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A versatile bioink for three-dimensional printing of cellular scaffolds based on thermally and photo-triggered tandem gelation

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ABSTRACT

Layer-by-layer bioprinting is a logical choice for the fabrication of stratified tissues like articular cartilage. Printing of viable organ replacements, however, is dependent on bioinks with appropriate rheological and cytocompatible properties. In cartilage engineering, photocrosslinkable glycosaminoglycan-based hydrogels are chondrogenic, but alone have generally poor printing properties. By blending the thermoresponsive polymer poly(*N*-isopropylacrylamide) grafted hyaluronan (HA-pNIPAAM) with methacrylated hyaluronan (HAMA), high-resolution scaffolds with good viability were printed. HA-pNIPAAM provided fast gelation and immediate post-printing structural fidelity, while HAMA ensured long-term mechanical stability upon photocrosslinking. The bioink was evaluated for rheological properties, swelling behavior, printability and biocompatibility of encapsulated bovine chondrocytes. Elution of HA-pNIPAAM from the scaffold was necessary to obtain good viability. HA-pNIPAAM can therefore be used to support extrusion of a range of biopolymers which undergo tandem gelation, thereby facilitating the printing of cell-laden, stratified cartilage constructs with zonally varying composition and stiffness.

1. Introduction

Healthy articular cartilage allows almost frictionless movement of the synovial joints. Focal lesions in the tissue resulting from high impact mechanical loading from sports injuries or trauma are not repaired spontaneously due to the limited healing capabilities of cartilage and may progress to include degeneration of the surrounding tissue [1,2]. Hence, surgical interventions are necessary to repair damage of the articular cartilage surface. One clinical treatment for large cartilage defects is osteochondral transplantation or mosaicplasty, where the cartilage lesion is filled with an array of tightly packed cylinders of osteochondral grafts. Although this technique may allow increased weight-bearing [3,4], it is hampered by several disadvantages, including scarcity of tissue, donor site morbidity (for autologous grafts), potential transfer of disease (for allogenic grafts) and poor integration with the host tissue and between cylinders. Another main disadvantage is the unavoidable mismatch in thickness and curvature between the donor cylinders and implantation site. The fabrication of individual cell-laden osteochondral grafts whose geometry is obtained from magnetic resonance imaging data would have enormous advantages over the current clinical option.

Bioprinting is a natural fabrication method given the stratified nature of articular cartilage, and could improve the functionality of tissue engineered scaffolds by allowing the placement of cells [5–7], biomaterials [8–10] and bioactive cues [11–13] in three-dimensional (3-D) space to mimic the native tissue. However, the search for bioinks for use in tissue printing applications [14,15] which have both good biocompatibility and tailored viscosity transitions for effective printing and handling continues [16,17]. For an extrusion-based process, the bioink should show shear thinning to allow extrusion through a needle and also immediate cessation of flow upon deposition on the substrate to retain its plotted shape. Such a material would allow the fabrication of complex structures with high resolution [18,19]. The final mechanical properties of the printed construct should also be sufficient for manipulation. The different stages of a biofabrication process and the viscosity requirements for an ideal bioink are shown in Fig. 1b. Bioinks such as alginate-gelatin blends [20,21] or pure alginate [20] have been used to generate 3-D scaffold structures. These materials have the advantage of good biocompatibility and cell-friendly ionic/thermal

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crosslinking without further chemical modifications of the biopolymers. However, these materials are usually soft and their mechanical properties decrease over time due to the reversibility of the non-covalent crosslink. It has been reported that the initial modulus of alginate-gelatin (10 kPa) scaffolds decreased over 60% in 7 days in culture [20]. Due to the limitation of these materials, interest in covalently crosslinked hydrogels has been increasing. Bioinks comprising polymers modified with (meth-)acrylates or diacetate have been extensively studied for biofabrication purposes. These modifications have been conjugated to both natural and synthetic polymers to create methacrylated gelatin (GelMA) [18,22] and acrylated versions of polymers such as poloxamer [23] and poly(ethylene glycol) [24]. The biggest advantage of the GelMA based bioinks is the good biocompatibility and improved mechanical properties with a compression modulus of ~180 kPa after 30 min of UV exposure [22]. For a more extensive review on the subject, the reader is referred to the recent review from Malda et al. [25].

As natural components of articular cartilage, hyaluronan (HA) and chondroitin sulfate are logical material choices for cartilage tissue engineering [26–29]. Both are used clinically as injections or food supplements and have purported anti-inflammatory [30–32] and/or immunomodulatory effects [33]. Bioprinting of glycosaminoglycan-based hydrogels, however, is challenging as the precursor solutions of these materials often have low viscosity and slow gelation kinetics, which result in flow before gelation. The goal of this study was to investigate the possibility of utilizing natural cartilage components HA and chondroitin sulfate in bioprinting. We hypothesized that a fast, reversible gelling component could be added to methacrylated HA and chondroitin sulfate to enhance their printing. Poly(*N*-isopropylacrylamide) (pNIPAAM) conjugated to HA was chosen as a thermoresponsive polymer as good cytocompatibility has been reported with nucleus pulposus cells [34], mesenchymal stromal cells [35] and a fibroblast cell line [36]. By grafting pNIPAAM onto the HA backbone (Fig. 1c), thermoresponsive hydrogels were created that are liquid at room temperature and gel at body temperature [37,38]. The basis of this gelation is the lower critical solution temperature (LCST) of pNIPAAM, which is ~32 °C [39]. The liquid state allows easy loading of the cartridges of the bioprinter and simplifies the mixing of cells, additional polymers and growth factors, whereas rapid gelation upon deposition onto a heated substrate ensured the maintenance of the shape of the printed 3-D structure until the further crosslinking (Fig. 2). After stabilization of the biopolymer with free radical polymerization of the methacrylated hyaluronan (HAMA), the HA-pNIPAAM can be eluted leaving a glycosaminoglycan-based scaffold (Fig. 2).

