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ScienceDirect
Biomaterials 29 (2008) 1739–1749
Biomaterials
www.elsevier.com/locate/biomaterials

Influence of the degree of methacrylation on hyaluronic acid hydrogels properties

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Received 9 August 2007; accepted 7 November 2007
Available online 30 January 2008

Abstract

The properties of hyaluronic acid (HA) hydrogels having a broad range of methacrylation are presented. Increasing solubility of glycidyl methacrylate (GM) in a co-solvent mixture during the methacrylation of HA with GM was shown to produce photopolymerizable HAGM conjugates with various degree of methacrylation (DM) ranging from 14% up to 90%. Aqueous solutions of HAGM macromonomers were photo-cross-linked to yield hydrogels with nearly full vinyl group conversions after 10 min exposure under ultraviolet light (UV). Hydrogels were characterized by uniaxial compression and volumetric swelling measurements. Keeping the DM constant, the shear modulus was varied from 16 kPa up to 73 kPa by varying the macromonomer concentration. However, at a given macromonomer concentration while varying the DM, similarly the shear modulus varied from 22 kPa up to 65 kPa. Preliminary *in-vitro* cell culture studies showed that GRGDS modified HAGM hydrogels promoted similarly cell interaction at both low and high DMs, 32% and 60%, respectively. Densely cross-linked hydrogels with a high DM have been shown to be more mechanically robust while maintaining cytocompability and cell adhesion.

Keywords: Hyaluronic acid; Cross-linking; Hydrogels; Mechanical properties; Cell response

1. Introduction

Natural polysaccharides such as hyaluronic acid (HA, hyaluronan) have been extensively studied in medical applications since they can provide intrinsic biological activity when used as the basis for biomaterials [1,2]. HA is a naturally occurring, biocompatible, and biodegradable linear polysaccharide composed of unbranched repeating units of glucuronic acid and *N*-acetyl glucosamine linked by β 1–3 and β 1–4 glycosidic bonds. Furthermore, HA is an important component of synovial fluid and extracellular matrices, therefore, it could be an attractive building block for new biocompatible and biodegradable polymers for drug delivery, tissue engineering, and viscosupplementation [3,4].

HA plays a prominent role in lubrication, cellular processes, wound healing, and it is naturally angiogenic when enzymatically degraded to small fragments [5–7]. Cellular interactions with HA occur through cell surface receptors and influence tissue formation, inflammation, and morphogenesis [8–11]. This evidence suggests that HA is an ideal candidate material for promoting wound healing and tissue regeneration if it can be modified to improve its mechanical properties.

A variety of modifications of native hyaluronan have been devised to provide mechanically and chemically robust materials through chemical cross-linking. For example, modification of HA can be achieved by covalent derivatization of either the carboxylic acid or hydroxyl functionalities of the polymer [5,7,12–16]. The resulting hyaluronan derivatives

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doi:10.1016/j.biomaterials.2007.11.047

Table 1
Photopolymerizable methacrylated-HA derivatives reported in literature

References	MW HA (10^6 g mol^{-1})	Degree of substitution (%)	Concentration (wt %)	Modulus (Pa)
[5,15]	2	≤11	2	≤155
[7]	0.35–1.1	≤7	≤5	$\leq 25 \times 10^3$
[12]	0.49–1.3	≤25	2	$\leq 5.5 \times 10^3$
[13]	1.7	≤30	2	$\leq 10.5 \times 10^3$

have physicochemical properties that are significantly different from native polymer, yet most derivatives are biocompatible and biodegradable.

Reducing the degradation rates of HA-based materials is necessary for many biomedical applications. HA is enzymatically degraded by hyaluronidase and is resorbable through multiple metabolic pathways [17]. Although HA polymer is broken down *in-vivo*, cross-linking individual HA polymer chains decreases their degradation rates. An effective strategy for cross-linking HA is photopolymerization [17]. Photopolymerization may increase spatial and temporal control over cross-linking and biocompability with possible *in-situ* polymerization [18].

