300 µm base area with a height of 80 µm was fabricated in a resin containing an embedded living C. elegans. 10 lines per layer and a total of 10 layers were fabricated at a writing speed of 300 µm/s with a laser power of 35 mW (measured past the objective). The whole process took 10 min. The movement of the tail of the animal indicated its survival of the process. The body part caught in the scaffold was limited in its movement. The animal was prevented from lateral bending to either side and could not escape from the scaffold.

This is the first time additive manufacturing was performed directly onto living tissue. With the further development of water-based biocompatible and biodegradable hydrogels together with water soluble, near-infrared initiators suitable for 2PP, this technique might play a role in tissue engineering and in a variety of medical applications like osteoporosis treatment.

5. Acknowledgement

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