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## Viability of Human Mesenchymal Stem Cells Seeded on Crosslinked Entropy-Elastic Gelatin-Based Hydrogels

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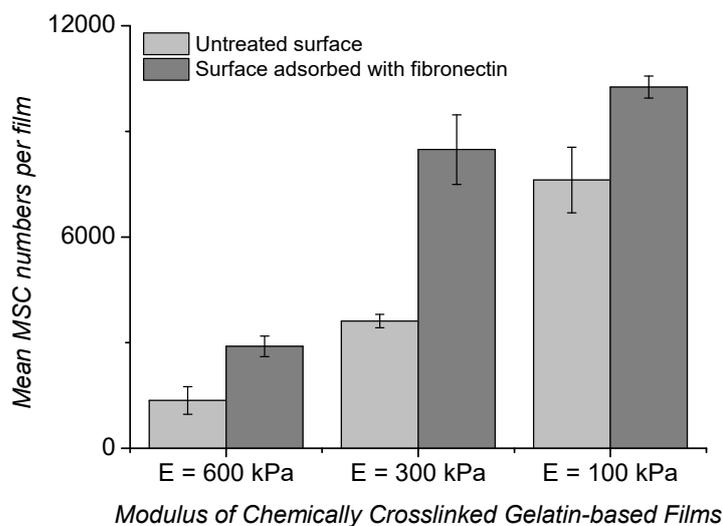
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### Abstract graphic:



## **Abstract**

Biomimetic polymer network systems with tailorable properties based on biopolymers represent a class of degradable hydrogels that provides sequences for protein adsorption and cell adhesion. Such materials show potential for in vitro mesenchymal stem cell (MSC) proliferation as well as in vivo applications and were obtained by crosslinking different concentrations of gelatin using varying amounts of ethyl lysine diisocyanate in the presence of a surfactant in pH 7.4 PBS buffer solution. Material extracts, which were tested for cytotoxic effects using L929 mouse fibroblasts, were non-toxic. The hydrogels were seeded with human bone marrow-derived MSCs and supported viable MSCs for the incubation time of 9 d. Preadsorption of fibronectin on materials improved this biofunctionality.

## **Introduction**

The therapeutic potential of mesenchymal stem cells (MSCs) is being investigated in biomaterial-supported regenerative therapies<sup>[1,2]</sup> such as in orthopedic surgery.<sup>[3]</sup> MSCs possess the ability to differentiate into multiple lineages, such as bone (osteogenic), cartilage (chondrogenic), and adipocyte (adipogenic) cells.<sup>[4,5]</sup> MSC viability and differentiation has been determined to depend largely on underlying 2-D substrate elasticity,<sup>[6]</sup> hydrophobicity,<sup>[7]</sup> and the presence of soluble or immobilized factors.<sup>[8]</sup> However, decoupling the effects from each of these environmental factors has been a great challenge in MSC-based studies, and the ability to finely control MSC fate and understand MSC attachability and viability has not yet been realized. Therefore, studying

the interaction of MSCs with a class of materials that allows a systematic variation of properties is an advantageous strategy to better understand the effects of material substrates on MSC behavior and fate. Conversely, the results of such a study can be used to assess the material capabilities as MSC substrates or as MSC-containing implantable devices.

Common polymeric bulk materials such as polystyrene, polycarbonate, and polypropylene have been successfully applied as substrates for MSC attachment, proliferation, and differentiation. However, these common plastics are not ideal as implantable systems for applications in regenerative therapies. More specific requirements need to be fulfilled for the next generation of biomaterials, such as degradability. In addition, properties that mimic the extracellular matrix (ECM) (e.g. high water uptake capabilities) are desirable.<sup>[9]</sup> Indeed, *ex vivo* studies have shown that ECM-based environments can serve as artificial niches for MSCs,<sup>[10]</sup> especially those that contain collagen as a main component. For example, calcified bone contains triple helical fibrils of type I collagen, while bone marrow contains multiple types of collagen (e.g. I, III, IV). Therefore, collagen-based materials have been extensively investigated as substrates for MSC culturing studies,<sup>[11]</sup> which have shown that collagen possesses positive attributes such as improved MSC attachment.<sup>[6]</sup> However, crosslinked collagen-based systems rarely exhibit tailorable material properties.<sup>[12]</sup> Moreover, collagen-based biomaterials can display drawbacks such as irreproducible mechanical properties, form instability,<sup>[13]</sup> and immunogenicity<sup>[14]</sup> upon implantation.

An alternative biopolymer basis for MSC substrates is gelatin, which is the product of partially hydrolyzed collagen. Like collagen, gelatin is degradable and

possesses cell adhesion sequences such as DGEA, which has been shown to improve MSC attachment and spreading.<sup>[15]</sup> However, unlike collagen, gelatin is easily water-soluble ( $T > 37\text{ }^{\circ}\text{C}$ ) and non-immunogenic. Recently, gelatin-containing hybrid materials have been shown to be useful as substrates for MSCs, and the presence of gelatin was shown to stimulate their adhesion and osteogenic potential,<sup>[16,17]</sup> although systematic studies that focus on the influence of mechanical properties and degradation kinetics on MSC viability are still lacking. The concept of developing a biopolymer system, i.e. a family of gelatin-based polymers, which have adjustable macroscopic properties that can be varied in a wide range by only small changes in their chemical composition, is an important approach to produce candidate materials for applications in biomaterial-induced autoregeneration.<sup>[18,19]</sup> The knowledge-based design of such biopolymer systems can be derived from corresponding synthetic polymer systems.<sup>[20-22]</sup> Recently, such an approach was used by our group to prepare chemically-crosslinked gelatin-based hydrogels, which exhibited tailorable mechanical properties that were controlled by hindering the tendency of the gelatin chains to naturally associate into triple helices.<sup>[23]</sup> The crosslinking reaction was performed in water in the presence of a tenside to confer solubility to the bifunctional crosslinkers (hexamethylene diisocyanate and ethyl lysine diisocyanate), which resulted in direct crosslinks and hydrophobic grafts that stabilized the hydrogels. Synthesizing hydrogels with reduced helical contents was advantageous over alternative gelatin-based hydrogels possessing mechanical properties largely reliant on their helical contents.<sup>[24-28]</sup>

Importantly, the biological evaluation of these gelatin-based hydrogels with systematically varied parameters is needed to gain a fundamental understanding of their

biofunctionality. Therefore, in this report, the synthesis procedure for these biopolymer networks was adapted to allow the production of putatively non-cytotoxic hydrogels. Films were prepared from solutions of varying amounts of gelatin (7, 10, and 13 wt%) and ethyl lysine diisocyanate (LDI) as crosslinker (3, 5, and 8 NCO/amino group molar ratio) in aqueous solutions under clean room conditions. Indirect cytotoxicity tests were conducted according to the EN DIN ISO standard 10993-5 using L929 mouse fibroblasts. The viability of MSCs was evaluated and analyzed in 2D cultures with regard to the swelling degrees and mechanical properties of the gelatin-based hydrogels. This study was expanded to include gelatin-based polymer network films with fibronectin adsorbed to their surfaces prior to cell seeding in order to evaluate how the adhered protein affects the overall MSC viability. Potentially, this class of materials may be useful as MSC stem cell substrates, niches, or implantable MSC carriers.

