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Printable Glycosaminoglycan Graded Gelatin Methacryloyl Acetyl Hydrogels

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Abstract

Hydrogels are considered as appropriate scaffold materials for cell encapsulation. This is due to their high water binding capacity similar to the native extracellular matrix. However, the equilibrium degree of swelling of simple hydrogels is related to the cross-linking degree of the hydrogels and thereby not freely adjustable. We decoupled the correlation of equilibrium degree of swelling and cross-linking density by chemical modification of the biopolymer gelatin and sophisticated hydrogel formulations. These formulations contained different amounts of chemical modified glycosaminoglycans, genuine components of native extracellular matrix of cartilage. We created glycosaminoglycan-graded hydrogels by layer-wise dispensing three hydrogel precursor solutions on top of each other. We investigated the viability of the encapsulated chondrocytes 28 days after printing and evaluated the production of newly synthesized extracellular matrix.

Motivation

Composition and properties of extracellular matrix (ECM) vary in a tissue-specific manner thereby influencing and regulating cellular behavior. [1] The future vision are biological implants that resemble the properties of specific tissues such as bone, cartilage, fatty tissue. We aim to provide tailored matrix materials, which mimic the ECM of native tissue as three-dimensional scaffolds for tissue specific cells. We hypothesize that tissue substitutes can be produced *in vitro* if isolated cells are enabled to re-produce genuine tissue matrix

Problem

Hydrogels adsorb large quantities of water without dissolving, which makes them an auspicious candidate for ECM imitation. However, the equilibrium degree of swelling (*EDS*), as a measure for adsorbed water within the hydrogel, is dependent on the cross-linking degree of the hydrogel: The higher the cross-linking degree the lower the *EDS* and *vice versa*. [2] It is desirable to tailor hydrogel properties such as biochemical composition, *EDS*, cross-linking density and polymer content independently. Thereby adjusting the tissue-specific differences in material properties such as composition and mechanical properties.

Approach

We used biopolymers occurring in the natural cartilage ECM namely gelatin (hydrolysate of collagen), hyaluronic acid and chondroitin sulfate. We modified all biopolymers with methacryl groups, which can be cross-linked by chemical reaction. In the case of gelatin, we did an additional functionalization with inert acetyl groups, yielding in gelatin methacryloyl acetyl (GMA). [3] Thereby, it is possible to tune the degree of total modification, which determines the viscosity, independently of the degree of methacryloylation, which determines the cross-linking degree. [4, 5]

We investigated two sets of formulation, out of three compositions in each case. [6] The polymer content rose from 10.6% via 11.5% to finally 13.0% in these three compositions as the gelatin concentration was kept equal while the glycosaminoglycan (GAG) content rose from composition 1 (or 1′) to composition 3 or (3′). In the first set (composition 1, 2 and 3), the degree of methacryloylation of the gelatin fraction was constant and thus the concentration of cross-linkable groups increased as the concentration of biopolymers increased.

In the second set (composition 1', 2' and 3'), the degree of methacryloylation was lowered in parallel to the increase of biopolymer content. Thereby, the number of cross-linking sites was reduced in spite of the increase in polymer concentration. Furthermore, the usability of the hydrogel precursor solutions for bioprinting was assessed. Formulations 1'-3' were bioprinted on top of each other containing chondrocytes, yielding in a GAG gradient as in the cartilage. It was investigated whether chondrocytes produced cartilage specific matrix within such biomimetic hydrogel scaffolds.

Results

The *EDS* and the storage moduli (G'_{LVER}) as measure for the gel stiffness of all formulations were investigated. [6] The *EDS* decreased and G'_{LVER} increased in formulations 1-3, whereby a constant gelatin fraction was used. The material properties of formulations 1'-3' are shown in Figure 1. In contrast to the other set of formulation (1-3, data not shown), both parameters G'_{LVER} and *EDS* increased. This was due to the trick on tuning the cross-link density by use of gelatin derivatives with various degrees of methacryloylation.

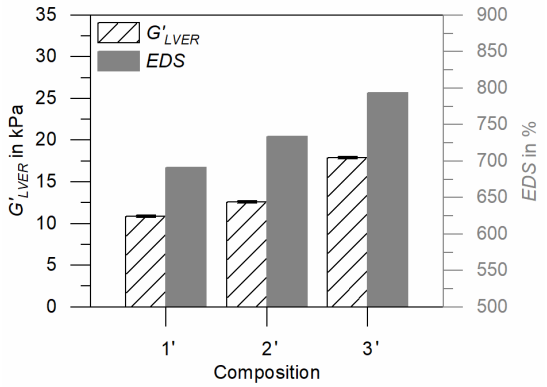

***Figure 1**. Storage moduli within the linear viscoelastic range (G'_{LVER}) and equilibrium degree of swelling (EDS) of gelatin methacryloyl acetyl hydrogels with a varying degree of methacryloylation. Data was adapted from [6].*

Based on these promising results of the advanced hydrogel formulations 1'-3', we investigated their suitability for bioprinting. The surface tension as well as the dynamic viscosity (see Figure 2) of the three hydrogel precursor solutions were determined.

