Development of Mechanically Tailored Gelatin-Chondroitin Sulphate Hydrogel Films

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Summary: The aim of the present work was to develop and characterize biohybrid hydrogels containing combinations of gelatin and chondroitin sulphate. Two approaches have been compared. First, physisorption of chondroitin sulphate to gelatin hydrogels was investigated using surface plasmon resonance measurements. Due to the limited interaction between both biopolymers, we developed in a second approach methacrylate-modified chondroitin sulphate hydrogels as such or in combination with methacrylamide-modified gelatin. Rheology measurements indicated that following this approach, mechanically stable hydrogel films could be obtained after UV irradiation in the presence of Irgacure 2959 as UV photo-initiator. When keeping the gelatin concentration and the modification degree constant, an increase in derivatization of the chondroitin sulphate component resulted in a high increase of the storage modulus.

Keywords: characterization; chondroitin sulphate; gelatin; hydrogel; modification

Introduction

Extracellular matrix (ECM) constituents including collagen (i.e. starting product for gelatin synthesis) and glycosaminoglycans (GAGs) are among the most abundant in the body and play key roles in a number of biological processes. They are widely utilized to fabricate scaffolds, serving as an active analogue of native ECM.[1]

Hydrogels are three-dimensional insoluble polymeric networks from which biochemical factors can be delivered and through which diffusion of vital cell nutrients and waste products can occur.[2] Previous research already studied the application of photopolymerized hydrogels for tissue engineering applications.[3–5] Photopolymerization provides a fast and efficient method to crosslink polymers forming a hydrogel with significant temporal and spatial control. These hydrogels have already demonstrated their potential as three dimensional structures suitable for tissue engineering applications.[6,7] Very frequently, photopolymerised hydrogels are composed of synthetic polymers. In order to enhance their bioactivity and biocompatibility, biopolymers can be included.

As an example, collagen-GAG scaffolds have been used extensively for *in vitro* studies of cell-ECM interactions and as a platform for tissue biosynthesis including *in vivo* studies of tissue or organ regeneration.[8,9] Favourable characteristics of scaffolds from these natural materials stimulate host cells to repopulate and form new tissues that closely simulate the native organization.[10,11] In addition, they enhance biological interactions with cells and speed up tissue regeneration by introducing cell-specific ligands or extracellular signalling molecules, such as peptides and oligosaccharides.[12]

In the present work, gelatin type B (i.e. processed from collagen via an alkali treatment) and chondroitin sulphate (CS) were selected as starting materials for the development of ECM mimicking materials. Gelatin has previously been successfully applied for a wide application range

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including burn dressings, cardiovascular surgery and 3D scaffolds for tissue engineering of skin, bone, cartilage and other tissues.[13] The combination of gelatin and GAGs is often used in skin regeneration since gelatin or GAGs as such cannot heal full thickness wounds.[14] GAGs have also been reported to significantly affect cellular response, morphology and stiffness of the biohybrid scaffolds.[15,16] In literature, the interaction between proteoglycans and extracellular matrix molecules was reported earlier.[17] Most of the interactions appeared to be ionic and probably mediated by the highly charged glycosaminoglycan chains of the proteoglycans.[18]

With the aim to evaluate the possibility to combine chondroitin sulphate and gelatin for the production of hydrogel materials, two alternative methods have been compared. First, the possibility of chondroitin sulphate physisorption to gelatin-based hydrogels was screened. Alternatively, a chondroitin sulphate derivative possessing methacrylate moieties (CS-MOD) has been combined with methacrylamide-modified