## **Experimental Part**

### *Materials*

Gelatin (Type A, Bloom 200,<sup>[29]</sup> porcine) was purchased from Gelita (Eberbach, Germany). LDI was purchased from Shanghai Infine Chemical Co., Ltd. (Shanghai, China) Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO, poloxamer F-108) and all other chemicals were purchased from Sigma Aldrich (Munich, Germany) unless otherwise noted.

### *Film synthesis*

All glassware pieces used for the film synthesis were autoclaved before their introduction into the cleanroom. In the cleanroom, low endotoxin-containing gelatin was dissolved in autoclaved, sterile-filtered Millipore water at 45 °C in a round bottom flask under magnetic stirring to form a 10 wt% solution (e.g. 5 g gelatin in 45 g water). PEO-PPO-PEO was added (0.5 g) to the solution, which was stirred for an additional 20 min at 45 °C. Freshly distilled ethyl lysine diisocyanate (LDI) was added and allowed to react under these conditions. Following 5 min, the crosslinking mixture was casted in Petri dishes, covered, and allowed to dry at room temperature overnight. Films were washed with autoclaved, sterile-filtered water for several days in the cleanroom to remove water-soluble impurities.

#### *Sterilization*

Samples were sterilized by Medicoplast (Illingen, Germany) according to DIN EN ISO 13485:2003 standards using ethylene oxide (EO). The sterilization step consisted of 3 h of contact time with 600 mg/L ethylene oxide at 44 °C, which was followed by a desorption step (35 – 45 °C for 3 d). Sterilized samples were stored in sealed, sterilized Stericlin<sup>®</sup> bags (VP Group, Feuchtwangen, Germany) in the cleanroom at 23 °C prior to seeding experiments.

#### *Swelling and Degradation studies*

The degree of swelling ( $Q$ ) at 37 °C in pH 7.4 PBS was calculated according to equation 1 from the ratios of sample weights of the swollen ( $m_s$ ) and dry ( $m_0$ ) material. The density of the polymer network  $\rho_p$  was determined by measuring the thickness of dogbone samples (area = 85.7 mm<sup>2</sup>) and  $\rho_s$  is the specific density of water.

$$Q = \left( 1 + \frac{\rho_p}{\rho_s} \left( \frac{m_s}{m_0} - 1 \right) \right) \times 100 \quad (1)$$

The remaining mass loss ( $\mu_{rel}$ ) was calculated according to

$$\mu_{rel} = \frac{m_d}{m_0} \times 100\% \quad (2)$$

where  $m_0$  is the initial dry sample weight and  $m_d$  is the dry sample weight following incubation in pH 7.4 PBS buffer solution at 37 °C at the desired time point. Measurements were performed in triplicate, and the calculated values are reported as average  $\pm$  standard deviation.

#### *Mechanical Tests*

Mechanical properties at 37 °C in water were assessed by tensile tests using a Zwick Z2.5 tensile tester (Zwick Roell GmbH, Ulm, Germany) equipped with a temperature controlled glass water tank. The deformation rate was 5 mm·min<sup>-1</sup>. Bone-shaped samples with dimensions of 30 mm x 3 mm and a thickness of 0.1 mm to 0.3 mm in the dry state and a free length of the clamped samples of 20 mm were used. The Young's modulus ( $E$ ) of all samples was calculated from the linear region of the stress-strain curve generally between 1 – 5% strain. Measurements were performed in triplicate, and the calculated values are reported as average  $\pm$  one standard deviation.

#### *Structural Analyses*

Wide-angle X-ray scattering (WAXS) measurements were carried out using a Bruker D8 Discover with GADDS. The X-ray generator operated at a voltage of 40 kV and a current of 40 mA, producing Cu K $\alpha$ -radiation with a wavelength  $\lambda = 0.154$  nm. The detector was a Hi-Star in 1024  $\times$  1024 pixel mode and was set at a distance of 15 cm covering  $2\theta$  from 5 to 40°. The collimeter was 0.8 mm and the samples were oscillated in the beam  $\pm$  1 mm to average. The exposure time was 120 s per frame.

#### *Cytotoxicity tests with mouse fibroblasts*

Indirect cytotoxicity tests were conducted according to the EN DIN ISO standard 10993-5 using L929 mouse fibroblasts. Cells were exposed to material eluate following 72 h incubation time in fresh cell culture medium (without serum supplementation) at 37 °C and changes in cell morphology was assessed after 48 h according to USP 23-NF18 using a transmitted light microscope in phase contrast mode (Zeiss, Germany). The integrity of the cell plasma membrane was tested by measuring the activity of the enzyme lactate dehydrogenase (LDH, Cytotoxicity Detection Kit LDH, Roche, Germany), which is released by damaged cells in the cell culture supernatant. The activity of the cells was measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by mitochondrial dehydrogenase to a formazan product that adsorbs at  $\lambda = 490$  nm, which was quantified (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega, Germany) at 48 h culturing time using pure cell culture medium as a control.

#### *Immunophenotypic analysis of Human Mesenchymal Stem Cells*

Cell surface antigen phenotyping of bone marrow-derived MSCs was performed at passage 3. The following cell-surface epitopes were marked with the anti-human antibodies CD29-APC, CD34-PerCP-Cy5.5, CD45-V500, CD73-PE, CD90-PE (Becton Dickinson, Heidelberg, Germany) and CD105-Alexa488 (AbD Serotec, Oxford, UK). Mouse isotype antibodies served as a control.  $2 \times 10^4$  labelled cells were acquired and analyzed using a FACS Scan flow cytometer (LSRII) using CellQuest-Software (Becton Dickinson, Heidelberg, Germany).

