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Dispensing of hydrogel ink onto electrospun biodegradable paper for biomedical applications

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Abstract

In this contribution we present the development of a biobased hybrid material made from a mechanically stable, paper-like fleece combined with a hydrogel, which mimics the extracellular matrix (ECM) of soft tissue. We used methacryl-modified gelatin as described earlier for the formulation of dispensable hydrogel precursor-solutions (bioinks). By variation of the degree of modification we provided viscous liquid inks and physically gelling inks. After deposition, such bioinks could be chemically crosslinkend by UVB irradiation to form non-soluble hydrogels. We produced cell-free hydrogels as well as hydrogels loaded with human fibroblasts from skin biopsies. Freeform fabrication of thin hydrogel-coatings or of three-dimensional (3D) hydrogel constructs was achieved by application of digital 3D printing (bioprinting). For stabilization of the hydrogels we used fleece substrates produced from the biodegradable polymer poly (L-lactide) (PLA) and the modified biopolymer gelatin by electrospinning. The presence of the biomolecule component in the fleece added a more hydrophilic character to the PLA non-wovens, which was crucial for stable bonding of hydrogel and fleece.

Introduction

The benefits of digital direct printing methods are increasingly transferred to the processing of new functional materials such as cytocompatible plastics and biofunctional inks. This inspires and supports new developments in terms of printable and crosslinkable biobased materials (bioinks). Simultaneously, additive manufacturing techniques such as printing, plotting, laser-based curing or electrospinning are being adjusted to the processing of biological materials and cells, three-dimensional tissue engineering, and fabrication of biological implants [1, 2].

The use of printing robots within the operation theatre is still a vision today. But the translation of bioprinting techniques to clinical applications such as in situ assembly of biological implants or wound-covers in a patient-specific manner may be part of medical care in future.

Hydrogels are non-soluble polymer networks based on hydrophilic synthetic polymers or on biopolymers that contain a high amount of water. Hydrogel wound covers based on synthetic polymers such as polyethylene glycol (PEG) or glycerin, and seaweed-derived alginate are used to provide a moist wound environment and to absorb wound excudate, respectively. They relief pain by cooling, facilitate exchange of dressing, and assist in autolytic debridement. On the other hand, collagen, which is the most abundant component of the natural human tissue matrix, is applied to form hydrogels in contact with wound excudate. Collagen gels remain within the wound, are degraded by natural enzymatic processes, and are assumed to attract skin cells [3].

Collagens and other biopolymers from the extracellular matrix (ECM) of native soft tissue such as gelatin, hyaluronic acid, chondroitin sulphate are also applied as scaffold material for cells in engineering of bioartificial tissue [4].Such biobased hydrogels have the potential to be used as a scaffold to administer cells or biopharmaceuticals e. g. to wound sites: Delivering of additional cells to wounds is expected to support the reconstruction of new skin [5]. Skin cells, including cells from blood capillaries, fatty cells and stem cells can be harvested from small biopsates from the patient´s healthy skin and expanded *in vitro* if necessary. Subsequently, they can be encapsulated into a hydrogel matrix resembling the ECM of native tissue, and then be delivered back to the patient.

Currently, first applications of matrix assisted cell administration using chondrocytes and collagens (matrix-induced autologous chondrocyte implant) are in the course of clinical validation (Vericel Corporation, USA, (former Aastrom Biosciences), MACI™).

To date the therapeutic use of cells is still very much in its infancy and regulatory issues have to be considered in order to develop a broad range of secure treatments [5, 6]. Once cell based therapies become mature to be transferred to the clinics, 3D bioprinting of cells and matrices can help to reconstruct tissue analogues in a patient specific geometry.

Our objective is to provide materials for individualized patient care including biological, biobased and biodegradable components that are tailored to be administered by automated and digitally addressed liquid handling techniques such as bioprinting. We aim at the development of bio-based materials with tunable properties for combination of biofabrication techniques in the biomedical field. Therefore we develop various kinds of modification of biomolecules in order to tailor their viscosity and their gelling behavior, to achieve stimulus-initiated chemical crosslinking, and to control the physico-chemical properties of the materials after crosslinking. We focus on the use of biobased hydrogels, which mimic the ECM of soft tissue and can be used for encapsulation of cells or drug-release. The hydrogels precursor materials we present here are based on methacryl-modified photo-crosslinkable gelatin [7]. Layerwise crosslinking of the hydrogel precursor inks and combination of different matrix materials and cells will enable the delivery of different cell types in a spatially ordered manner.