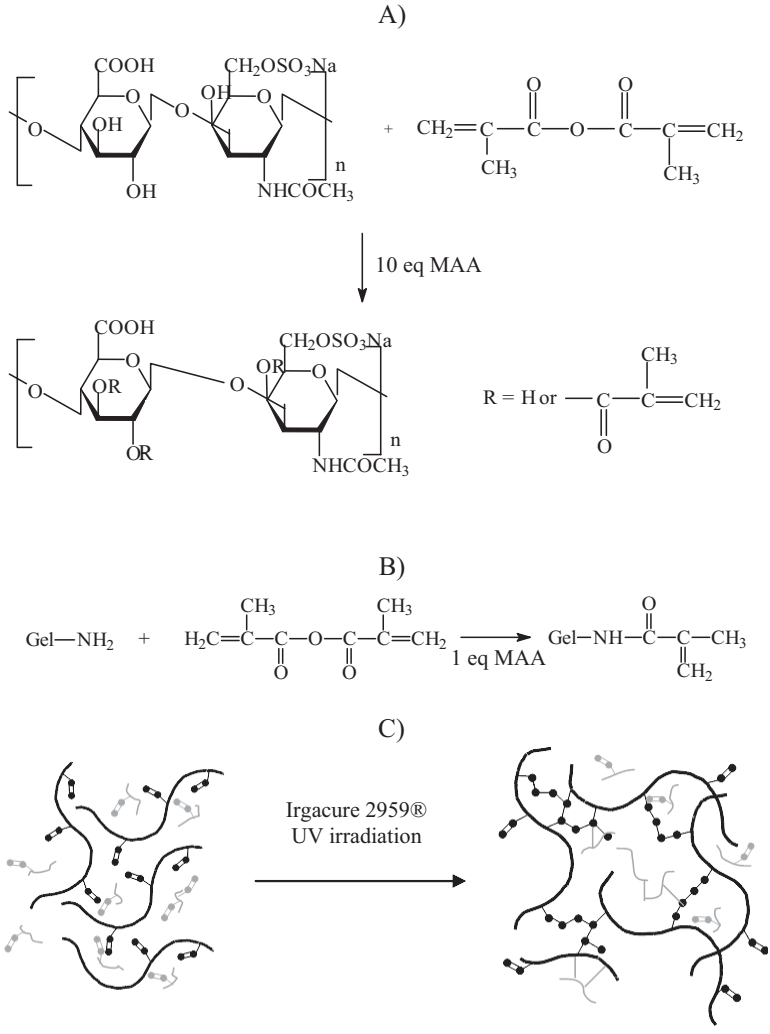

Figure 1.
Synthesis of CS-MOD (A) and Gel-MOD (B); Chemical crosslinking of biohybrid networks starting from Gel-MOD (black) and CS-MOD (grey) (C).

gelatin (Gel-MOD) to develop biohybrid chemically crosslinked hydrogels (see Figure 1). The effect of the applied polymer concentration and the hydrogel precursor modification degree on the final properties of the hydrogel films has been evaluated using rheology.

Materials and Methods

Materials

Gelatin (type B), isolated from bovine skin by an alkaline process, was kindly supplied by Rousselot, Ghent, Belgium. Gelatin samples with an approximate iso-electric point of 5, a Bloom strength of 257 and a viscosity (6.67%, 60 °C) of 4.88 mPa.s were used. Methacrylic anhydride (MAA), chondroitin sulphate C, (sodium salt, from shark cartilage), chondroitin sulphate A (sodium salt, from bovine trachea) and monoclonal anti-chondroitin sulphate (clone CS-56, from mouse ascites fluid), were acquired from Sigma-Aldrich (Bornem, Belgium). 1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure® 2959) was a kind gift from Ciba Speciality Chemicals N.V. (Groot-Bijgaarden, Belgium).

Surface Plasmon Resonance Measurements

The interaction between gelatin and CS was measured using a Biacore-X (GE Healthcare Europe, Diegem, Belgium) equipped with an internal 500 μl Hamilton syringe. All SPR measurements were performed at 25 °C using a phosphate buffer (0.05 M, pH = 7.4). The flow rate was set to 50 μl/min. The sensor surface was spincoated using 90 μl of an aqueous 5 w/v% Gel-MOD (degree of substitution 60%) solution at a speed of 6000 rpm during 90 seconds. After spincoating, the gelatin coated sensor was inserted into the SPR apparatus. After stabilisation of the baseline, 50 μl of various concentrations of CS solutions was injected. In a final step, after stabilisation of the signal, 50 μl of an chondroitin sulphate antibody solution (200× dilution of the stock) was injected. All values reported are relative to a reference flow channel.