#### *In vitro functional differentiation assay*

To assess their differentiation capacity, MSCs were induced to differentiate to adipocytes and chondrocytes at passage 3 employing a previously described method.<sup>[30]</sup> To induce adipogenic differentiation, MSCs were seeded at a density of  $3 \times 10^3$  cells per  $\text{cm}^2$  and cultured for up to 3 weeks in cell culture medium supplemented with  $10^{-8}$  M dexamethasone, 2.5  $\mu\text{g}/\text{mL}$  insulin, and 100  $\mu\text{g}$  indomethacin. To induce chondrogenic differentiation,  $3 \times 10^5$  cells were cultured in 1 mL of chondrogenic induction medium (cell culture medium supplemented with 0.1  $\mu\text{M}$  dexamethasone, 1 mM sodium pyruvate, 0.17 mM L-ascorbic acid 2-phosphate, 0.35 mM L-proline, 6.25  $\mu\text{g}/\text{mL}$  insulin, 6.25  $\mu\text{g}/\text{mL}$  transferrin, 6.25 ng/mL selenite, 5.33  $\mu\text{g}/\text{mL}$  linolic acid, 1.25 mg/mL bovine serum albumin, and 0.01  $\mu\text{g}/\text{mL}$  transforming growth factor  $\beta 3$ ) in the tip of a 15-mL conical tube to allow aggregation of the cells in suspension culture. The induction of chondrogenic differentiation was performed for 4 weeks. The differentiation capacity towards different cell lineages was verified by morphology changes and immunostaining for specific markers (aggrecan for chondrocytes and fatty acid binding protein (FABP-4) for adipocytes).

#### *Human Mesenchymal Stem Cell in vitro studies*

Human mesenchymal stem cells (hMSC) were isolated and prepared as previously described in detail by Gaebel.<sup>[30]</sup> All medical procedures dealing with human products were revised and approved by an independent institutional research ethics committee of the University of Rostock (approval no. A21/2007) and all patients provided informed consent. Three bone marrow samples were obtained by sternal aspiration from patients undergoing coronary artery bypass graft surgery at Rostock University Hospital, Rostock, Germany. After the puncture of patients sternum, 10 ml bone marrow aspirate was mixed

with 15 ml supplemented MSCBM and seeded in 175 cm<sup>2</sup> culture flask. For hMSC culture, Mesenchymal Stem Cell Basal Medium (MSCBM®-2) supplemented with L-glutamine, mesenchymal growth supplement, GA-1000 (Lonza, Basel, SWI), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA, Pasching, AUT) was utilized. Medium was changed every 2-3 days. For this study, cells were used after 3-4 medium changes and by subculturing passage 6.

Gelatin-based hydrogels (n = 8 – 10) were pre-wetted with PBS for 2 hours at room temperature prior to the proliferation experiments. Then, films were cut into disc-shaped pieces with a base area of ~0.75 cm<sup>2</sup>. For the protein adsorption studies with cells and material characterization, the materials were incubated with 5 µg/ml human plasma fibronectin in a total volume of 300 µL at 37 °C for 3 hours (Millipore, Billerica, MA, USA). The fibronectin solution was then removed and the materials adsorbed with fibronectin were washed once with PBS buffer solution. Next, 2×10<sup>4</sup> hMSC were seeded on the material and incubated for 9 days because 7-10 d of incubation time are usually required to reach confluency on tissue-culture plastics.<sup>[5]</sup> Supplemented MSCBM®-2 was used to maintain cells in culture. The medium was changed every 3 days.

At 9 days after seeding, cells were washed with PBS for three times and fixed with PBS/4% PFA (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Next, fixed cells were washed three times with PBS and stained with Hoechst 33258 (5 µg/ml) for 20 min at room temperature. After staining, films were washed three times with PBS and put on slides for analysis with confocal laser scanning microscope (LSM780 ELYRA PS.1, Carl Zeiss, Jena, GER). For cell quantification, cells were counted in randomly chosen high power fields. One power field had a surface area of ~0.41 mm<sup>2</sup>.

### *Statistical analysis*

All values are shown as mean  $\pm$  standard error of the mean. For statistical analyses, Sigma Stat software version 3.5 (SPSS Inc., Chicago, USA) was used. One-way ANOVA was used to compare numeric data between the two experimental groups. A level of  $p < 0.05$  was considered as significantly different.

### **Results and Discussion**

This study focused on the viability of MSCs derived from human bone marrow on the surfaces of degradable chemically-crosslinked gelatin-based hydrogels. The requirements for biomaterials suitable as MSC substrates are that they possess high purity, tailorable properties, and cell adhesion capabilities. Therefore, chemically crosslinked gelatin based films were prepared according to a method described in reference [23], but with slight adaptations in the starting materials to yield hydrogels based on components that are well tolerated by cells. The material components used to prepare these hydrogels included low endotoxin-containing starting gelatin (Type A, 200 Bloom), LDI, and PEO-PPO-PEO as a detergent. A systematic variation in material composition and, therefore, material properties, was achieved by synthesizing crosslinked gelatin films, which were prepared from solutions of varying amounts of gelatin (7, 10, and 13 wt%) and LDI crosslinker (3, 5, and 8 NCO/amino group molar ratio). The samples reported here are referred to as GX\_LNCOY, with X = gelatin concentration in wt% and Z = the molar excess of isocyanate groups. These particular compositions were chosen based on the concentration limits of aqueous gelatin solutions and the tailorability achieved when varying the crosslinker excess within this range.<sup>[23]</sup> However, G7\_LNCO3

and G7\_LNCO8 were not evaluated as substrates for MSC culturing because they degraded completely during cell seeding studies (9 d).

Gelatin generally contains about 3 mol% lysine/hydroxylysine residues,<sup>[31]</sup> and amino groups were quantified in the low endotoxin-containing starting gelatin using a trinitrobenzene sulfonate (TNBS) based assay ( $3.1131 \times 10^{-4}$  mol amino group/g gelatin).<sup>[32]</sup> The free amine groups of gelatin can react with isocyanates<sup>[33]</sup> and therefore may be crosslinked using a diisocyanate. LDI was used as chemical crosslinker and derives from the naturally occurring amino acid lysine, which is potentially formed as a degradation product and should not induce cytotoxic effects. LDI was added in 3 to 8 molar excess with respect to gelatin amino group content taking into account the complex reaction mechanism in water,<sup>[23]</sup> in which not only direct crosslinks are formed but also oligomeric crosslinks, grafts, or blended oligomers may be formed. LDI was reacted with gelatin in Millipore water that was sterilized (steam) and filtered ( $\varnothing = 0.2 \mu\text{m}$ ) immediately before each synthesis to ensure that the presence of contaminants were avoided. With the exception of distilling LDI, all synthetic steps were carried out in a cleanroom.

A surfactant needed to be included in the synthesis of the hydrogels to mediate an oil-in-water suspension that would confer the solubility of the hydrophobic diisocyanate crosslinker. PEO-PPO-PEO was used as a surfactant for the synthesized films instead of the earlier reported saponin.<sup>[23]</sup> PEO-PPO-PEO surfactants have gained a great deal of attention for the design of compatible biomaterials since they are FDA approved for in vivo injection.<sup>[34,35]</sup> Here, PEO-PPO-PEO (20 mol.-% PPO, 80 mol.-% PEO,  $M_n = 14.6$  kg/mol) was used in the synthesis of gelatin-based hydrogels because of its relatively

high molecular weight and decreased concentration of terminal hydroxyl groups, which reduced the probability of their reaction with the reactive diisocyanate crosslinker.