We used biodegradable electrospun fleece materials which can serve as stable substrates and facilitate hydrogel handling and fixation. Non-woven materials produced by electrospinning are increasingly applied in tissue engineering applications due to their fibrous structure in the nanometer scale similar to e.g. collagen fibers [8, 9]. They are known to provide higher stability than hydrogels.

Materials and Methods

Synthesis of photo-crosslinkable biopolymers

Photo-crosslinkable derivatives of the ECM biopolymers gelatin (Gelita AG, Germany) and sodium hyaluronate (research grade, 41KDa-65KDa, Lifecore Biomedicals LLC, USA) were prepared by derivatization with methacrylic anhydride (MAAnh; Sigma-Aldrich, Germany) as described elsewhere [6-8]. Methacryl-modified gelatin (GM) was prepared with three different degrees of methacryl-functionalization by varying the amount of added MAAnh to give a two¬fold (GM_2, lots SynGM22 and SynGM29), fivefold (GM_5, lot SynGM25), or tenfold molar excess (GM_{10}, 22.08.2014) with respect to free amino groups of gelatin [10]. Alternatively, twofold modification was performed using fivefold molar excess of MAAnh and additionally fivefold molar excess of acetic acid anhydride (AcAnh) to achieve masking of amino acid sidechains for low viscous gelatine solutions without increasing the crosslinking potential as compared to GM_5 [7]. Methacryl-modified hyaluronic acid (HAM) was prepared using a fivefold (HAM_5) molar excess of MAAnh with respect to free hydroxyl groups of unmodified HA. The amount of methacryl-function per gram biopolymer was determined by 1H NMR spectroscopy according to [11].

Preparation of hydrogel precursor inks

Solutions of methacryl-modified biopolymers were prepared and formulated to achieve low viscous bioinks and physically gelling bioinks for extrusion based dispensing. For low viscous inks GM_5 or GM_{10} (10 wt%) were dissolved in phosphate buffered saline (PBS, 0.01M, pH 7) at 37 °C. For physically gelling inks blends of GM_2 and HAM_5 were used. Stock solutions of HAM_5 (5 wt%) in PBS were prepared and added to GM_2 solutions to achieve GM_2 (10 wt%)/HAM_5(0.2 wt%) inks. To prepare photo-crosslinkable inks, stock solutions (0.7 wt%) of the photoinitiator were prepared and added to achieve final concentrations of 0.135 % to 0.4 % (w/w relating to biopolymer mass), depending on the sensitivity of cells. As cytocompatible photoinitiators 2-hydroxy-4'-hydroxyethoxy-2-methylpropiophenone (Irgacure 2959 (I2959), kind gift from Bodo Moeller Chemie GmbH,Germany) or phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, synthesis according [12]) were applied.

Rheological characterization of hydrogel precursor inks

A rotational rheometer with concentric cylinder system (Physica MCR 301, Anton Paar, Germany) was used for viscosity measurements of low viscous, non-gelling inks. The dynamic viscosity of GM_{10} inks was determined at 37°C at shear rates in the range of 1 s^{-1} to 400 s^{-1}.
Measurements of storage modulus G` and loss modulus G`` of physically gelling ink were performed with a cone-plate system in oscillating mode to characterize the sensitive physical hydrogel inks: GM_2(10 wt%)/HAM_5(0.2 wt%) gels were applied to the rheometer at 40°C in a liquid state, cooled down to 10°C and then warmed up to 20°C at 0.9°K /10 s. After 90 min of equilibration at an oscillating frequency of 2 Hz and 1% amplitude, G` and G`` were determined at 2 Hz / 1% amplitude (low shear stress) and 2 Hz / 500 % amplitude (elavated shear stress) in turns.

Hydrogel bioplotting

For manual investigations of ink processability standard syringes and cannulas (luer lock, 0,33 µm, Vieweg GmbH, Germany) were used. For automated printing of the hydrogel precursor inks GM_5 (10 wt%) and GM_2 (10 wt%)/HAM_5(0.2 wt%) the table-top extrusion plotter System 30 (Hyrel 3D, USA) was applied. For the generation of printing patterns, slicing and the generation of g-code FreeCAD 0.15 and Slic3r 1.2.9 freeware was used. Dispensing of cell-laden bioink (GM_5 (10 wt%)) with primary human fibroblasts from skin (300.000 mL^{-1}) was performed using a table-top robot for pneumatic dispensing (TR300, Unitechnologies, CH). The choice of printing systems was due to the close proximity of the TR300 to the cell laboratories. Both printers were equipped with the same cannula as used for manual experiments (luer lock, 0,33 µm, Vieweg GmbH, Germany).