Chemical modification of HA with methacrylated derivatives in aqueous environment has been used to yield photopolymerizable conjugates for gel preparation [2,5,7,12,13,15,16]. However, as shown in Table 1, low percentage gels (≤5%) and low degree of modification (≤30%) for high molecular weight macromonomer precursors have resulted in low cross-linking density for HA-based hydrogels, limiting mechanical properties and stability over a long period of time against enzymatic degradation [5,7,12,13,15]. There is a general consensus that such material properties strongly affect the cell response. A typical approach to control hydrogel mechanical properties is to tailor the network cross-link density. For example, Bryant and Anseth have demonstrated the benefits of cross-linking density on mechanical properties and cell differentiation on the chondrocytes' ability to produce cartilaginous tissues [19]. This suggests that a broader range of cross-linking density on the gel properties could result in a corresponding increase in the range of cellular responses.

In this article, we describe the effect of cross-link density via the DM on the properties of HAGM hydrogels (swelling and mechanics) and evaluate cell response. To this end, we have developed and characterized a series of photopolymerizable HAGM macromonomers with various DM. Proton nuclear magnetic resonance (^{1}H NMR) was used to investigate the mechanism of methacrylation of HA, and to calculate the DM and vinyl conversion during photopolymerization. In addition, an evaluation of the effects of the DM on the swelling behavior, mechanical strength, and cellular responses were conducted on the gels.

2. Experimental methods

2.1. Materials

Hyaluronic acid ($\sim 1.6 \times 10^6$ g/mol), monomethoxy-PEG ($\sim 5 \times 10^3$ g/mol), glycidyl methacrylate (GM), and triethylamine (TEA) were purchased from Sigma–Aldrich and used as received. Monomethoxy-PEG-carboxymethyl ($\sim 5 \times 10^3$ g/mol) was purchased from Laysan Bio, Inc. Acryloyl–PEG–*N*-hydroxysuccinimide (ACRL–PEG–NHS, 3400 g/mol) was purchased from Nektar Therapeutics. GRGDS peptide was purchased from Bachem Bioscience Inc. Photoinitiator Irgacure 2959 (I2959) was obtained from Ciba Specialty Chemicals and used as received. All other chemicals were of reagent grade and were used without further purification. C2C12 mouse myoblast cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with fetal bovine serum and penicillin/streptomycin, all obtained from Invitrogen (Carlsbad, CA). Live/Dead® Viability/Cytotoxicity Kit were purchased from Invitrogen – Molecular Probes, Inc. (Eugene, OR).

Table 2
Reaction conditions for the synthesis of HAGM macromers with a broad range of degrees of methacrylation (from 14% up to 90%)

Reactions		Solvent (vol. %)		Temp (°C)	Time (days)	DM (%)
	HA/GM	PBS	DMF			
1	1/50	100	0	45	10	14
2	1/50	100	0	25	10	21
3	1/50	75	25	25	10	32
4	1/50	50	50	25	5	60
5	1/100	50	50	25	10	90

2.2. Model reactions: GM with monomethoxy-PEG and monomethoxy-PEG-carboxymethyl

In two separate vials, monomethoxy-PEG and monomethoxy-PEG-carboxymethyl (1 g, 0.2 mmol) were reacted with glycidyl methacrylate (0.71 g, 5 mmol) and TEA (0.25 g) in 5 mL of PBS:DMF co-solvent (50:50) for 6 d at room temperature. Detailed experimental protocol, reaction scheme, characterization, and analysis of resulting oligomeric products are described in the supporting information.

2.3. Synthesis of HAGM conjugates

Photopolymerizable methacrylate groups were added to HA to yield HA–glycidyl methacrylate (HAGM) conjugates. As reported in Table 2, we prepared a series of HAGM polymers by treating a 0.5% wt/v solution of fermentation-derived HA ($\sim 1.6 \times 10^6$ Da) in phosphate buffer saline (PBS), and dimethylformamide (DMF) with a 50- or 100-fold molar excess of GM in the presence of excess triethylamine. The reactions were carried out at two different temperatures (25 °C or 45 °C) for 5–10 d. An example of the synthesis of HAGM with a DM of 32% is as follows (reaction 3). HA (1.0 g) was first dissolved in 200 mL phosphate buffer saline (PBS, pH ~7.4) and 67 mL of dimethylformamide (DMF), and subsequently mixed with 13.3 g of GM and 6.7 g of TEA. After 10 d reaction, the solution was precipitated twice in a large