The internal structure of dried chemically crosslinked gelatin-based hydrogels was investigated using wide-angle x-ray scattering (WAXS) obtained at room temperature, which showed a peak at approximately  $2\theta = 20^\circ$  representing the amorphous organization of chains.<sup>[36]</sup> Peaks representing single or triple helices within gelatin, which may be found at  $2\theta = 31^\circ$  (corresponding to the distance between prolines in a turn in the polyproline-II-helical single helices) or  $8^\circ$  (corresponding to the diameter of a triple helix perpendicular to the chain direction), respectively, were also observed. By dividing the integrated intensities of the peaks that accounted for the lateral distances between single collagen chains in triple helices by the amorphous peak areas, it was possible to quantify the degree of renaturation or relative amount of triple helices ( $X_{c,t}$ ) or single helices ( $X_{c,s}$ ) (Table 1). The amount of single helices present in these samples was independent of composition ( $X_{c,s} = 1.0 - 2.3\%$ ). The contents of triple helices for the G10 samples decreased with increasing LDI content. This observation shows the importance of performing the synthesis above the gel-sol transition temperature of gelatin ( $37^\circ\text{C}$ ) and using chemical crosslinking to hinder triple (re)helicalization within gelatin matrices in order to yield materials with tailorable mechanical properties.<sup>[23,36]</sup> G7\_LNCO5, G10\_LNCO3, and G10\_LNCO5 contained relatively high contents of triple helices ( $X_{c,t} = 3.6 - 4.1\%$ ), which indicated that the amount of crosslinking obtained in these materials was lower than for the other samples since the helical contents were not as well suppressed due to the lower amounts of crosslinker and gelatin chain density. However, these values were still relatively lower than triple helical contents that have been

observed in physically crosslinked gelatin-based hydrogels dried at a temperature below the sol-gel transition ( $T < 37\text{ }^{\circ}\text{C}$ ;  $X_{c,t} = 8 - 10\%$ ).<sup>[36]</sup>

The biomaterial properties that are exhibited under physiological conditions such as their mechanical properties and swelling behavior must be understood in order to correlate the results of cell behavior as tested across a system of substrates. An increasing number of studies has supported that the wettability or hydrophilicity of materials dictates how stem cells respond when seeded on a material surface.<sup>[7]</sup> The mechanical properties of the hydrogels under physiological conditions were obtained using tensile tests at  $37\text{ }^{\circ}\text{C}$  for hydrogels swollen in pH 7.4 PBS buffer for 24 h given that this time has been observed to be important for initial MSC attachment (Table 1).<sup>[37]</sup> The G13 samples possessed the lowest Young's moduli (60 – 300 kPa) and the G10 samples possessed the highest Young's moduli (330 – 740 kPa) amongst the studied materials. The G10 samples were not significantly less elastic than the G13 samples and the maximum stress values were insignificantly different, with the exception of G10\_LNCO5, which possessed the highest maximum stress value ( $\sigma_{\max} = 265 \pm 65\text{ kPa}$ ) of the networks and G13\_LNCO5, which possessed the lowest maximum stress value ( $\sigma_{\max} = 45 \pm 5\text{ kPa}$ ) during their tensile measurements. In order to assess the substrate mechanical properties, which would be experienced by MSCs during this study, the hydrogels were additionally characterized for their mechanical properties after 9 d incubation time in pH 7.4 PBS buffer solution. The mechanical properties of the hydrogels diminished with the exception of G13\_LNCO5 and G13\_LNCO8 during this time, which correlated well with the observed changes in mass loss during the *in vitro* hydrolytic degradation experiments (Figure 1).

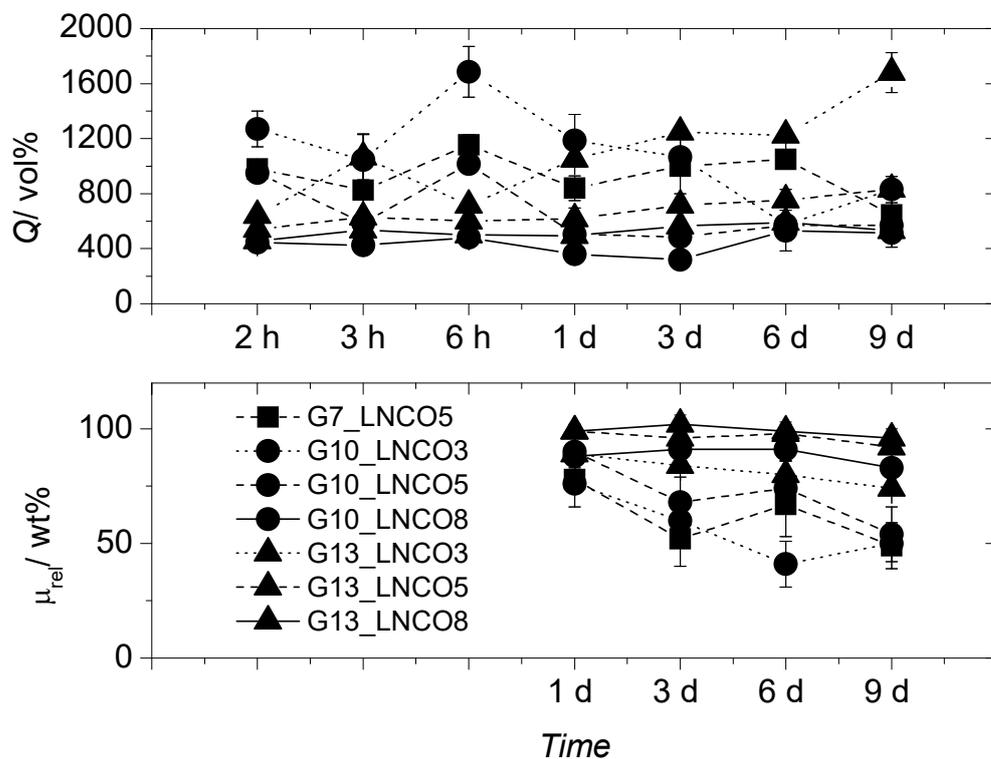
**Table 1. Relative single ( $X_{c,s}$ ) and triple ( $X_{c,t}$ ) helix content for dry networks and mechanical properties of LDI-crosslinked Gelatin films swollen in pH 7.4 PBS buffer solution at 37 °C ( $t = 24 \text{ h}^a$  and  $9 \text{ d}^b$ ).**

