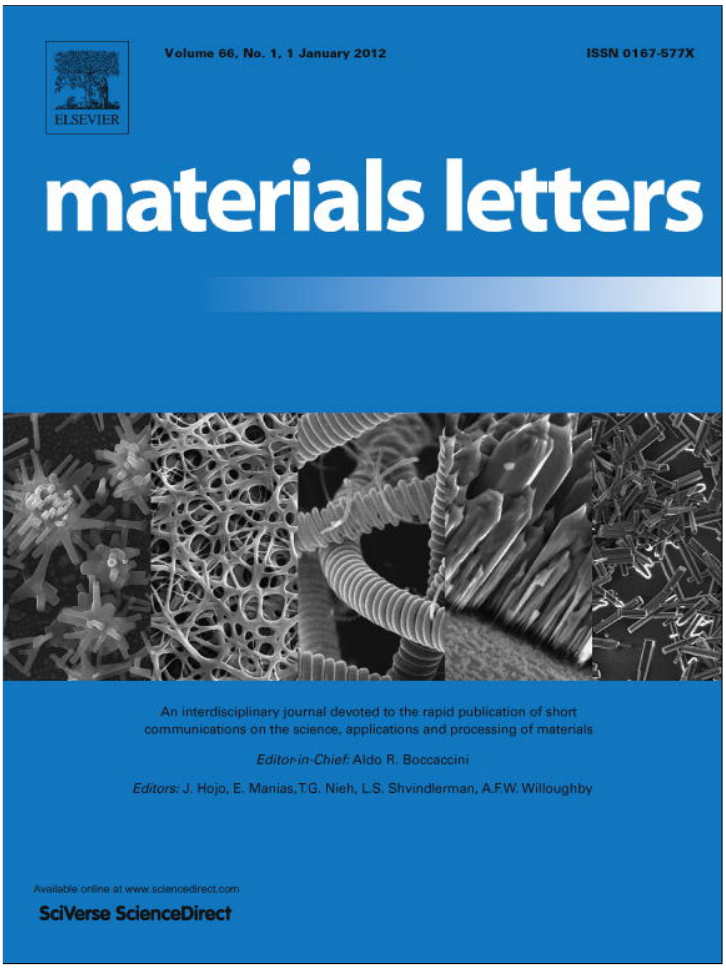

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Riboflavin and collagen: New crosslinking methods to tailor the stiffness of hydrogels

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ABSTRACT

Fabricating materials with tailored mechanical properties is a challenge and crucial for their successful application in a variety of fields such as tissue engineering. Here collagen and riboflavin were used to create hydrogels with controlled mechanical properties mimicking those of soft tissues (e.g. liver). Collagen-based hydrogels were obtained using a two-step gelation method. Firstly a physical gelation step (i.e. modulation of temperature and pH) was used to fix a specific shape; then photo-initiated cross-links were formed to increase the stiffness. Specifically the chemical cross-linking step was initiated with UV (ultra-violet) radiation to obtain riboflavin derivatised radical polymerization of collagen chains. Cylindrical shaped samples with controlled dimensions were fabricated, and then tested using compressive loading. We show that the compressive elastic modulus of collagen-based hydrogels can be tuned between 0.9 and 3.6 kPa by changing collagen concentration, irradiation with UV in the presence of riboflavin and freeze-drying.

