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A thermo-responsive and photo-polymerizable chondroitin sulfate-based hydrogel for 3D printing applications

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ABSTRACT

The aim of this study was to design a hydrogel system based on methacrylated chondroitin sulfate (CSMA) and a thermo-sensitive poly(*N*-(2-hydroxypropyl) methacrylamide-mono/dilactate)-polyethylene glycol triblock copolymer ($M_{15}P_{10}$) as a suitable material for additive manufacturing of scaffolds. CSMA was synthesized by reaction of chondroitin sulfate with glycidyl methacrylate (GMA) in dimethylsulfoxide at 50 °C and its degree of methacrylation was tunable up to 48.5%, by changing reaction time and GMA feed. Unlike polymer solutions composed of CSMA alone (20% w/w), mixtures based on 2% w/w of CSMA and 18% of $M_{15}P_{10}$ showed strain-softening, thermo-sensitive and shear-thinning properties more pronounced than those found for polymer solutions based on $M_{15}P_{10}$ alone. Additionally, they displayed a yield stress of 19.2 ± 7.0 Pa. The 3D printing of this hydrogel resulted in the generation of constructs with tailorable porosity and good handling properties. Finally, embedded chondrogenic cells remained viable and proliferating over a culture period of 6 days. The hydrogel described herein represents a promising biomaterial for cartilage 3D printing applications.

1. Introduction

Tissue-engineered constructs are currently under investigation for the regeneration of several types of tissue, including bony, cartilaginous and vascular tissues. A tissue engineering (TE) approach is of particular relevance for damaged tissues that have a poor capability to regenerate spontaneously, such as articular cartilage defects of critical sizes. Biomimetic hydrogels composed of naturally occurring polysaccharides, *e.g.* chondroitin sulfate (CS) and hyaluronic acid (HA) have already shown significant chondrogenic potential for encapsulated chondrocytes and mesenchymal stem cells (Chung, Beecham, Mauck, & Burdick, 2009; Erickson et al., 2009; Hu, Li, Zhou, & Gao, 2011; Ko, Huang, Huang, & Chu, 2009; Levett et al., 2014; Na et al., 2007; Toh, Lim, Kurisawa, & Spector, 2012; Yoo, Lee, Yoon, & Park, 2005). Hence, they are promising biopolymers for the development of implantable scaffolds in TE. In native tissue, CS is predominantly present as part of aggrecan and this natural polymer is involved in several biological mechanisms for the physiological maintenance of cartilage and its role in the resistance to compressive loading. More specifically, due to its hydrophilic nature and abundant negative charges, CS is responsible for retaining a large amount of water in the extracellular matrix (ECM), which is partially released upon compression and re-absorbed when the load is removed (Roughley & Mort, 2014). This mechanism not only provides mechanical resistance, but also contributes to the nutrients/waste products exchange, and thereby also to functioning/performance of the embedded chondrocytes.

Abbreviations: bFGF, recombinant human Fibroblast Growth Factor-basic; CS, chondroitin sulfate; CS-TBA, CS in form of TBA salt; CSMA, methacrylated CS; CP, Cloud Point; DAPI, 4′,6-diamidino-2-phenylindole; DM, degree of methacrylation; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; DMEM/F-12, Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 supplemented with GlutaMax-1 31331; DMSO, dimethyl sulfoxide; EdU, 5-ethynyl-2′-deoxyuridine; GMA, glycidyl methacrylate; ^{1}H NMR, ^{1}H-Nuclear Magnetic Resonance; HA, hyaluronic acid; MA, methacrylic anhydride; $M_{15}P_{10}$, methacrylated poly(*N*-(2-hydroxypropyl) methacrylamide-mono/dilactate)-PEG triblock; Mn, number average molecular weight; Mp, peak molecular weight; PBS, Phosphate buffered saline; PDI, polydispersity index; PEG, polyethylene glycol; pen/strep, penicillin/streptomycin; pHPMAlac, poly(*N*-(2-hydroxypropyl) methacrylamide-mono/dilactate); ratio TBA, CS molar ratio of TBA per disaccharide units of CS; TBA, tetrabutylammonium; T_{gel}, temperature of gelation.