<i>Material</i>	$X_{c,s}$	$X_{c,t}$	$E$	$\sigma_{\max}$	$\epsilon_B$	$E$	$\sigma_{\max}$	$\epsilon_B$
	[%]	[%]	[kPa] <sup>a</sup>	[kPa] <sup>a</sup>	[%] <sup>a</sup>	[kPa] <sup>b</sup>	[kPa] <sup>b</sup>	[%] <sup>b</sup>
G7_LNCO5	1.6	3.6	330 ± 175	120 ± 5	55 ± 15	n.m.	n.m.	n.m.
G10_LNCO3	1.7	4.1	495 ± 165	120 ± 15	50 ± 25	n.m.	n.m.	n.m.
G10_LNCO5	2.1	3.8	635 ± 105	265 ± 65	40 ± 20	n.m.	n.m.	n.m.
G10_LNCO8	1.8	1.7	590 ± 30	125 ± 55	25 ± 10	290 ± 65	115 ± 0	35 ± 0
G13_LNCO3	2.2	2.0	215 ± 10	135 ± 15	90 ± 15	110 ± 10	60 ± 20	55 ± 30
G13_LNCO5	1.0	3.0	90 ± 30	45 ± 5	35 ± 5	115 ± 25	90 ± 5	50 ± 5
G13_LNCO8	2.3	1.9	290 ± 10	115 ± 40	45 ± 15	245 ± 25	80 ± 30	45 ± 20

n.m. = not measurable

The material swelling ( $Q$ ) and mass loss of the hydrogels in PBS buffer solution at 37 °C were quantified (Figure 1). The swelling properties for the substrates at 2 h, 6 h, 24 h, and 72 h of incubation time in pH 7.4 PBS buffer are reported because these times corresponded to the swelling equilibration times for the materials (e.g. 6 h and 24 h) or to the important initial time points of the biological tests (e.g. the 2 h time point corresponded to the wetting pre-conditions used before MSC seeding and the 72 h time point corresponded with the EN DIN ISO standard 10993-5 used to assess indirect fibroblast response). The G10 and G13 samples displayed tailored amounts of degrees of swelling following 2 h incubation time in pH 7.4 PBS buffer solution at 37 °C. G7\_LNCO5, G10\_LNCO3, and G10\_LNCO5 showed maximum degrees of swelling after 6 h incubation time, after which time the values decreased. This corresponded to a

loss in mass, which is the third step in hydrolytic bulk degradation of materials (1<sup>st</sup> step: water uptake, 2<sup>nd</sup> step: bond hydrolysis, 3<sup>rd</sup> step: loss of material).<sup>[22]</sup> G10\_LNCO8 and the G13 samples showed nearly equilibrated swelling properties starting from 24 h. At 6 h and 24 h of incubation time, the swelling degrees decreased with increasing amounts of gelatin concentrations at equal LDI ratios (e.g. G7\_LNCO5, G10\_LNCO5, and G13\_LNCO5). The decrease in swelling of G7\_LNCO5, G10\_LNCO3, and G10\_LNCO5 indicated a slight mass loss between 6 h and 72 h, whereas the swelling degrees of G10\_LNCO8, G13\_LNCO5, and G13\_LNCO8 remained nearly stable from 24 h to 9 d. Films with lower amounts of gelatin concentrations degraded more rapidly because their crosslinking densities per volume were decreased.<sup>[38]</sup> This corresponded to the higher  $X_{c,t}$  values measured for G7\_LNCO5, G10\_LNCO3, and G10\_LNCO5 (3.6 – 4.1%), which indicated a higher amount of unstable triple helices, compared with the  $X_{c,t}$  values measured for G10\_LNCO8, G13\_LNCO3, G13\_LNCO5, and G13\_LNCO8 ( $X_{c,t}$  = 1.7 – 3.0%), which indicated that their material properties were ruled by the crosslinking density of the materials. Particularly, the stability of the G13 samples highlighted that, in addition to varying the crosslinker amounts, the degradation kinetics of LDI-crosslinked gelatin-based hydrogels can be controlled by varying the gelatin concentrations.



**Figure 1.**  $Q$  values and  $\mu_{\text{rel}}$  for networks in pH 7.4 PBS buffer solution at 37 °C up to  $t = 9$  d.

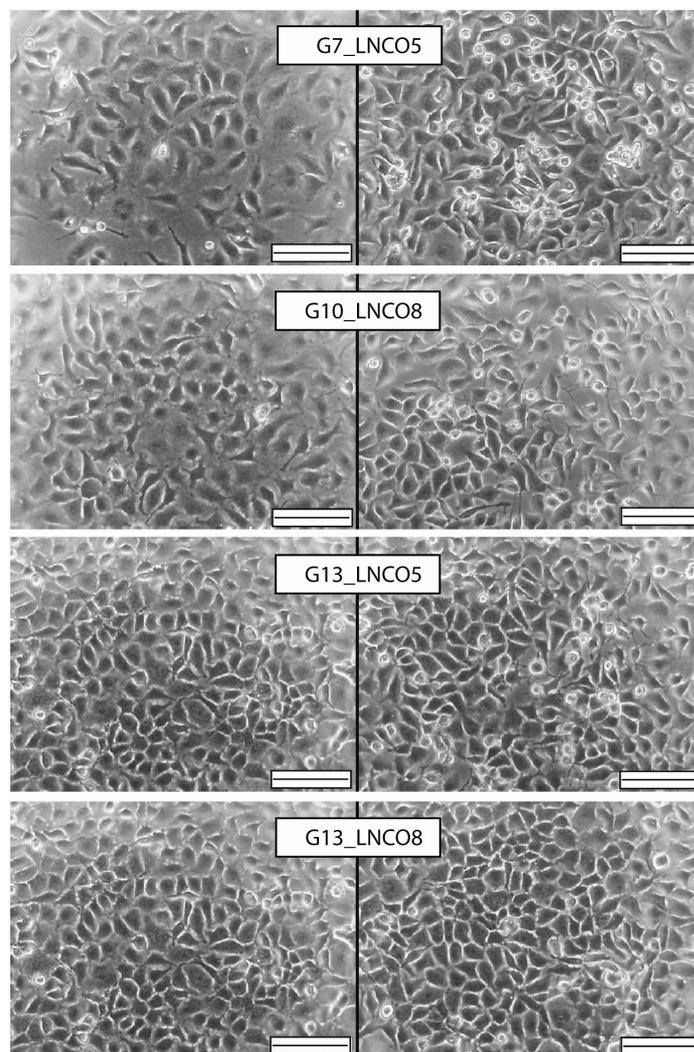
The results obtained from the swelling experiments corresponded to the mechanical characterization of hydrogels incubated in pH 7.4 PBS buffer solution for 24 h as well as for 9 d. For example, the mechanical properties of swollen G7\_LNCO5, G10\_LNCO3, and G10\_LNCO5 were quantified at times longer (24 h) than the time needed to achieve maximum swelling (6 h). This indicated that the moduli of G7\_LNCO5, G10\_LNCO3, and G10\_LNCO5 were quantified at times following the initiation of sample degradation, which included random chain scission of gelatin chains. Indeed, *in vitro* degradation experiments in pH 7.4 PBS buffer demonstrated that G7\_LNCO5 ( $\mu_{\text{rel}} = 78 \pm 12$  wt%) and G10\_LNCO3 ( $\mu_{\text{rel}} = 76 \pm 3$  wt%) degraded following 24 h incubation time, while G10\_LNCO5 showed lower mass loss under the

