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Bioink Development and Bioprinting Bio-based Matrices

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Introduction

The future vision of medical care comprises the generation of biological implants. Thus, formulations based on biological or biocompatible matrices with or without cells are needed for automated generation of tissue engineered products. Such so called bioinks have to match the technical requirements of the deposition processes, and at the same time fulfill the biological needs of the cells and mimic the properties of native tissue.

We introduce gelatin-based biomaterials for the manufacturing of flexible structures by freeform fabrication methods: printable non-gelling precursor solutions and crosslinked hydrogels with tunable physico-chemical properties constitute biomimetic matrices with adjustable properties for engineering of specific tissue models such as cartilage or bone. Currently, computer-controlled 3D manufacturing techniques are being successfully adapted for tissue engineering (TE) applications in order to enable sophisticated manufacturing of artificial tissue substitutes.[1] One approach called bioprinting particularly aims for the direct deposition of biological and biologically relevant materials, such as biomolecules and living cells, into spatial orientations and geometries.[2]

Development of bioinks based on double-modified gelatin

Generally, various techniques are being adapted for spatially defined transfer of cells including extrusion based techniques for cells within a viscous matrix or hydrogels, laser-induced transfer of single cells, or inkjet printing of cells in suspension.

Each technique requires suitable cell-loaded matrices, so called bioinks that match with the requirements of the process on the one hand, and meet the needs of the cells on the other hand.

We provide bioinks based on formulations of biomolecules from the native extracellular matrix. Gelatin, a hydrolysed form of collagen, is soluble in water at pH 7.4 and 37°C. Gelatin solutions form physical hydrogels at temperatures below their gel point. The gel point depends on the gelatin concentration and can be influenced by chemical modification of the gelatin molecules. This makes gelatin a versatile candidate for formulation of bioinks. Yet, it has to be chemically crosslinked to form a stable matrix at physiological pH and 37°C. Crosslinking of gelatin molecules can be achieved by modification of the biomolecule e.g. with methacrylic functions which can be radically crosslinked triggered by a light stimulus in presence of a photoinitiator (e.g Irgacue 2050)[3].

We introduced a well-controlled procedure to produce gelatin derivatives with defined degrees of modification either solely by radically crosslinkable methacrylic functions or by double functionalization with methacrylic functions and non-crosslinkable acetic residues.[4] Thereby we provide biobased matrix materials for bioprinting of cells that can be adapted in terms of solution viscosity to match different deposition techniques, and that additionally provide flexibility in the degree of crosslinking achieved during hydrogel formation.

Double chemical functionalization of gelatin by methacryloylation and acetylation of free amino and hydroxyl groups of the biomolecules enables control over both, the viscous behavior of its solutions and the mechanical properties of the resulting hydrogels after photochemical crosslinking. The total degree of modification determines the viscosity of the resulting solutions of the gelatin derivative. The ratio of coupled methacrylic functions to coupled acetic functions determine the crosslinking potential of the resulting gelatin derivative and thus the mechanical and rheological properties of the resulting crosslinked hydrogels.[5]

Double chemical modification of gelatin and determination of degree of modification

We used 2D NMR and ^{1}H-NMR spectra of gelatin derivatives, and sodium 3-trimethylsilyl-propionate-2,2,3,3-d4 (TMSP) as external standard to quantify the amounts of methacrylate functions (methacrylic acid coupling to free hydroxyl groups of the biomolecule), methacrylamide functions (methacrylic acid coupling to free amino groups of the biomolecule) and acetic functions.[4] Tenfold molar excess of MAAnh with respect to the nominal amount of free amino functions of gelatin [6] lead to gelatin derivatives with high degree of metharcyloylation (DM) (GM10), fivefold excess of MAAnh lead to gelatin derivatives with medium DM (GM5), twofold excess of MAAnh lead to derivatives with significantly lower DM (GM2) (Figure 1, left). Addition of acetic anhydride (AcAnh) using either twofold excess of MAAnh and eightfold excess of AcAnh (GM2A8) or fivefold excess of both (GM5A5) lead to the same DM as for GM2 and GM5, respectively, while the total degrees of modification (DMA) were equal to the DMA of GM10.

Viscosity of gelatin derivatives

The mean dynamic viscosity ($10s^{-1}$-$1000s^{-1}$) decreased in correlation with increasing DMA.GM2 solutions yielded 6.17 ± 2.20 mPa s, while GM10, GM2A8, and GM5A5 solutions yielded 2.97 ± 0.194 mPa s, 2.42 ± 0.205 mPa s, and 2.96 ± 0.666 mPa s, respectively. The differences in viscosities became even larger when higher concentrations were applied; ranging from 39.3 ± 3.6 mPa s and 110.2 ± 31.5 mPa for GM2, to 5.2 ± 0.5 mPa s and 9.0 ± 0.4 mPa s for GM2A8 at 15 % (w/w) and 20 % (w/w), respectively (Figure 1, right).