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Hydrogel systems based on cross-linked CS offer a suitable *in vitro* platform in which encapsulated cells, particularly chondrocytes and mesenchymal stem cells, can survive, proliferate, as well as produce cartilage-like ECM (Guo et al., 2012; Huang et al., 2015; Ingavle, Dormer, Gehrke, & Detamore, 2012; Sawatjui et al., 2015). Moreover, CS is able to confer desirable mechanical properties to implants (Khanlari, Suekama, Detamore, & Gehrke, 2015; Sawatjui, Damrongrungruang, Leeanansaksiri, Jearanaikoon, & Limpaiboon, 2014; Zhang et al., 2011). As a result, CS is currently one of the main components of several recently developed hybrid scaffolds studied *in vitro* and *in vivo* (Kuo, Chen, Hsiao, & Chen, 2015; Liao, Qu, Chu, Zhang, & Qian, 2015; Ni et al., 2015; Wei, Wang, Su, Wang, & Qiu, 2015).

The clinical applicability of implantable scaffolds requires hydrogels with a tunable shape and size to match the space of the tissue defect. Moreover, the regenerative potential likely depends on the capacity of the scaffold to mimic the inner structural complexity of tissue. For cartilage regeneration, the possibility to create implants having a multi-layer organization, typical of native tissue is believed to be beneficial, but the feasibility of this approach still represents a challenge (Schuurman et al., 2015). These aspects highlight the need for a sophisticated engineering-based approach that guarantees customized scaffolds with tunable degree of complexity. Bioprinting of hydrogels is an attractive technique to generate 3D scaffolds with reproducible and complex structures. It is based on computer aided deposition of hydrogel filaments in a layer-by-layer fashion (Malda et al., 2013). By changing certain parameters in the 3D printing settings, it is possible to tune the porosity of printed scaffolds. Porosity is an important parameter that can affect cell survival and activity, because porosity provides higher contact area between the implant and the surrounding fluids and, is thus responsible for better oxygen and nutrient supply to encapsulated cells (Malda et al., 2013). The implantation of porous scaffolds may also facilitate cell migration from neighboring tissues that, in turn could offer opportunities to enrich the implant with ECM producing cells (Hunziker & Rosenberg, 1996; Liu, Shu, & Prestwich, 2006). Furthermore, 3D bioprinting offers the opportunity to generate customized hydrogel scaffolds with desired pattern, shape and size.

A 3D printable material needs to have rheological properties allowing its extrusion through a small needle and fast stabilization after deposition to guarantee shape fidelity of the extruded line (Billiet, Vandenhaute, Schelfhout, Van Vlierberghe, & Dubruel, 2012; Malda et al., 2013). This implies that hydrogel materials with shear thinning properties complimented with substantial yield stress behavior are attractive candidates.

In this study, we aimed to design a hydrogel based on UV-cross-linkable CS, *i.e.* methacrylated CS (CSMA) as a candidate biomaterial for cartilage 3D printing. As mentioned before, hydrogels composed of CS and/or other similar polysaccharides display high chondrogenic potential. Nevertheless, they usually lack essential mechanical properties needed for 3D printing applications. Therefore, CSMA was blended with a synthetic thermo-sensitive polymer which has an ABA architecture based on polyethylene glycol (PEG) and partially methacrylated poly(*N*-(2-hydroxypropyl) methacrylamide-mono/dilactate) (pHPMAlac), and has already been used for the development of 3D printable hydrogels (Censi et al., 2011). Moreover, hydrogels based on methacrylated pHPMAlac-PEG triblock copolymers have been demonstrated to be cytocompatible. In detail, Censi et al. have found an excellent cell survival of embedded equine articular chondrocytes, *i.e.* $94 \pm 3\%$ and $85 \pm 7\%$ after 1 and 3 days of culture, respectively (Censi et al., 2011). For similar hydrogels, adequate cell viability was also found for goat-derived mesenchymal stem cells cultured over a long culture period of 3 weeks (Vermonden et al., 2008). By combining CSMA with a pHPMAlac-PEG triblock copolymer, we aimed to improve the rheological profile, and thus the 3D printing potential of hydrogels based on CSMA, without compromising cytocompatibility. In this scenario, it is evident that the reproducibility of mechanical and 3D printing properties of the hydrogel depends on the reproducibility of the polymers' characteristics, and thus on the robustness of the synthetic procedure used to obtain the polymers (Kirchmajer, Gorkin III, & in het Panhuis, 2015).