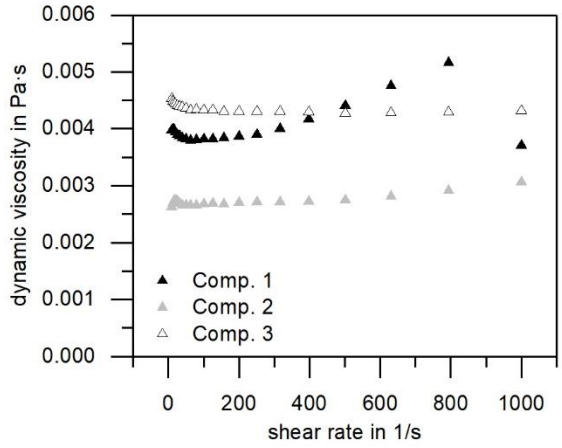

***Figure 2**. Dynamic viscosity of the three investigated hydrogel compositions.*

Furthermore, different UV-curing durations were tested for the generation of stable nine layer thick graded hydrogels (three layers per hydrogel formulation).

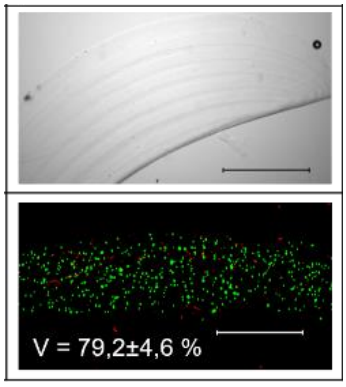

***Figure 3**. Bright field images of bioprinted hydrogels (up) and live-dead-staining staining of incorporated porcine chondrocytes. The viability (V) was calculated as number of living cells (green) relative to the total cell number (green and red); scale bar equals 1 mm. Figure was adapted from [6].*

It was shown that all hydrogel precursor solutions were suitable for bioprinting and graded, porcine chondrocyte containing hydrogels were fabricated using formulations 1'-3', see Figure 3. [6] The viability of encapsulated cells was high (~80%) for all tested UV-curing durations (10 s, 20 s and 30 s, data not shown), but 10 s UV-curing resulted in instable hydrogels and reduced hydrogel thickness (~1 mm) compared to 20 s and 30 s (~1.4 mm).

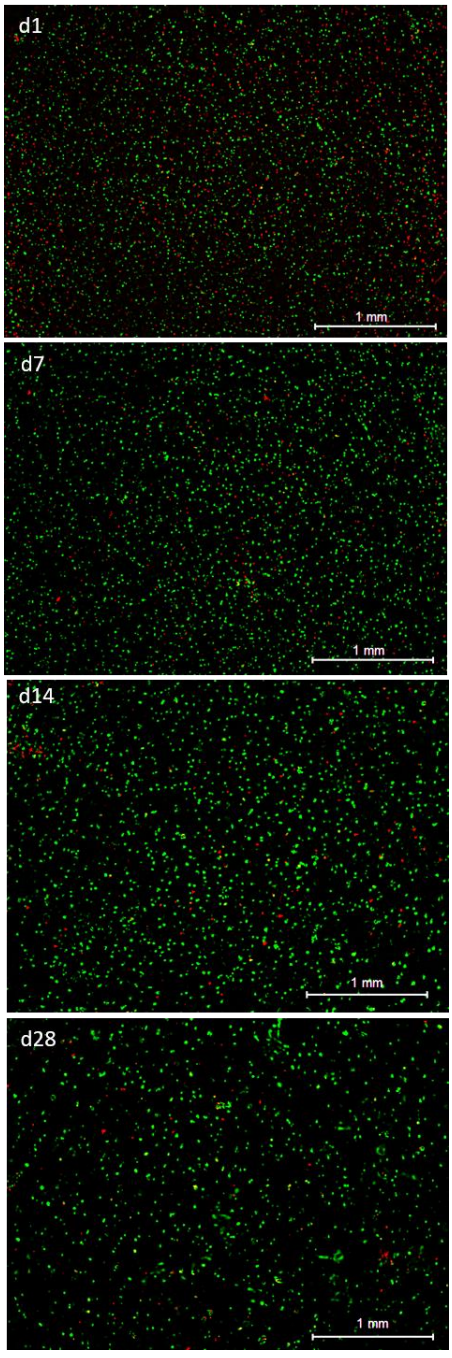

***Figure 4**. Live-Dead-Stainings of bioprinted biomimetic hydrogels over the duration of cultivation. Viable cells are stained green and dead cells are stained red. The scale bars represent 1 mm. Figure was adapted from [6].*