same time frame ( $\mu_{\text{rel}} = 90 \pm 7 \text{ wt}\%$ ). This may be a reason for the Young's moduli having larger errors for G7\_LNCO5 and G10\_LNCO3 than for G10\_LNCO8 and the G13 samples, which possessed  $\mu_{\text{rel}} = 90 - 100 \text{ wt}\%$  after 24 h degradation time. The absolute values of the Young's moduli after 24 h incubation time differed from the earlier reported data,<sup>[23]</sup> which were obtained for materials synthesized using a different tenside. A likely explanation is that this changes led to a different ratio between direct crosslinks, grafting, and potential blended oligomers in the system, with the values reported here indicating highly grafted materials and a relatively low number of covalent crosslinks.<sup>[23]</sup> The G13 samples possessed the lowest Young's moduli (60 – 300 kPa) within this class of materials, which was surprising given that the  $\mu_{\text{rel}}$  values of the G13 samples were relatively low following 24 h incubation time in pH 7.4 PBS buffer solution and the swelling degrees were not significantly different from the other networks. The mechanical properties of equilibrated wet gelatin-based hydrogels at room temperature were shown to increase with increasing amount of LDI crosslinker for G10 samples ( $E = 70 - 230 \text{ kPa}$ ).<sup>[23]</sup> However, the mechanical properties showed no such trends according to the amount of LDI crosslinker following 24 h incubation time in pH 7.4 PBS buffer solution. This highlights the challenging endeavor of assessing degradable substrates and their influence on MSC morphology.<sup>[39,40]</sup>

Indirect cytotoxicity tests were conducted according to EN DIN ISO standard 10993-5 using L929 fibroblasts (Figure 2). EO-sterilized samples were incubated with medium for 72 h at 37 °C, and the toxicity of the medium extract was evaluated by examining the cell morphology, the release of extracellular lactate dehydrogenase, and the cell mitochondrial activity following 48 h of culturing time. The eluates from all

samples were well-tolerated by the L929 cells and showed a non-toxic response for each sample eluate, with the L929 cells showing the slightest change in morphology when incubated with the eluates from G10\_LNCO8 and the G13 samples. Figure 1 shows the images of L929 cells incubated with eluates from samples G7\_LNCO5, G10\_LNCO5, G10\_LNCO8, and G13\_LNCO8. The fibroblasts were confluent and showed tight or nearly tight cell layer with minimal lysis. The images show non-adherent yet vital cells that are rounded in shape and possess a white region near their edge. LDH assays were used to determine the amount of cell proliferation by measuring cell number via total cytoplasmic LDH released upon cell lysis. Overall, the hydrogels did not negatively affect the functional integrity of the outer cell membrane with respect to the control samples, with the exception of the extract of G13\_LNCO5, which showed only a slightly elevated LDH level with respect to the control values for  $n = 8$  samples. The cell proliferation of L929 fibroblasts based on LDH analyses for the LDI-crosslinked gelatin-based hydrogels were superior to those based on glutaraldehyde-crosslinked gelatin materials.<sup>[41]</sup> The mitochondrial activities were slightly lower for G7\_LNCO5, G10\_LNCO3, and G10\_LNCO5, whereas the extract of G13\_LNCO3 did not significantly affect cell activity, and G10\_LNCO8, G13\_LNCO5, and G13\_LNCO8 extracts all improved cell mitochondrial activity. These results indicated that the presence of degraded byproducts affected cell activity, although to an overall minor extent. Because the L929 cells remained viable, the morphological changes of the L929 cells were most likely due to adhesion-related issues, which were most prevalent in G7\_LNCO5 and G10\_LNCO3. This indicated that the extracts of gelatin-based hydrogels with sufficient amounts of stable netpoints and gelatin densities, which released the

lowest concentrations of degraded gelatin fragments, least affected the adhesion capabilities of mouse fibroblasts. The extracts from the faster degrading materials contained higher concentrations of gelatin fragments, which possess bioactive domains, such as RGD or DGEA peptide sequences, that can facilitate fibroblast attachment to gelatin-based substrates<sup>[42]</sup> and disrupted their attachment to tissue culture polystyrene surfaces.