Hence, to obtain CS with a controllable and reproducible degree of methacrylation (DM), we firstly focused on the investigation of an efficient method for the synthesis of CSMA. The two most frequently used methods for methacrylating CS consist of reactions in aqueous solutions using methacrylic anhydride (MA) or glycidyl methacrylate (GMA). When MA is chosen, a large excess of this compound is necessary to compensate for its hydrolysis in water-based medium (Bryant et al., 2004; Guo et al., 2012; Kesti et al., 2015; Steinmetz & Bryant, 2012; Wang, Shen, & Lu, 2003). Moreover, the adjustement of the pH to basic conditions is crucial for the reaction to proceed. The drawback of adding a basic solution to maintain the pH, is that when not accurately dosed this can cause chain scission of the polysaccharide and hydrolysis of the aimed ester bonds after their formation, as was found by Wang and co-workers (Wang et al., 2003). On the other hand, the protocol that employs GMA for the methacylation of CS in aqueous solution is more efficient (Li, Wang, & Elisseeff, 2003). Nevertheless, it leads to the synthesis of a mixture of products originating from trans-esterification and ring-opening mechanisms, and requires a reaction time of 15 days. Our aim was to develop a GMA trans-esterification procedure, similar as used for the methacrylation of dextran in an aprotic and polar solvent, *i.e.* dimethyl sulfoxide (DMSO) (van Dijk-Wolthuis, Kettenes-van den Bosch, van der Kerk-van Hoof, & Hennink, 1997; van Dijk-Wolthuis et al., 1995), to obtain CS modified with methacrylic functionalities in a fast, efficient, and reproducible manner.

Subsequently, rheological properties of hydrogels composed of CSMA and partially methacrylated pHPMAlac-PEG triblock copolymer were compared with those of hydrogels only composed of CSMA or partially methacrylated pHPMAlac-PEG triblock. 3D printability of the proposed hydrogel was investigated with the aim to generate 3D printed scaffolds with tunable porosity. Finally, viability and proliferation of chondrogenic cells embedded in the described hydrogel were evaluated.

2. Experimental

2.1. Materials

All chemicals and solvents were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands) and Biosolve (Valkenswaard, the Netherlands), respectively, unless indicated otherwise. Chemicals and solvents were used as received. Chondroitin sulfate A sodium salt from bovine trachea ($\geq$ 60% type A (Scheme 1a), balanced with type C), further referred to as CS, was purchased from Sigma-Aldrich. CS was analyzed by Gel Permeation Chromatography (GPC), which showed the presence of three molecular weight distributions. Because of the high polydispersity of the sample, the peak molecular weights (Mp) are reported. The Mp values found for CS were 189 kDa, 13 kDa and 3 kDa, when using dextrans as standards (Fig. S1a). A thermo-sensitive triblock copolymer consisting of a PEG (10000 Da, abbreviated as PEG_{10000}) mid-block flanked with two partially methacrylated pHPMAlac outer blocks (Scheme 1b) was synthesized and characterized as previously reported (Vermonden et al., 2008). The thermo-sensitive polymer is further referred to as $M_{15}P_{10}$ (M_{15} refers to a degree of methacrylation, DM of 15% and P_{10} refers to a PEG chain length of 10 kDa). Characteristics of $M_{15}P_{10}$ are reported in Table 1. Irgacure 2959