Gelatin derivative	DM	DA	DMA
GM2	0.338 ± 0.022		
GM2A8	0.379 ± 0.013	0.450 ± 0.022	0.858 ± 0.011
GM5	0.618 ± 0.032		
GM5A5	0.638 ± 0.023	0.263 ± 0.016	0.935 ± 0.047
GM10	0.958 ± 0.068		

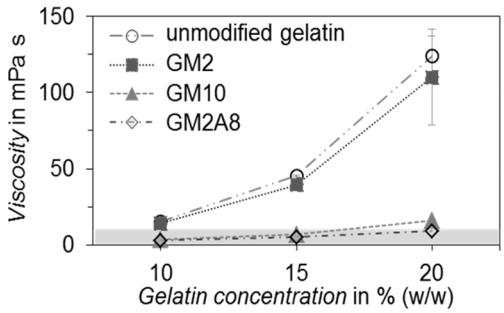

Figure 1 *Left: Degree of total methacyloylation (DM, sum of methacylate and methacrylamide functions), degree of acetylation (DA), and total degree of modification (DMA) for gelatin derivatives determined via ^{1}H-NMR spectroscopy. Values are presented in mmol g^{-1} as mean ± standard deviation of three independent synthesis batches. Adapted from [4]. Right: Viscosities of solutions of GM2, GM10 and GM2A8 compared to unmodified gelatin measured at 37°C. The lines are added for guidance of the eye only. 1 ≤n≤5. Adapted from [5].*

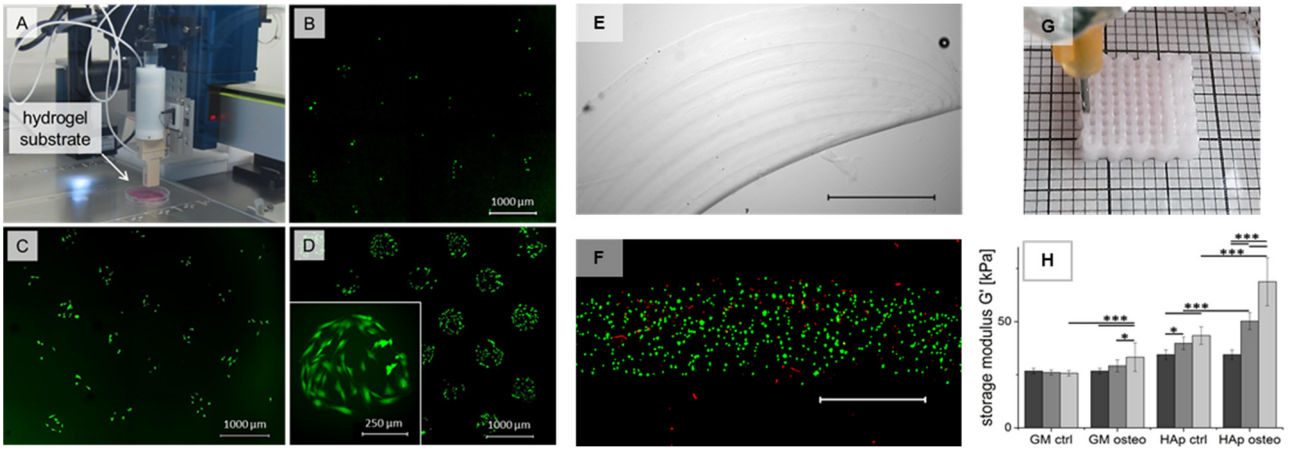

Figure 2 *Inkjet printing of porcine chondrocytes: (A) Cells suspended in 10 % (w/w) GM10 bioink (1x10^6 cells mL^{-1}) were printed onto hydrogel substrates and subsequently incubated in cell culture medium. FDA (live cells, displayed in green)/PI (dead cells, displayed in red) staining allowed for determination of cell viability of printed cells (B) 3 h, (C) 24 h and (D) 72 h after printing (taken from [5]); (E) layer-by-layer dispensing and UVA (385 nm) curing of bioink based on 10% (w/w) GM2A8 and GM5A5, and loaded with porcine chondrocytes (3.75x10^6 cells mL^{-1}; 16 µL ink per layer), (F) which survived dispensing and irradiation (submitted to Journal of Material Science: Materials for Medicine); (G) extrusion-printed grid structures (10 mm x 10 mm x 5 mm) based on cell-laden bioink with hydroxyapatite (HAp), (H) enhanced matrix production in HAp containing matrix indicated the biofunctional formulation of bioink (modified from [7]). Scale bars 1 mm if not stated otherwise.*