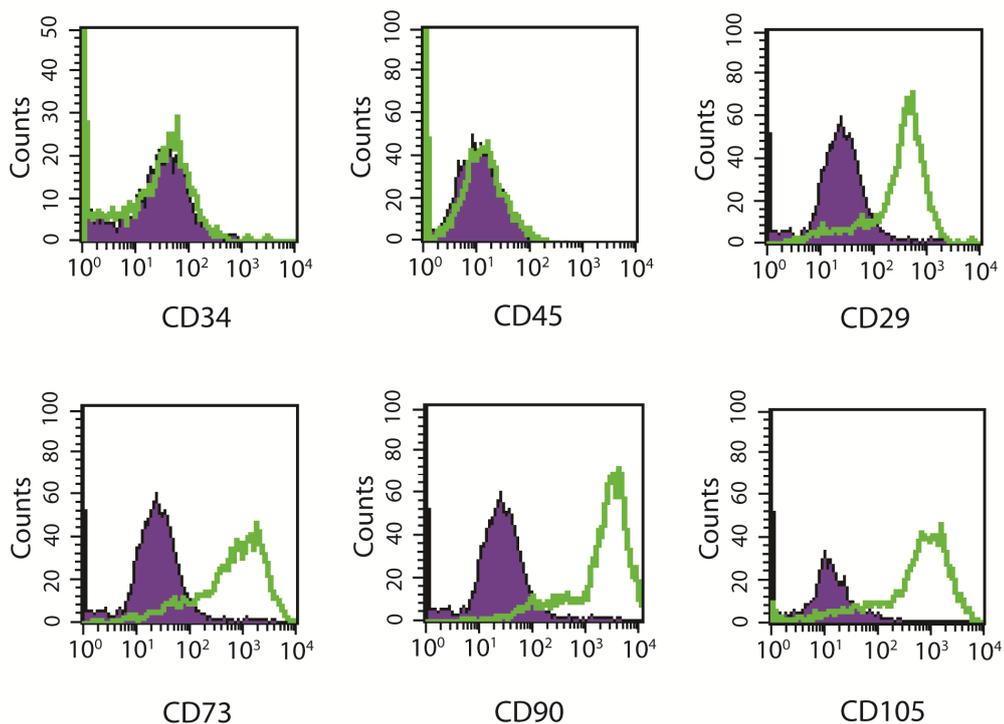


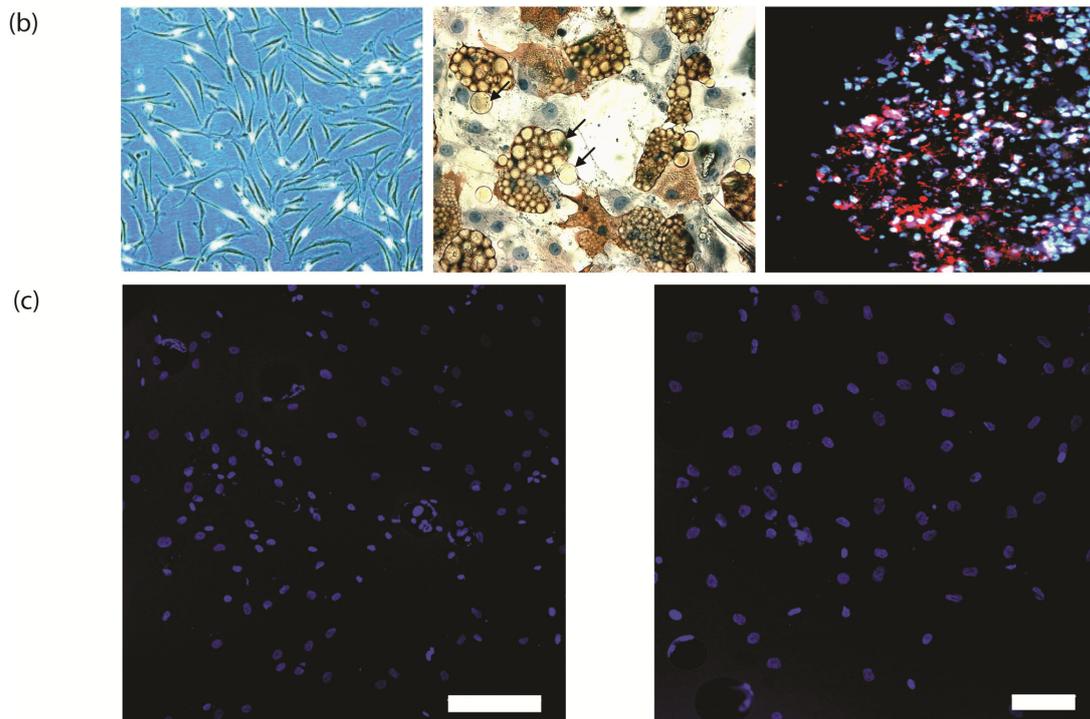
**Figure 2. The morphology of L929 mouse fibroblast cells following 48 h culturing time following 72 h incubation with sample extracts at 37 °C in cell culture medium (G7\_LNCO5, G10\_LNCO8, G13\_LNCO5, and G13\_LNCO8 (top to bottom, right); control media (left)). Scale bars = 100  $\mu$ m.**

Primary mesenchymal stem cells derived from human bone marrow were seeded on the gelatin-based substrates following 2 h of incubation time with medium and allowed to proliferate for 9 d (Figures 3 and 4). At 9 d culturing time, nuclei were stained and counted to quantify the proliferation. All material substrates supported MSC viability and proliferation. The mean number of MSCs grown on films were highest for G13\_LNCO5 ( $7617 \pm 933$  cells/film) and least viable on G10\_LNCO8 ( $1359 \pm 393$  cells/film). Cells remained viable on all other surfaces, but there were no significant differences in cell viability for the other substrates (mean cell numbers = 3100 cells/film). The influence of the material swelling properties and mechanical properties on the MSC behavior was closely examined. G13\_LNCO5 possessed the lowest elastic modulus ( $E = 92 \pm 28$  kPa) after 24 h at 37 °C in pH 7.4 PBS buffer solution and remained stable up to 9 d, which may have contributed to the excellent MSC viability observed using this composition as a substrate. The G13 substrates showed the highest stability during the swelling studies and during the cell seeding studies. After 24 h at 37 °C in aqueous buffer, G13\_LNCO5 exhibited a lower modulus ( $E = 92 \pm 28$  kPa) compared to G13\_LNCO3 ( $E = 217 \pm 12$  kPa) and G13\_LNCO8 ( $E = 289 \pm 8$  kPa), and these moduli were only minimally affected following 9 d incubation time. Correspondingly, the viability results of MSCs on these three substrates showed that G13\_LNCO5 was a preferable substrate in terms of viable cells, which may be due to its lower yet stable modulus.<sup>[43]</sup> Similarly, G10\_LNCO8 showed less ability to support MSCs, while maintaining a higher modulus following 9 d incubation time ( $E = 290 \pm 65$  kPa). MSCs on substrates with low moduli (e.g. the elasticity of bone marrow) can become quiescent

but remain responsive to other stimuli.<sup>[43]</sup> Therefore, materials with lower moduli that support viable MSCs may be used as a niche, which is an environment that controls differentiation potential of surrounding cells. G10\_LNCO8, which supported the lowest numbers of viable MSCs, possessed a relatively high modulus ( $E = 592 \pm 31$  kPa), although the modulus of G10\_LNCO5 was higher. A possible explanation for this is that G10\_LNCO5 required only 6 h to reach maximum swelling capacity, which was followed by material degradation and yielded a softer substrate surface for the MSC cells during the 9 d seeding time, whereas the modulus of G10\_LNCO8 may have remained higher for a longer time (Table 1), thereby reducing the viability of the seeded MSCs. Previous studies have shown that extremely hydrophilic substrates may not be ideal MSC substrates,<sup>[7]</sup> yet MSC viability was clearly observed on the chemically crosslinked gelatin-based hydrogels. This may be due to the presence of binding motifs such as DGEA along the gelatin chains.<sup>[15]</sup> Indeed, blends containing gelatin components have previously been shown to exhibit preferable interactions with fibroblasts as well as with MSCs.<sup>[44,45]</sup>

(a)