Synthesis and Characterization of Hydrogel Precursors

Chondroitin sulphate methacrylate (CS-MOD) and gelatin-methacrylamide (Gel-MOD) were synthesized as described earlier.[7,19]

In brief, 1 g chondroitin sulphate was dissolved in 50 ml double distilled water at room temperature. Next, an excess methacrylic anhydride (0.06 mol, 8.94 ml) was added dropwise. Simultaneously, the pH of the reaction mixture was adjusted to 8, by adding NaOH (5 N). The ratio between the added amounts of methacrylic anhydride and NaOH was 1 to 1.12. Next, the mixture was stirred at room temperature for 2 hours. Finally, the solution was diluted with 50 ml double distilled water and transferred to dialysis membranes (Spectra/Por® 3, MWCO 3,500 Da, 3 days), followed by lyophilization. Chondroitin sulphate methacrylates with lower modification degrees were obtained by adding lower amounts of methacrylic anhydride.

^{1}H NMR-spectra of modified chondroitin sulphate were recorded at room temperature in deuterated water. The degree of substitution could be obtained after comparison of the integrations of the characteristic peaks of the methacrylate-substituent (1.95 ppm, 5.76 ppm and 6.19 ppm) and the integration of the characteristic peak corresponding to the methyl groups in native CS (2.04 ppm). Consequently, the degree of substitution could be calculated, as indicated by the following equation:

$$DS\,(\%) = 100 \times (I_{5.7\,ppm})/((I_{1.95\,ppm} + I_{2.04\,ppm} - 3 \times (I_{5.7\,ppm}))/3)$$

Hydrogel Preparation

CS-MOD (0.1–1 g) with various modification degrees (degree of substitution, DS) (5–40%) was dissolved in 20 ml double distilled water at room temperature. For the biohybrid hydrogels, Gel-MOD (1.4–3 g, DS 65%) was added and the mixture was stirred at 40 °C. Next, the photoinitiator Irgacure® 2959 (2 mol% relative to the methacrylates and methacrylamides of

respectively CS-MOD and Gel-MOD) was added, followed by injection of the mixture between silanized glass plates, separated by a 1 mm thick silicone spacer. Silanization of the glass plates occurred by incubating the glass plates overnight into an aqueous solution of 2 v/v% H_2SO_4/HNO_3, followed by an overnight incubation in toluene, containing 10 v/v% trimethylsilylchloride.

Finally, the hydrogel formed was UV irradiated (276 nm, 10 mW/cm^2) for 20 minutes on both sides or crosslinked in situ during rheology. To enable the crosslinking prior to the rheological evaluation, an LWUV-lamp model VL-400L (Vilber Lourmat, Marne La Vallée, France) with an intensity of 10 mW/cm^2 and a wavelength range of 250–450 nm, was applied for sample curing. The crosslinked hydrogels were stored at 5 °C until further evaluation.

Hydrogel Characterization

The visco-elastic properties of the hydrogels were evaluated using a rheometer type Physica MCR-301 (Anton Paar, Sint-Martens-Latem, Belgium). First, the linear visco-elastic range was determined as a function of the deformation (0.01–1%) and at constant frequency (1 Hz) (data not shown). Next, the effect of the UV irradiation applied, on the final mechanical properties was monitored by performing a time scan using the following parameters: 1 Hz, 0.5% strain, $F_N = 0.01$ N and 21 °C.

Results and Discussion

Evaluation of the Gelatin Chondroitin Sulphate Affinity

Literature data previously indicated that chondroitin sulphate E possesses specific affinity for type V collagen.[18] The binding requires a sequence of repeating units, consisting of one glucuronic acid and one N-acetyl-galactosamine, sulphated at carbon-4 and carbon-6. Alternative oligosaccharides, consisting of other sequences, however possessing the same charge, do not interact with type V collagen.[20] The latter demonstrates that the interaction between chondroitin sulphate and gelatin might also depend on various parameters. Therefore, the interaction between gelatin type B and two types of chondroitin sulphate (i.e. type A and C) was studied in the present work using SPR.

In a first part of the SPR experiment, chondroitin sulphate A (CSA) solutions of different concentrations were rinsed over the sensor chip (100–1000 μg/ml), previously spincoated with methacrylamide modified gelatin type B (gel-MOD). In a second part, an antibody specific for CSA was injected in order to verify that the response was related to the deposition of the GAG onto the gelatin-coated SPR chip. The response signal plotted as a function of incubation time, giving an idea on the interaction between gel-MOD and CSA, is given in Figure 2. As a reference, the antibody was rinsed over a gel-MOD coated chip. As anticipated, the SPR sensorgram did not show any response (data not shown).