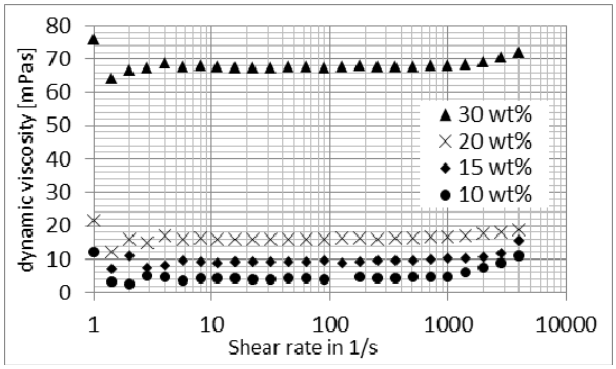
Cell staining

After printing the hydrogels were crosslinked by UVB irradiation (365 nm, 8.5 % mW cm^{-2}, 2 min) and were then incubated in DMEM medium with fetal calw serum (FCS, 10%) and Penicillin/Streptomycin (1%) in a humid atmosphere of 5 % CO_2 in air at 37 °C for 24 h. Subsequently, cell viability was monitored by fluorescence based live/dead staining with fluorescein diacetate (FDA, Sigma Aldrich, Germany) and propidium iodid (PI, Sigma Aldrich, Germany) (10 µL of 5 µg mL^{-1} FDA in acetone, 10 µL of 0.5 µg mL^{-1} PI in 980 µL PBS).

Results and Discussion

Viscoelastic behavior of hydrogel precursor inks

Solutions (10 wt%) of gelatin with high degrees of modification (GM_5, GM_{10}, GM_5A_5) are viscous liquids that do not show physical gelling at 20°C due to the masking of functional groups of the amino acid side chains. In the contrary, solutions (10 wt%) of gelatin with low degrees of modification (GM_2) and combined bioinks (GM_2 (10 wt%)/HAM_5(0,2 wt%)) form physical gels at 20°C.



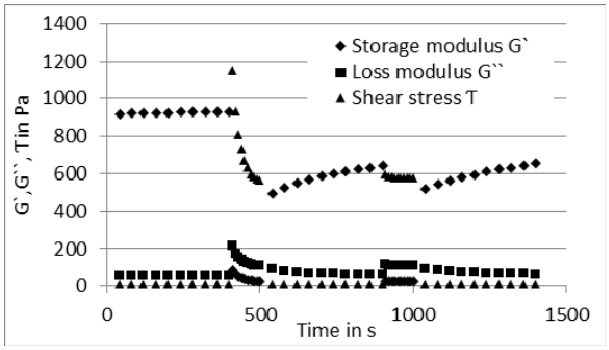


Figure 1 *Top: Viscosities of GM_{10} inks were measured by rotational viscosimetry. Bottom: Viscoelastic behavior of the physically gelling GM_2/HAM_5 inks characterized by alternately applying phases of elevated shear stress (shear rate maximum 62.8 s^{-1}) and phases for relaxation at low shear stress (shear rate maximum 0.162 s^{-1}) in order to simulate repeated ink dispensing and ink stopping during printing. The storage*

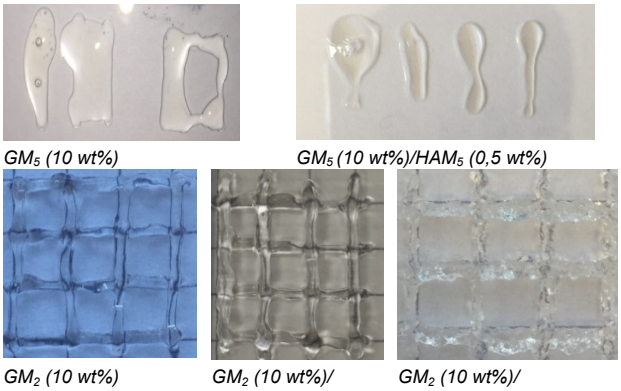
modulus G` and the loss modulus G`` were determined together with the resulting shear stress.

The viscoelastic behavior of the soluble gelatin is characterized by the solution viscosity. Viscosity measurements of GM_{10} (10 wt%-30 wt%) solutions revealed Newtonian fluid behavior at low shear rates up to approximately 1000 s^{-1} and slightly shear thickening behavior at higher shear rates up to 4000 s^{-1} ***(Figure 1, top)*** [13]. The increase in viscosity is assumed to be due to stretching and elongation and thus entanglement of the biopolymers upon shear stress [14].