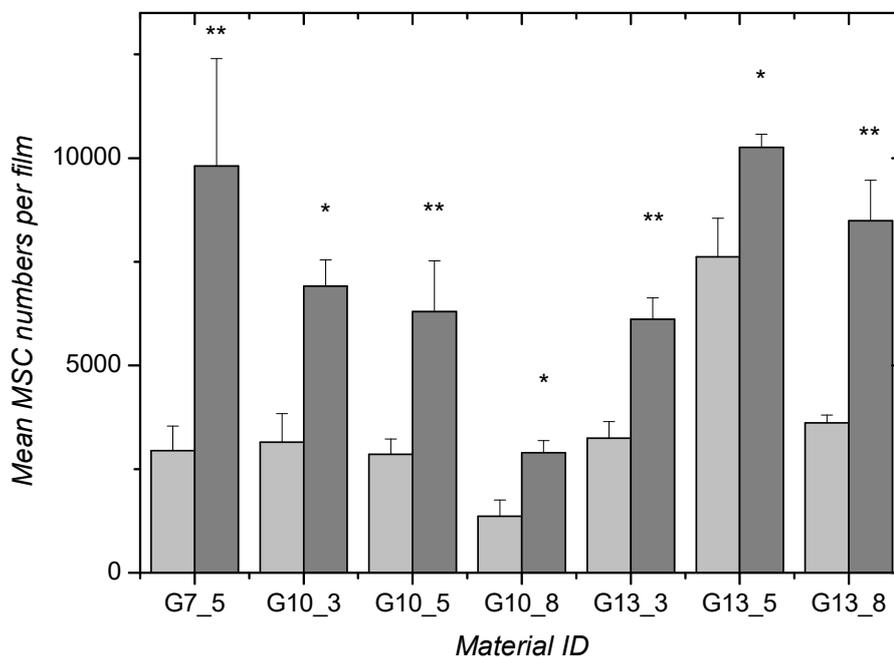


**Figure 3. (a) Immunophenotypic characterization of MSCs. (b) (left) Morphological appearance of MSCs, which maintained typical spindle shape (magnification: X100); (middle) adipogenic differentiation capacity of MSCs. The differentiation into adipocyte was revealed by the formation of liquid droplets (black arrows) stained with FABP-4 and slightly counterstained by hematoxin to reveal cell nuclei (blue); (right) chondrogenic differentiation capacity of MSCs. This differentiation was evaluated by aggrecan staining (red) the cell nucleus and counterstained by DAPI (blue). The magnification for the differentiation images was X400. (c) Representative image of viable MSCs after 9 d culture time seeded on G13\_LNCO5. The MSC nuclei have been stained with Hoechst 33258 (blue) for clarity. Scale bars = 200  $\mu\text{m}$  (left) and 100  $\mu\text{m}$  (right).**

The observed behavior of the MSCs seeded on the surface of the gelatin-based hydrogels indicated that these materials are suitable degradable substrates for MSCs. While such direct cell seeding studies are valuable to assess materials for their use in vitro, these studies are not indicative of how cells attach to the surfaces of implanted materials. A biomimetic approach that can be used to more closely resemble the in vivo situation is by first adsorbing fibronectin to the surfaces of gelatin hydrogels and then

seed MSCs because protein adsorption occurs before any cell-material interaction can take place. Therefore, the viability of MSCs was examined on surfaces adsorbed with fibronectin, which also has been shown to enhance MSC attachment on surfaces.<sup>[46]</sup> The viability of MSCs on human plasma fibronectin-coated surfaces was expected to increase for all compositions of crosslinked gelatin substrate. The substrates were treated with fibronectin by incubating them with 5  $\mu\text{g/ml}$  human fibronectin at 37 °C for 3 h, at which point the substrates were seeded with MSCs. Significantly enhanced MSC viability was observed for all of the fibronectin treated materials, where the largest increase in viability was observed for coated G7\_LNCO5 with adsorbed fibronectin (Figure 4). The highest proliferation of MSCs for fibronectin-adsorbed materials was observed for G13\_LNCO5 (mean cell number =  $10260 \pm 312$  cells/film) and the lowest cell count on any fibronectin-adsorbed substrate was for G10\_LNCO8 (mean cell number =  $2895 \pm 293$  cells/film). The cell growth results of the other fibronectin-adsorbed materials ranged from  $\sim 6100$  cells/film to  $\sim 9800$  cells/film. Interestingly, the viability of MSCs seeded on hydrogels that quickly degraded within 9 d (e.g.  $\mu_{\text{rel}} < 80$  wt% (i.e. G7\_LNCO5, G10\_LNCO3, G10\_LNCO5, and G13\_LNCO3)) after being initially adsorbed with fibronectin increased with increasing  $\mu_{\text{rel}}$ . In order to investigate the cause of this phenomenon, hydrogels adsorbed with fibronectin were characterized for their swelling and uptake properties, mechanical properties, and degradation behaviors identically to those without any adhered fibronectin (Figure 1). The initial fibronectin treatment insignificantly altered the swelling properties, mechanical properties, and degradation behaviors after 9 d incubation time in pH 7.4 PBS buffer solution (data not shown). Although there are some examples of reports describing the quantification of fibronectin

adhered to gelatin substrates following short incubation times,<sup>[47]</sup> studies that account for the role of this protein on degradable surfaces over longer incubation times and how it impacts hMSCs proliferation would likely aid in better understanding this phenomenon. The quantification of protein adhesion on these degradable hydrogels and its impact on cellular behavior will be the focus of future studies.



**Figure 4. Cell viability of MSCs seeded on gelatin-based hydrogels treated with fibronectin (dark grey) compared with untreated substrates (light grey). The nomenclature ‘LNCO’ has been removed from each sample for clarity; (\*  $p < 0.05$ ; \*\*  $p < 0.01$  significantly different).**

MSCs possess the ability to differentiate into multiple lineages, such as adipogenic, chondrogenic, and osteogenic. However, materials that do not influence the fate of stem cell differentiation are useful for in vitro culturing as well as in vivo delivery of undifferentiated cells. For the extent of these studies, for the untreated and fibronectin-treated materials, there were no signs of uncontrolled differentiation in any of the cell

populations, even when cell populations were expanded. These materials could form the basis to produce substrates that are able to sustain the multipotency of stem cells.<sup>[48]</sup>

## **Conclusion**

Gelatin-based hydrogels with tailorable mechanical properties as well as water uptake and degrees of swelling were prepared using an adapted synthetic strategy. Eluates from the materials were well tolerated by L929 mouse fibroblasts, and changes in morphology during the culturing can be attributed to some degradation/leaching of compounds which however did not affect proliferation or viability of the cells and did not lead to membrane disruption. Human MSCs derived from bone marrow remained viable on all substrates, whereby materials with relatively low mechanical properties (e.g. Young's modulus of 90 kPa), hydrogels with adsorbed fibronectin, as well as rapidly degrading surfaces previously adsorbed with fibronectin yielded the highest numbers of viable mesenchymal stem cells. Overall, this class of biopolymer-based hydrogels showed favorable interactions with fibroblasts as well as MSCs. These crosslinked gelatin-based substrates proved to be suitable for in vitro MSC cultures up to 9 d. Such materials are promising biomaterial candidates that merit further study as possible stem cell niches.

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**Keywords:** biomaterials, gelatin, mesenchymal stem cells (MSCs), hydrogels, structure-property relations

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## Table of Content Text and Graphics

**Human mesenchymal stem cells (MSC) show a high viability on gelatin-based networks** with tailorable properties. Gelatin crosslinked with lysine diisocyanate ethyl ester in water yielded degradable, sterilizable, and non-toxic hydrogels. The hydrogels have potential as implantable carriers for stem cells, which are needed in regenerative therapies.