From the figure, it can be derived that the affinity between chondroitin sulphate A and gel-MOD is relatively low. The amount of adsorbed polysaccharide increases slightly with increasing CS concentration. The limited response can be explained by the iso-electric point of the applied gelatin (i.e. 5). Consequently, both gelatin B and chondroitin sulphate A are negatively charged, excluding the possibility for ionic interactions. Literature data already revealed a weak interaction between collagen and chondroitin 4-sulphate.[21]

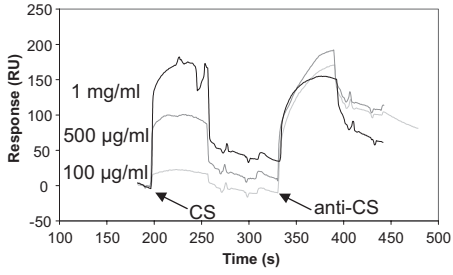

Figure 2.
SPR sensorgram showing the effect of the CS concentration (100 μg/l – 1 mg/ml) on the interaction between gelatin and chondroitin sulphate A.

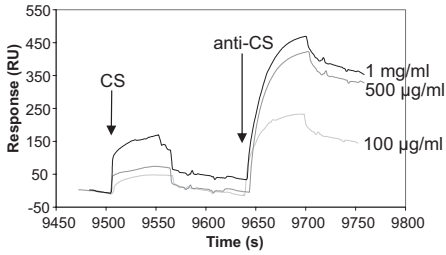

Figure 3.
SPR sensorgram showing the effect of the CS concentration (100 µg/l – 1 mg/ml) on the interaction between gelatin and chondroitin sulphate C.

Interestingly, the adsorbed amount of GAGs still enabled subsequent interaction with CS antibodies, which was reflected by the increased response signal after the antibody injection (i.e. second injection) (Figure 2).

In a following part of the SPR experiments, we also investigated the interaction between chondroitin sulphate type C and gelatin (Figure 3).

When comparing the affinity between both types of chondroitin sulphate and gelatin type B, no significant difference in response signal was observed. The SPR data obtained clearly show that a chemical modification of and a subsequent co-crosslinking of chondroitin sulphate with crosslinkable gelatin is essential to realize an efficient incorporation of CS into hydrogel films.

Synthesis and Characterization of Hydrogel Precursors

The synthesis and characterization of the methacrylate-modified chondroitin sulphate (CS-MOD) precursor has been described earlier.[7] In brief, part of the hydroxyl groups of CS were converted into methacrylate groups. As methacrylic acid (MA) is generated during the esterification, NaOH was added as neutralizing agent, avoiding possible acid catalysed degradation of the polysaccharide. The glycosaminoglycan containing crosslinkable methacrylate groups was purified by membrane dialysis against double distilled water for several days, followed by isolation via lyophilization.

The methacrylate substitution on CS was quantified using ^{1}H-NMR spectroscopy. The two distinctive peaks at 5.76 and 6.19 ppm can be attributed to the two protons on the double bond ($C{=}CH_2$), while the peak at 1.95 ppm can be ascribed to the methyl groups adjacent to the double bonds (CH_3-$C{=}CH_2$). The ^{1}H-NMR region from 1.6 to 2.5 ppm was expanded and the peaks corresponding to the two methyl groups were deconvoluted and integrated. The ratio of the peak intensities at 1.95 ppm to that at 2.04 ppm, corresponding to the methyl groups on native CS, was used to calculate the degree of substitution. In what follows, the degree of substitution will be expressed as the amount of modified repeating disaccharide units. The degree of substitution was calculated using the following equation:

$$DS\,(\%) = 100 \times (I_{5.7\,ppm})/((I_{1.95\,ppm} + I_{2.04\,ppm} - 3 \times (I_{5.7\,ppm}))/3)$$

We were able to show that the degree of substitution can be easily varied by adjusting the amount of added methacrylic anhydride. Figure 4 indeed shows the master curve for the CS modification in which the degree of substitution is plotted against the equivalents methacrylic anhydride added.