With these data we show, that by double functionalization of gelatin it was possible to achieve low solutions viscosities of gelatin derivatives independently of the degree of methacryloylation (DM) which defines the crosslinking potential of the modified gelatin. The upper viscosity limit required for inkjet printing is often reported to be approx. 10 mPa s. Double functionalization yielded solutions in the ink-jet printable range at low degree of methacryloylation (GM2A8) while this was not possible with pure methacryl-modification using comparable excess of MAAnh (GM2). Gelatin derivatives with low DM (GM2 and lower) show gel-like properties at e.g. 25°C and can then be used for extrusion based deposition.

Thus, by adjusting the degrees of methacryloylation and acetylation, bioink formulations with various viscosities and gelling behavior can be provided for different printing methods, independently from the crosslinking potential.

Inkjet printing of low viscous gelatin-based inks loaded with chondrocytes

With respect to the generation of cell-laden hydrogels by drop-on-demand inkjet bioprinting we undertook proof-of-principle trials by printing viable porcine chondrocytes suspended in GM(A) bioinks (Figure 2, A-D). Spot patterns were printed onto hydrogel substrates using 10 wt% GM10 or GM2A8 including 1 000 000 porcine chondrocytes per mL bioink, applying a piezoelectric inkjet printing system (Nanoplotter, GeSim mbH, Germany). Cell printing experiments were performed at 25 °C. Despite lower viscosity of bioinks at 37°C clogging of nozzles occurred more frequently probably due to elevated evaporation. Printed porcine chondrocytes adhered to the substrate, developed outstretched morphology as in typical 2D culture, and showed proliferation. The results indicate that the present printing system caused no significant mechanical damage to living chondrocytes and that the presented bioink is generally applicable for processing viable cells.[5]

Extrusion-based printing of low viscous inks chondrocytes with interim UV curing of bioinks

We also printed chondrocytes in bioinks composed of GM5A5, GM2A8, methacryl-modified chondroitin sulfate (CSM) and hyaluronic acid HAM) (11.6 %-13.0 % (w/w) in sum) by extrusion-based dispensing in layer-by-layer constructs using a prototype table-top robot (TR300; Unitechnologies SA, Switzerland) (Figure 2 E,F). The ink contained lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) as photoinitiator for photoinduced radical crosslinking and hydrogels were stable if each layer was irradiated for 15 s (385 nm, 8.6 mW cm^{-2}) after printing. The top layer was lost, probably due to impaired radical crosslinking oxygen.

Cell survival after 24 h of culture within printed hydrogels indicated that bioink formulations, extrusion based printing, and curing were applicable for assembly of artificial tissue.

Extrusion-based printing of mesenchymal stem cells in hydroxyapatite containing inks

We modified formulations of GM2, GM5 and HAM (7wt%, 5 wt% and 1 wt%, respectively) [7] with hydroxyapatite (HAp) particles and mesenchymal stemcells, representing the composite of natural bone. The bioinks were printed to form free standing containing grids (Figure 2, G). The storage modulus G' of the hydrogels on day 1 was 26.8 ± 1.27 kPa for GM-gels without HAp, and 34.5 ± 2.28 kPa for HAp-containing gels. After 28 days the mechanical properties of the GM-gels with cells cultivated under control conditions remained nearly constant, while HAp-containing gels showed significantly increased values for G' after cultivation under control conditions (43.5 ± 4.13 kPa), and even more pronounced increase under osteogenic conditions (68.8 ± 1.13 kPa) (Figure 2, H). The respective increase of 26% or 99% showed that the HAp containing bioink significantly promoted matrix production and osteogenic differentiation.[7]

Conclusion

We developed procedures for production and chemical analysis of methacryl-modified and double-modified gelatin and provide materials with high specifications concerning the degree of modification. This new generation of modified gelatins provides the base for improved gelatin processing, new formulations, and development of biobased assays and biofunctional products.

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Keywords

Methacryl modified biomolecules, bioinks, tissue engineering

Biography

Kirsten Borchers obtained her PhD degree at the University of Stuttgart in 2007 for her investigations on the use of biofunctionalized SiOx nanoparticles for printing of microarrays for protein detection. Since then she continued her scientific work with Fraunhofer in Stuttgart were she established the bioprinting topic within the interdisciplinary environment of the Fraunhofer-Institute for Interfacial Engineering and Biotechnology IGB. She is engaged in biomaterials development with regard to applications with additive manufacturing techniques. Her focus is on bio-based materials and formulation of bioinks which can be used to fabricate bioartificial tissue equivalents and coatings that mimic the native tissue matrix.

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