2. Materials and methods

2.1. Materials

2,2-Azobisisobutyronitrile (AIBN), *N*-isopropylacrylamide (NIPAAM) monomer, *N*,*N*-dimethylformamide (DMF), 2-aminoethanethiol hydrochloride, diethyl ether, Dowex Resin M-31, tetrabutylammonium hydroxide (TBA-OH), dimethyl sulfoxide (DMSO), carbonyl diimidazole (CDI), sodium bromide (NaBr), pronase, collagenase, methacrylic anhydride, L-ascorbic acid and sodium hydroxide (NaOH) were all purchased from Sigma Aldrich (Buchs, Switzerland). Chondroitin sulfate (CS, DS_S (degree of substitution (sulfate groups)) = 0.9, M_W = 20 kDa, polydispersion index M_w/M_n = 1.55) was purchased from Kraeber (Ellerbek, Germany). Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was prepared following the procedure reported by Fairbanks et al. [40]. Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), antibiotic-antimycotic, Live/Dead Assay and trypsin/EDTA were all purchased from Life Technologies (Zug, Switzerland). High-molecular-weight HA sodium salt (HANa, 1506 kDa, M_w/M_n = 1.53) and low-molecular-weight HANa (293 kDa, M_w/M_n = 1.86) were obtained from Contipro Biotech s.r.o. (Dolni Dobrouc, Czech Republic). All dialysis membranes used were from SpectrumLabs (Breda, Netherlands) and the 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (MTS) assay was from Promega (Madison, USA). Methanesulfonic acid was obtained from Fluka (Buchs, Switzerland). Alginate (ProNova UP-LVG) was purchased from NovaMatrix (Sandvika, Norway).

All concentrations are given in weight/volume percentages (% w/v) unless indicated otherwise.

2.2. Synthesis of polymers

2.2.1. pNIPAAM-NH2 synthesis

Amino-terminated pNIPAAM (pNIPAAM-NH2) was synthesized by radical polymerization using AIBN as a radical initiator [41]. Briefly, NIPAAM monomer (1.13 g) was dissolved in degassed DMF together with AIBN (1.6 mg) and 2-aminoethanethiol hydrochloride (11.4 mg). The polymerization was carried out at 70 °C for 6 h under argon atmosphere. The reactant was precipitated in an excess of diethyl ether and dried under vacuum at room temperature. The precipitate was dissolved in ultrapure water and dialyzed for 48 h (Spectra/Por 7; molecular weight cutoff (MWCO), 8000 Da) in ultrapure water. The dialyzed product was then lyophilized, characterized and stored until further use.

The molecular weight of the synthesized pNIPAAM was determined using a multi-detector chromatographic system (GPCV 2000, Waters, Milford, USA) equipped with three online detectors: a differential viscometer, a differential refractometer and a multi-angle light scattering (MALS) Dawn DSP-F photometer (Wyatt, Santa Barbara, USA). The mobile phase was DMF + 0.1 M LiCl with a flow rate of 0.8 ml min^{-1}, 50 °C and an injection volume of 218.5 µl. The sample concentration was 2 mg ml^{-1}. Evaluation of the molecular weight of the synthesized polymer was 24 kDa for the pNIPAAM.

2.2.2. HA-pNIPAAM synthesis

15 g of HANa was dissolved in 1.1 l of ultrapure water under constant stirring. Dowex Resin M-31 (120 g) was washed with ultrapure water until the washing solutions were clear and further soaked overnight in ultrapure water. Water was then removed from the resin and covered with 70 g of a 40% TBA-OH water solution. The solution with the resin was placed on an orbital shaker for 1 h, after which the supernatant was removed and replaced with 80 g of fresh TBA-OH solution and placed overnight on an orbital shaker. After that, the resin was rinsed with large amounts of ultrapure water until the flow through was clear and the pH was below 10. The resin was then moved into a gooch filter (No. 2) and water was removed without drying out the resin. The filter containing the resin was then filled with the HANa solution. The solution was left to flow through the resin and finally collected and frozen at −20 °C. The product was freeze-dried and subsequently further dried at 42 °C under vacuum.

2 g of HA-TBA was then dissolved in 160 ml of DMSO. pNIPAAM-NH2 (3.5 g) was dissolved in 40 ml of DMSO. First, 230 µl of methanesulfonic acid and then 540 mg of CDI were added to the dissolved HA-TBA and stirred at 42 °C for 1 h. After that, the pNIPAAM-NH2 solution was added and the mixture was cooled down to room temperature and the reaction was let to proceed for 48 h under constant stirring. Then 20 ml of saturated NaBr solution was added dropwise until the reaction mixture turned cloudy and stirred for another 2 h at room temperature. The solution was then transferred into dialysis tubing (Spectra/Por 6 RC, MWCO 50 kDa) and dialyzed against cold (<13 °C) tap water, which was changed every 30 min within the first 4 h. The dialysis was contin-