For rheological characterization of gelling inks the storage modulus G` and loss modulus G`` of the materials were measured in an alternating stress-release mode. The physical gels were probed in oscillating shear mode at 2 Hz, 1 % amplitude (low shear stress for equilibration and relaxation) for 400 s and subsequently at 2 Hz, 500 % amplitude (elavated shear stress for simulation of dispensing) for 100 s. This set up was chosen to simulate the repeated shearing during the dispensing process (***Figure 1, bottom)***. At low shear stress the storage modulus G` exceeded the loss modulus G`` which is the characteristic behavior of a gel *per definitionem* [15]. During increased shear stress the loss modulus G`` was higher then G` indicating that during shearing the viscous behaviour of the ink became predominant.The decrease in shear stress during the first application of shear and the reduced storage modulus after the first application of shear showed that part of the structural integrity of the gel was destroyed by shearing. Yet, repeated recovery of the storage modulus to the reduced level indicated that the physical gel repeatedly formed when the shearing stopped.

Dispensing of hydrogel precursor inks

The dispensing of hydrogel precursor inks based on crosslinkable biopolymers from the native ECM was first probed manually. We compared solutions (10wt%) of GM_5 and GM_2 and gradually added HAM_5 as additional ECM component. Inks based on gelatine with high degrees of modification (GM5, GM10) were non-gelling viscous solutions, that were rather forming films than defined threedimensional filaments. Gelatine solutions with low degree of modification (GM_2) formed stable gel filaments upon dispensing (***Figure 2***).
Addition of small amounts of methacryl-modified sodium hyaluronan (HAM_5) resulted in stronger gels producing less smooth filaments then GM_2 alone or solutions with sligthly increased viscosity then GM_5 alone, but did not change the overall gelling behaviour of the inks.



GM_5 (10 wt%) *GM_5 (10 wt%)/HAM_5 (0,5 wt%)*

GM_2 (10 wt%) *GM_2 (10 wt%)/ HAM_5 (0,2 wt%)* *GM_2 (10 wt%)/ HAM_5 (0,5 wt%)*

Figure 2 *Manually dispensed hydrogel precursor inks based on methacryl-modified gelatin with high degree of modification (GM_5) and low degree of modification (GM_2). Addition of methacryl-modified hyaluronan (HAM_5) did not change the gelling behavior of the inks in general, but resulted in increase of viscosity in the case of non-gelling GM_5 solutions and produced less smooth gel filaments in the case of the physically gelling GM_2 inks.*

Combination of electrospun fleeces and hydrogel bioinks

In order to provide a stable support to the hydrogel materials we used electrospun fleeces that were provided from the Eberhard Karls University Tuebingen, as a substrate to print on. Wetting of the electrospun fibers by the hydrogel precursor solutions was crucial in order to enable stable bonding of the fleece substrate and the hydrogel coatings. While hydrogel precursors showed dewetting behavior on hydrophobic pure PLA fleeces, stable adhesion was achieved when we used the combined PLA/GM_2 fleece materials (***Figure 3, top)***.

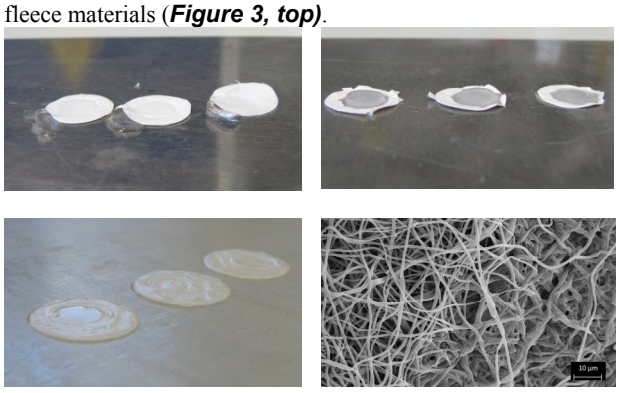


Figure 3 *Hybrid materials composed of electrospun fleeces and hydrogel coatings. Top: The fleece material had to be sufficiently hydrophilic to enable wetting by the hydrogel precursor inks. The left photograph shows that hydrogel precursor ink (HAM_5) dewetted from hydrophobic PLA fleece. The right photograph shows the same ink adhering to more hydrophilic fleeces fabricated from PLA/GM_2. Bottom left: After UV crosslinking of the printed hydrogel precursor ink, the fleece-hydrogel hybrid materials remained stable. The photograph shows hydrogels on fleeces after 24 h incubation in PBS at 37°C.Bottomright: SEM analysis of hydrogel-fleece hybrid materials. The left half shows uncoated fleece, the right half shows fleece infiltrated with GM_2.*