1. Introduction

Collagen is the most abundant structural protein of the extra cellular matrix (ECM) and has been studied for several biomedical applications [1]. It has a high density of RGD sequences for cell adhesion and differentiation [2–4] and other functional groups which can be further used to form a three-dimensional (3D) matrix or promote specific cell processes [5], [6]. Due to its biocompatibility and structure, collagen is commonly used in biological and tissue engineering (TE) applications as a scaffold for cell adhesion and in-vitro regeneration of tissues such as skin, cornea and vascular tissue [7–12]. From a TE perspective the stability and mechanical resistance of collagen-based biomaterials need improvement prior to their fabrication as scaffolds in in-vitro and in-vivo studies. As demonstrated by several reports over the last decade, substrate stiffness can influence and control cell function and fate [13–15]. Therefore, in designing collagen scaffolds for TE or other applications, it is also necessary to match the mechanical properties of the target tissue. To do so it is necessary to understand and control the physical and chemical factors involved in collagen fibre alignment and interaction. Several studies have investigated methods for enhancing the mechanical strength of collagen-based hydrogels (e.g. controlling cross-link density) using different gelation and cross-linking methods [6,12]. Riboflavin (RF) is widely used in ophthalmic applications to enhance corneal stroma strength through UV irradiation in a completely biocompatible and non-toxic manner [16–18], but there is only one report of its use in a biomaterial for cell encapsulation [19]. The authors demonstrate that RF incorporated collagen gels do not induce cell toxicity. However, when irradiated, the production of free radicals may damage cells encapsulated or seeded prior to cross-linking. Indeed cytotoxic effects were observed using a 1.8% w/v RF solution and 40 s of UV exposure on collagen encapsulated fibroblasts and chondrocytes. Therefore RF is nontoxic if used to cross-link collagen prior to cell seeding, unlike potentially cytotoxic cross-linking agents such as glutaraldehyde or ethyl-dimethylaminopropyl-carbodiimide, which are known to induce cytotoxicity [20–22]. In this paper we propose a similar approach to tailor the stiffness of hydrogel-based scaffolds for TE applications. RF is used as photo-initiated species to promote the formation of chemical cross-links in a 3D hydrogel network. The photochemical reaction was used to generate radicals that induce the formation of covalent cross-links between amino acid groups in collagen chains. To obtain a homogeneous 3D matrix with a controlled shape and dimensions, collagen was cross-linked using a two-step method: physical gelation was first used to fix scaffold shape and the photo-chemical reaction was then used to obtain a stable 3D cross-linked network (Fig. 1).

2. Materials and methods

Type I collagen was extracted from rat tails using the method described by Elsdale and Bard [8]. Riboflavin, glutaraldehyde, M199 cell culture medium and acetic acid were purchased from Sigma-Aldrich (Milan, Italy).

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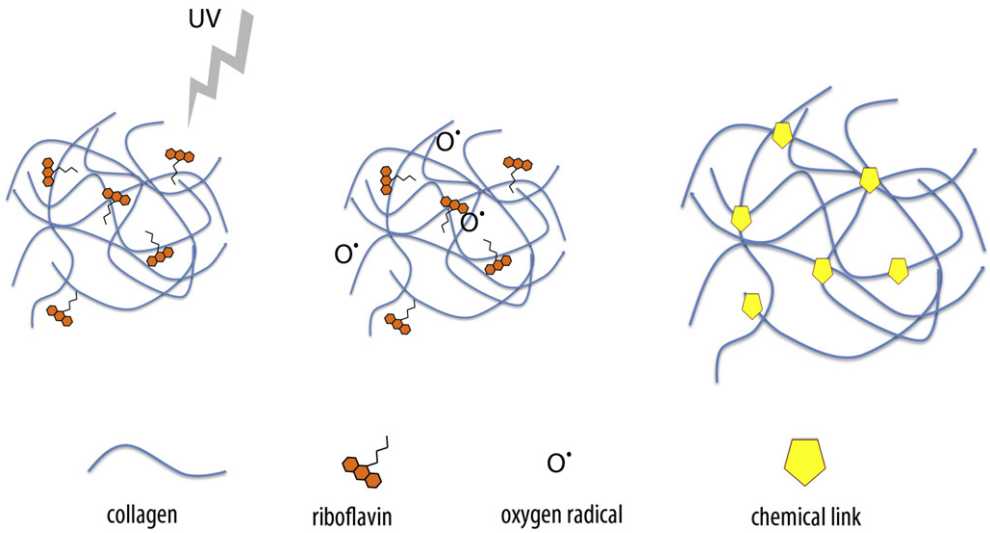

Fig. 1. Photo-initiated free radical cross-linking reaction induced by riboflavin. Once irradiated with UV radiation an H+ ion is released, producing a radical active oxygen 0. Collagen amine groups can then react generating covalent bonds.

2.1. Preparation of hydrated collagen (HC) hydrogels

Type I collagen was diluted to final concentrations of 3 and 5 mg/mL with a 0.02 N solution of acetic acid. A solution of RF 0.02% w/v (W Luo, Muller & Burrows 2001) was prepared using distilled water. Collagen (850 μL) and RF (50 μL) were then pipetted and gently mixed in 24 multiwell plates in contact with ice. Finally, 100 μL of M199 10X (pH 9.4) was added (giving 9:1 collagen:medium volume ratio). To obtain gelled samples they were kept at 37 °C for 1 h. After this first step, chemical cross-links were promoted by exposing samples to UV (wavelength 265 nm and intensity 10 mJ/cm2) for 5 min (CL-1000, UVP, UK). Non-irradiated and 1% v/v glutaraldehyde cross-linked samples were used as controls.