The cytocompatibility range of the used photoinitaitor lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was determined.[6] Therefore, porcine chondrocytes were treated with LAP-concentrations between 0 mg mL^{-1}-0.8 mg mL^{-1} in the presence of 0 %, 5 % or 10 % gelatin. Furthermore, UV-curing duration was varied between 0 min and 2 min.

Without irradiation, no effect on chondrocyte vitality was observed, but UV-irradiation lead to a significant decrease in chondrocyte viability. The highest impact on LAP cytocompatibility was shown for the gelatin concentration, which was present during UV-curing. By addition of 10 % gelatin during irradiation all tested LAP-concentration and curing durations were interpreted as cytocompatible (viability >70 % relative to untreated control). Furthermore, this protecting effect could be correlated with the gelatin concentration, whereby the cytotoxic effect decreased with increasing gelatin concentration.

The viability over the duration of cultivation for 28 days was investigated using live-dead-stainings (see Figure 4). Viable cells could be detected at all time points. Compared to day one the cell number decreased from 2,700 to 1,800 cells at day seven, however less dead cells were detected at day seven compared to day one. Furthermore, the amount of living cells was stable until day 28. This result suggested that the process of hydrogel fabrication has the highest impact on chondrocyte viability. That should be taken into account for future investigations with the goal to minimize the stress during the fabrication process resulting in higher cell viabilities.

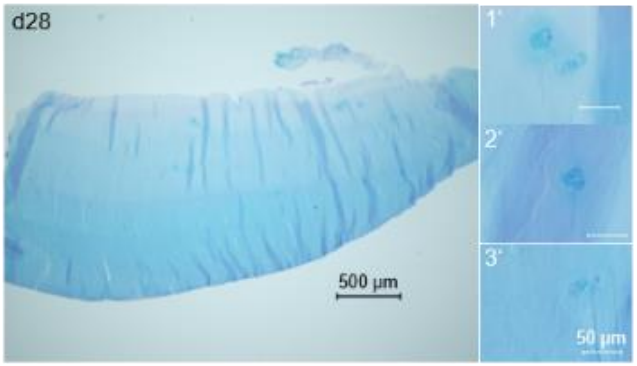

***Figure 5**. Alcian blue staining of glycosaminoglycan graded hydrogels 28 days after bioprinting. The increase in dye concentration over the cross-section indicates the increase in glycosaminoglycan concentration. Figure was adapted from [6].*

The positively charged dye alcian blue was used to stain the bioprinted, cell-containing hydrogels out of hydrogel precursor solutions 1'-3'.[6] This staining is commonly used to verify the presence of glycosaminoglycans, which are typical for cartilage ECM. In Figure 5 a hydrogel is shown which was cultivated under cell culture conditions for 28 days.

The alcian blue staining proofed the glycosaminoglycan gradient of the hydrogel, since an increasing intensity of blue staining from the top to the bottom could be observed. This was explained by the incorporation of negatively charged glycosaminoglycans, which lead to an increased negative charge in the formulations 2' and 3' compared to 1'.

Furthermore, a bright blue staining around the encapsulated chondrocytes was observed (see higher magnifications on the left in Figure 5). This staining indicated new ECM synthesis of the encapsulated porcine chondrocytes during the cultivation of 28 days within the hydrogels.