The synthesis and characterization of Gel-MOD as second hydrogel building block was reported earlier by Van Den Bulcke et al.[19]

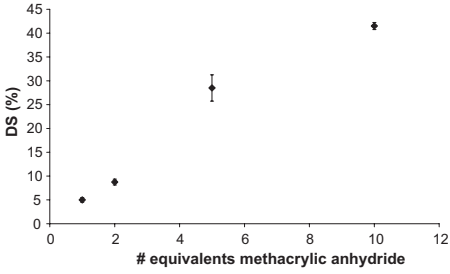

Figure 4.
Master curve of CS showing the degree of substitution as a function of the amount of methacrylic anhydride added.

Hydrogel Preparation and Characterization

Both the biohybrid hydrogels and the hydrogels composed of CS-MOD only were prepared and evaluated as thin films. The hydrogel films were prepared by mixing an aqueous solution of the hydrogel precursors (at 40 °C) in the presence of the photo-initiator, followed by injection between two silanized glass plates, separated by a 1 mm silicone spacer. Chemical crosslinking occurred via UV-irradiation ($\lambda_{ex} = 279$ nm). In the present work, Irgacure 2959 was selected as a photo-initiator since previous research already indicated its biocompatibility.[6,7] Dubruel et al have already shown that applying 2 mol% Irgacure 2959 to the methacrylamides present in Gel-MOD resulted in scaffolds suitable to support the attachment and proliferation of a large panel of human cells including endothelial cells, glial cells, osteoblasts, fibroblasts and epithelial cells.[6] We therefore do not anticipate any problems regarding the material toxicity when applying Irgacure 2959 as a photo-initiator. The major advantage of combining CS-MOD and Gel-MOD in stead of applying carbodiimide chemistry to couple amines and carboxylic acids is the possibility to introduce additional growth factors, if needed, without affecting their biological activity. When applying carbodiimide chemistry, the growth factors present would also react, while using the proposed approach, only compounds containing double bonds are coupled.

First, the linear visco-elastic range of the hydrogels developed was determined using an amplitude scan (data not shown). Next, mechanical spectra were recorded, from which it could be concluded that well-structured networks were obtained following the applied procedure (data not shown).

CS-MOD with varying modification degrees were crosslinked *in situ* during rheological evaluation. In contrast to gelatin-based hydrogels,[22,23] where the total hydrogel network strength is the sum of both the physical and the chemical crosslinking, the strength of chondroitin sulphate hydrogels only depends on the chemical contribution, since CS has no gelling properties. However, by derivatization and subsequent irradiation, hydrogels with storage moduli up to 20,000 could be obtained (Figure 5).

The mechanical strength of the biohybrid hydrogels (containing both gelatin and chondroitin sulphate) was also studied using rheology. In this case, the total hydrogel network strength was the sum of different contributions: (1) the physical gelation of gelatin as a consequence of triple helix formation, (2) the chemical network strength caused by Gel-MOD and

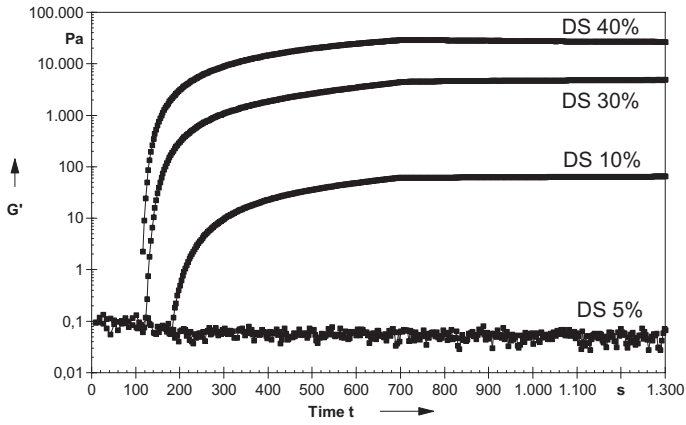

Figure 5.
Influence of the modification degree of CS-MOD on the mechanical properties of the hydrogels developed (0.5% strain, 1 Hz, $F_N = 0.01$ N, T = 21 °C).

Table 1.
Composition of the gelatin-chondroitin sulphate hydrogels and their mechanical strength, obtained by means of rheology (0.5% strain, 1 Hz, $F_N = 0.01$ N, $T = 21\ ^\circ C$).