2.2. Preparation of freeze-dried collagen (FDC) hydrogels

FDC scaffolds were obtained by a controlled freeze-drying method. Samples were first kept at 4 °C for 2 h and then transferred to −20 °C for 24 h. Completely frozen samples were freeze-dried (temperature −50 °C, pressure 180 mbar). For each subsequent use the sponges were rehydrated using 1 mL of PBS for 1 h at room temperature before use.

2.3. Observation of cross-sectional morphology

The morphology of FDC samples was observed with SEM (LEO-1430, Germany). Samples were observed in cross-section in the centre of the scaffold.

2.4. Compressive tests

The mechanical properties of HC and FDC samples were analysed with the Zwick-Roell Z005 (Zwick Testing Machines Ltd., UK). Cylindrical shaped samples (14 mm diameter and 4 mm height) immersed in PBS were used for the compression tests. The cylindrical probe was positioned 2 mm above samples, avoiding any contact before the start of the measurement. A compressive strain rate of 0.1 mm/s was set and a maximum strain of 80% was achieved. The compressive modulus was calculated from the slope of the stress–strain curve in the linear region. A first analysis was performed to evaluate the influence of UV exposure by testing irradiated and non-irradiated samples. A second analysis was used to evaluate the influence of the freeze-drying process.

3. Results and discussion

Here we propose a novel and non-toxic two-step gelation method to obtain stable and tailored collagen-based hydrogel biomaterials. RF was used as the initiator of a free-radical photochemically activated cross-linking reaction. Glutaraldehyde cross-linked samples were chosen as controls as it is generally accepted that aldehydes react with collagen chains to generate covalent cross-links [23–25] giving rise to a 3D porous network. The SEM images of freeze-dried collagen show the 3D network obtained in uncross-linked (Fig. 2a) and glutaraldehyde (Fig. 2b) and RF (Fig. 2c) treated samples. Both have interconnected pores with a random pore structure (pore sizes from 50 to 500 μm), evidence of the formation of covalent cross-links within

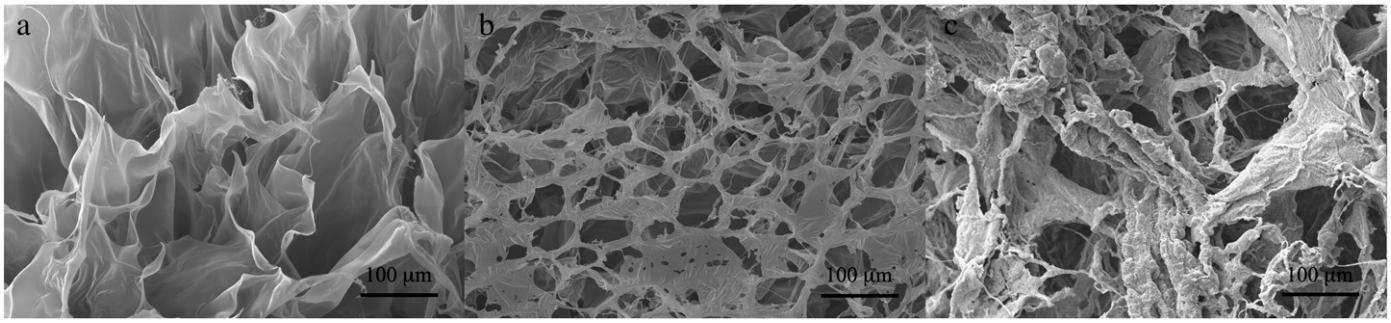

Fig. 2. SEM images of FDC prepared using a 5 mg/mL solution: (a) physically cross-linked collagen, (b) collagen cross-linked with 1% v/v glutaraldehyde solution (chemical cross-link) and (c) collagen cross-linked with 0.02% w/v RF solution (UV induced photo-polymerisation).