The gelling behavior affected the dispensing process and thus the choice of the printing parameters, as well as the printing outcome. Viscous solutions of highly modified biomolecules (GM_5, GM_{10}) formed thin layers or were soaked into the hydrophilic and porous fleece materials. Small layer distances d of approximately 0. 1 mm were chosen for dispensing. Increased layer distances of approximately 0.13 mm were used on physically gelling materials (GM_2). This ink formed stable three-dimensional (3D) filaments, that could be stacked on top of each other to form 3D hydrogels in the course of several minutes. Printing onto PLA/GM blends worked well for both ink formulations. Low viscous GM_{10} inks were used for the impregnation of the fleece materials and formation of a thin hydrogel coating. GM_2/HAM_5 ink was used to prepare 3D hydrogel constructs of several millimeters in height (***Figure 4***). In both cases the fleece substrate was crucial for mechanical stability and the ability to handle the hydrogels. Figure

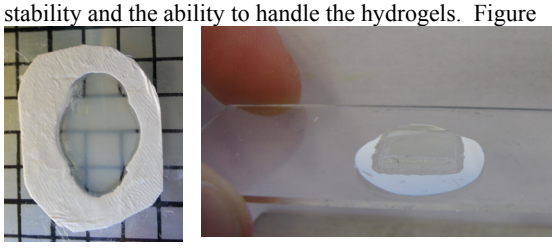


Figure 4 *Hydrogels printed onto fleece substrates. Left: low viscous bioink infiltrated the fleece substrates, right: printing physical gels resulted in 3D hydrogels of customized heights.*

Printing of cells

The bio-based hydrogel precursor inks have also been applied to print primary human fibroblastsderived onto PLA/GM_2 fleece substrate, have been photo-crosslinked in the presence of the cells. It has been proofed before that LAP [12] and I2959 [16] photoinitiators have been cytocompatible at cell type-specific concentrations and UVB irradiation (385 nm). In this case we used GM_5 (10 wt%) and 0,135% LAP (w/w to biopolymer mass). Photo-initiated crosslinking of the methacryl-modified biopolymers resulted in stable hydrogels and stable bonding of hydrogels and fleece. After 24h of incubation in DMEM medium, a live-dead staining was performed and revealed living fibroblasts well distributed within the hydrogel matrix (***Figure 5***).

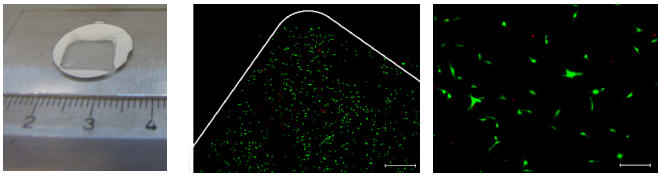


Figure 5 *Cell-laden hydrogel-fleece hybrid constructs. The liquid precursor ink including human primary fibroblasts was printed onto the fleece substrate and crosslinked by UV irradiation to form a stable hydrogel with the fibroblasts encapsulated. The cells were alive and spread out within the gel after 24 h incubation in cell culture media. Scale bars are 1 mm (center) and 200 µm (right).*

Conclusion

Methacryl-modified biopolymers can be used for the encapsulation of cells in biomimetic hydrogel matrices. The hydrogel precursor solutions can be tuned to form low viscous or physically gelling solutions. This opens the opportunity to apply the materials as bioinks for various options of digital production techniques such as 3D printing. Electrospun substrates composed of biodegradable PLA and hydrophilic methacryl-modified gelatin are versatile substrates to stabilize the hydrogels. Biocompatible, biodegradabe and biobased materials like the hybrid constructs presented here seem to be predestined as base for future therapies that can support the reconstruction of native tissue by providing to the patients their own expanded cells together with stabilized bioartificial extracellular matrix. With biobased and biodegradable materials we aim to contribute to the development of cell-free or cell-laden biological implants for the future [7, 10, 17]. Careful validation of materials and processes will now be the prerequisit to achieve new therapies for the future.

Author Biography

Sandra Stier *studied Pharmaceutical Biotechnology at the University of Applied Sciences Biberach and achieved her Master's Degree in Biomedical Sciences from the University of Applied Sciences Albstadt-Sigmaringen in 2015.*

Achim Weber*, studied Chemistry at the University of Stuttgart. In 2000 he joined the Fraunhofer Institute of Interfacial Engineering & Biotechnology (IGB) as a scientist and project manager, and the Institute for Interfacial Engineering (IGVT) at the University of Stuttgart. In 2006 he became a Group Manager and since 2011 he is Deputy Head of the Department of Interfacial Engineering and Material Science at the Fraunhofer IGB. His main interest is the forming and understanding of smart, nanoscopic materials and its surfaces for applications in Pharmacy, Medicine, Environment, Material and Biotechnology.*

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 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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