Composition		G' (20 °C) Pa
Gel-MOD	CS-MOD	
10 w/v%, DS 65%	0.5 w/v%, DS 40%	17500
10 w/v%, DS 65%	2 w/v%, DS 40%	42300
10 w/v%, DS 65%	5 w/v%, DS 40%	98100
10 w/v%, DS 65%	0.5 w/v%, DS 5%	9740
10 w/v%, DS 65%	2 w/v%, DS 5%	15800
10 w/v%, DS 65%	5 w/v%, DS 5%	44600
7 w/v%, DS 65%	5 w/v%, DS 40%	89400
10 w/v%, DS 65%	5 w/v%, DS 40%	101500
15 w/v%, DS 65%	5 w/v%, DS 40%	190000
7 w/v%, DS 65%	5 w/v%, DS 5%	27900
10 w/v%, DS 65%	5 w/v%, DS 5%	39400
15 w/v%, DS 65%	5 w/v%, DS 5%	61700

CS-MOD. The latter factor is referred to as the total chemical network strength since no distinction can be made between double bonds in protein or glycosaminoglycan side chains during the crosslinking process. This part of the network is thermo-stable.

In a following part of the work, a large variety of gelatin and chondroitin sulphate derivatives with different modification degrees were synthesized, enabling the production of a broad selection of hydrogel materials with varying mechanical properties. An overview of the polymer films developed and their resulting storage moduli are presented in Table 1. When keeping the gelatin concentration and the modification degrees constant, an increase in derivatization of the chondroitin sulphate component (DS 5% versus 40%) results in a high increase of the storage modulus ($G' \times 2$). All hydrogels developed were thus crosslinked very efficiently. The moduli remained unaltered at elevated temperatures (40 °C) (data not shown), implying that the physical contribution to the hydrogel network strength was minimal. Literature data already indicated that the presence of covalent bonds hinders the physical structuring of gelatin.[19,24]

Figure 6 shows the *in situ* crosslinking of CS-MOD hydrogels possessing varying polymer concentrations using rheology.

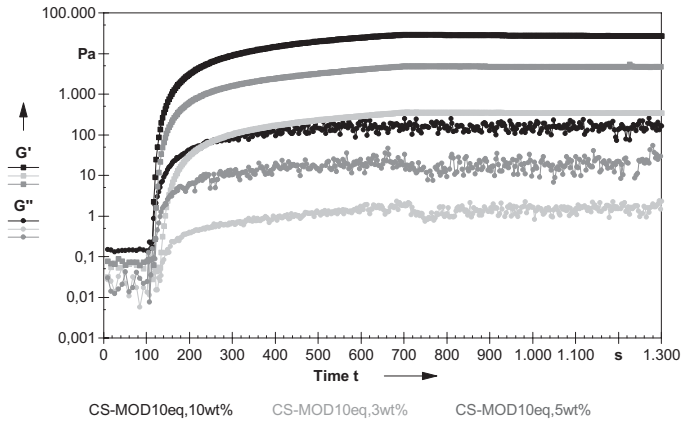

Figure 6.
Influence of the glycosaminoglycan concentration on the storage modulus (0.5% strain, 1 Hz, $F_N = 0.01$ N, $T = 21\ ^\circ C$).

The results indicate that both the crosslinking degree as well as the crosslinking rate increased for higher polymer concentrations. Interestingly, the increase in mechanical strength was higher than anticipated. The latter phenomenon was also observed for the biohybrid hydrogels composed of gelatin and chondroitin sulphate (Table 1).

Conclusion

In the present work, we have compared two approaches to develop biohybrid hydrogels containing both chondroitin sulphate (CS) and gelatin with the final aim to develop ECM mimicking hydrogels. Due to the limited interaction between CS and gelatin, as obtained by SPR, the physisorption of CS to gelatin hydrogels was not successful. An alternative method, in which cross-linkable CS and gelatin were copolymerized into one single biohybrid hydrogel proved to be successful. Finally, we showed that the mechanical properties of the hydrogels developed, depended on various parameters including the storage time, the polymer concentration and the hydrogel precursor modification degree. The final network strength of the biohybrid hydrogels is determined by both a physical gelation and a chemical crosslinking contribution. In follow-up research, the materials developed will be screened for their potential to act as cell scaffolds.

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