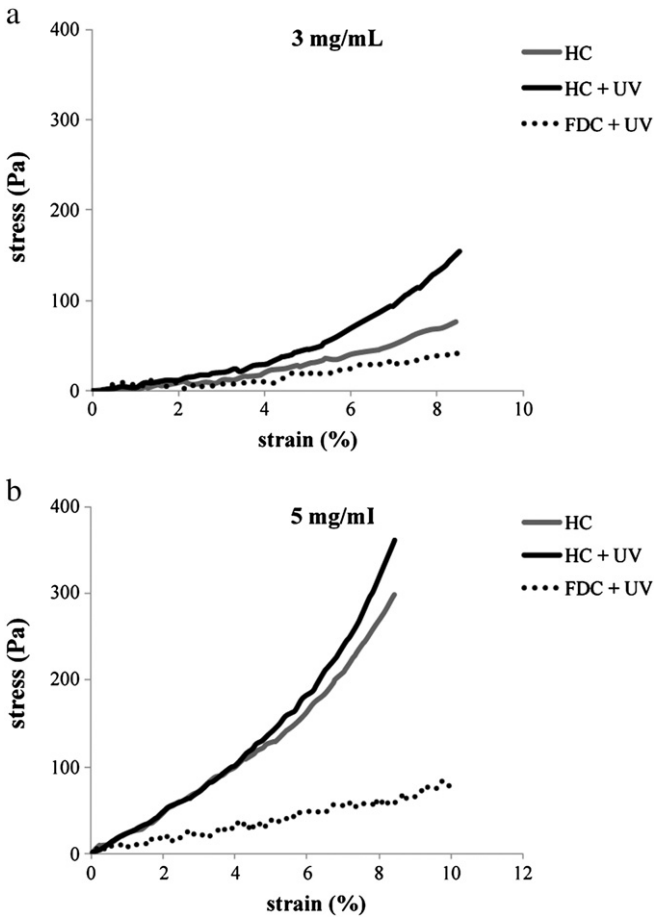

Fig. 3. Mechanical testing of collagen-RF hydrogels: influence of collagen concentration, UV exposure and freeze-drying.

adjacent fibres. The formation of chemical cross-links in Fig. 2c is therefore a consequence of UV exposure of RF, as confirmed in several studies directed at ophthalmic applications [17,26,27].

Having demonstrated that collagen hydrogel treated with UV radiation and RF gives rise to a micro-structure characteristic of chemical cross-linking, we focused on the feasibility of controlling and eventually tailoring the stiffness of the hydrogels. The influence of collagen concentration as well as UV exposure was measured using compressive tests. Collagen-RF hydrogels were tested before and after irradiation.

Typical stress–strain curves of FDC hydrogels respectively before and after UV exposure are shown in Fig. 3. In both exposed and unexposed samples the linear zone considered to evaluate the resultant stiffness was in the region beyond 5% compressive strain.

In the case of unexposed collagen, the protein chain mobility is probably due to the physical interactions between adjacent fibres. On the contrary, once exposed to UV radiation, collagen chain mobility is reduced by chemical cross-links, which is reflected in an increase of the elastic modulus of the scaffolds. Compressive tests showed that collagen concentration is relevant in terms of scaffold stiffness. A summary of the elastic moduli evaluated in the linear region of the stress–strain curve of different samples is reported in Table 1, these values are typical of soft tissues such as liver and lung. As shown collagen concentration and UV exposure are responsible for the increase of the elastic modulus of collagen-RF scaffolds. With higher collagen concentrations there is more interaction within adjacent polymer chains, and this entanglement increases scaffold stiffness [28]. Once exposed to UV, covalent cross-links are formed in the polymeric network further enhancing the mechanical resistance. Having assessed the increase of the elastic modulus of collagen-RF samples exposed to UV, other compressive mechanical tests were performed using different compressive strain rates in order to analyse the viscoelastic behaviour of cross-linked collagen. These tests demonstrated that FDC collagen scaffolds exhibit a more elastic behaviour than the hydrogels: in HC scaffolds the stiffness increases with the compressive velocity while it remains constant in the FDC (data not shown). Moreover FDC scaffolds possess a lower elastic modulus compared to the corresponding HC samples and this is probably due to the lower overall water content of the former. Hydrogel structure stability and degradation in time were also analysed by maintaining samples immersed in PBS for several days at room temperature. Compressive mechanical tests were performed at different time points; the measured stiffness does not significantly change over 2 weeks (data not shown).

Table 1
Elastic moduli of HC and FDC hydrogels varying collagen concentration, UV exposure and freeze-drying.

Sample	Elastic modulus (kPa)
HC 3 mg/mL	2.48 ± 0.14
HC 3 mg/mL + UV	3.04 ± 0.26
FDC 3 mg/mL + UV	0.90 ± 0.11
HC 5 mg/mL	3.38 ± 0.55
HC 5 mg/mL + UV	3.60 ± 0.40
FDC 5 mg/mL + UV	0.96 ± 0.04

4. Conclusions

In conclusion the two-step cross-linking method described can be used to control the stiffness of collagen-based scaffolds without the aid of toxic reagents. If the scaffolds are polymerised prior to cell seeding, as proposed here, HC and FDC matrices with appropriate forms and sizes and tailored stiffness can be fabricated for a variety of applications in soft tissue regeneration.

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