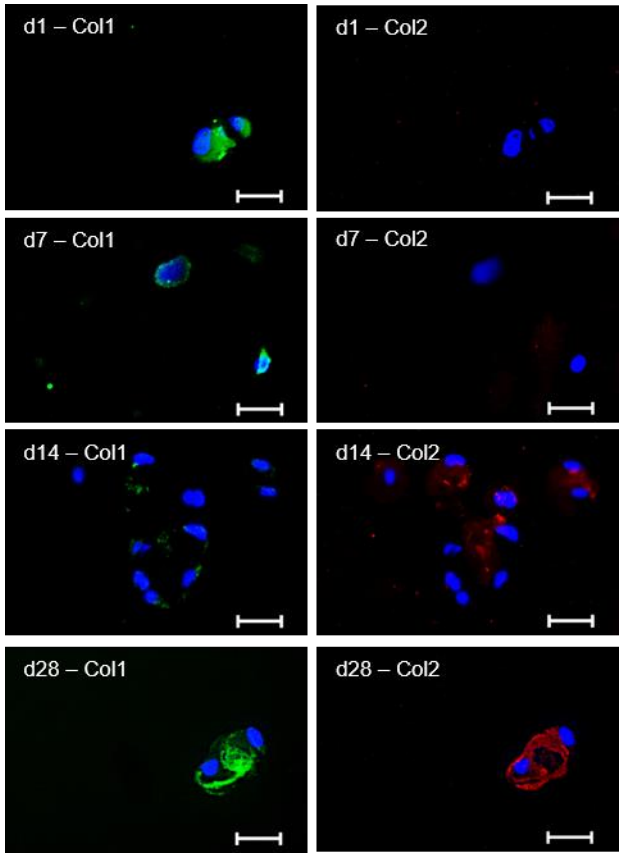

***Figure 6**. Antibody staining against collagen type I (Col1, left column, green) and collagen type II (Col2, right column, red) over cultivation duration (day 1 to day 28) of porcine chondrocytes encapsulated in bioprinted, biomimetic hydrogels. The scale bars represent 20 µm.*

To further characterize the newly synthesized ECM of the porcine chondrocytes within the biomimetic hydrogels, antibody stainings against collagen type I and collagen type II were performed (see Figure 6). Over the duration of cultivation, an increase in collagen type II was detected (see red staining in the right column, Figure 6). However, collagen type I was detectable over the investigated cultivation duration. Nevertheless, the alcian blue positive matrix around the encapsulated chondrocytes, which stains glycosaminoglycans, and the collagen type II staining after 28 days of cultivation suggest a redifferentiation of the chondrocytes. Since the used chondrocytes were dedifferentiated before 3D culture in the hydrogels for expansion, this shows that our biomimetic hydrogels are able to redifferentiate dedifferentiated chondrocytes.

Conclusions

We were able to show that it is possible to tune *EDS* and G'_{LVER} of hydrogels independently from each other by smart formulation of methacryl-acetyl-modified gelatin with different degrees of

methacryloylation and methacrylated glycosaminoglycans. These hydrogel precursor solutions were appropriate to bioprint thin layers. The printing process was adjusted so that on the one hand, all printed layers were stable and cross-linked and on the other hand the cross-linking conditions were cytocompatible. The yielded glycosaminoglycan graded hydrogels were stable over 28 days and encapsulated porcine chondrocytes synthesized were viable over this cultivation duration. Furthermore, the newly synthesized extracellular matrix of the encapsulated chondrocytes was rated as cartilage-specific, since it was stained with alcian blue and collagen type II was detectable. That suggested that the biomimetic hydrogel compositions were able to redifferentiate the prior dedifferentiated porcine chondrocytes. [6]

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Author Biography

Lisa Rebers is a PhD student at the University of Stuttgart. She graduated in Biomedical Engineering and is now investigating biomimetic hydrogels for cartilage tissue engineering in her PhD project.

Kirsten Borchers obtained her PhD at the University of Stuttgart in 2007 for her investigations on the use of biofunctionalized SiOx nanoparticles for microarray printing for protein detection. Since then she continued her scientific work with Fraunhofer IGB in Stuttgart were she established the bioprinting topic. Her focus is on development of bio-based materials and formulation of bioinks which can be used to fabricate bioartificial tissue equivalents and coatings that mimic the native tissue matrix.

Eva Hoch obtained her PhD at the University of Stuttgart in 2013. She investigated biopolymer-based hydrogel systems for inkjet printing. Afterwards, she continued as Post Doc at the University of Stuttgart and worked together with Kirsten Borchers in the field of printable bio-based hydrogels for cartilage tissue engineering. Since 2015 she is an Research Engineer at Robert Bosch GmbH.

Sandra Stier graduated in Biomedical Sciences. She did her master thesis at Fraunhofer IGB under the guidance of Kirsten Borchers and Eva Hoch. Since her graduation, she is working as a product specialist in E-xtra DESIGN ENGINEERING at JOTEC GmbH.

Veronika Schönhaar graduated in Applied Chemistry. Since then she is working as scientific employee at Fraunhofer IGB.

Achim Weber is group leader at Fraunhofer IGB since 2002. He obtained his PhD in 1999 at the University of Stuttgart in the field of Physical Chemistry. Since 2000, he has a Post Doc Position at Institute of Interfacial Process Engineering and Plasma Technology of the University of Stuttgart. With his group, he is working on printable materials and nanoparticles for controlled release applications.