SYNTHESIS AND CHARACTERIZATION OF HIGHLY CROSSLINKED HYALURONAN HYDROGELS

Newell R. Washburn[1], Sidi A. Bencherif[1], Abiraman Srinivasan[2], Jeffrey O. Hollinger[2], Ferenc Horkay[3], Krzysztof Matyjaszewski[1]

Departments of [1]Chemistry and [2]Biomedical Engineering
Carnegie Mellon University, Pittsburgh, PA 15213
[3]Laboratory of Integrative and Medical Biophysics
National Institutes of Health, NICHD
13 South Drive, Bethesda, MD 20892

Introduction

Methacrylation of hyaluronic acid (HA) with glycidyl methacrylate (GM) is a strategy for producing photocross-linkable macromers to generate biodegradable hydrogels by in-situ injection and light-induced polymerization. Facile synthesis and characterization by ^{1}H NMR spectroscopy of a series of photopolymerizable macromers based on HA-glycidyl methacrylate (HAGM) conjugates with various degrees of methacrylation (DM) ranging from 14% to 90% are described. Aqueous solutions of HAGM were photopolymerized to yield hydrogels with high vinyl group conversions up to 99% after 10 min exposure under ultraviolet light (UV). Uniaxial compression and volumetric swelling measurements showed that HAGM hydrogels were mechanically robust with shear moduli ranging from 17 kPa to 95 kPa, with a broad range of swelling ratios. Preliminary in-vitro cell culture studies showed that these HA-based hydrogels were cytocompatible, and the introduction of the GRGDS peptide promoted adhesion and proliferation of cells to confluence after 5 d of incubation. Densely cross-linked hydrogels with a DM of 60% have been shown to be stable in culture while maintaining cytocompability and bioactivity. These highly cross-linked HAGM hydrogel systems with improved mechanical properties appear to be very attractive for biomedical applications such as drug delivery systems and tissue engineering and could expand the range of properties of crosslinked hyaluronan [1].

Experimental

Materials. Hyaluronic acid (~ 1.6x10^6 g/mol), glycidyl methacrylate (GM), and triethylamine (TEA) were purchased from Sigma-Aldrich and used as received. 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).

Instrumentation. High-resolution, 300 MHz proton NMR spectra were taken on a Bruker Avance 300 spectrometer. Deuterium oxide was used as solvent, and the polymer concentrations were varied between 0.5 % and 3 % by mass fraction.

The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a PerSeptive Biosystems Voyager STR in reflectron mode. The MALDI matrix, dihydrobenzoic acid (DHB), and the macromer were dissolved in 1 mL of mix solvent methanol/water (50:50). All MALDI samples were hand spotted on the target.

Uniaxial compression measurements were used on hydrogels to assess the mechanical strength. The shear modulus was determined using uniaxial compression measurements performed by a TA.XT21 HR texture analyzer (Stable Micro Systems, UK). This apparatus measures the deformation (0.001 mm) as a function of an applied force (0.01 N). Cylindrical hydrogels (height = diameter = 5 mm) were deformed (at constant volume) between two parallel glass plates.

Synthesis of Methacrylated Hyaluronic Acid. Photopolymerizable methacrylate groups were added to HA to yield HA-glycidyl methacrylate (HAGM) conjugates. Briefly, we prepared a series of HAGM polymers by treating a 0.5% w/v solution of fermentation-derived HA (~ 1.6x106 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 6-10 d. An example of the synthesis of HAGM with a degree of methacrylation of 32% is as follows (reaction 3). 1.0 g of HA was first dissolved in 200 mL phosphate buffer saline (PBS, pH ~ 7.4) and 67 mL of dimethylformamide (DMF), and subsequently mixed with 13.3g of GM and 6.7g of TEA.

Synthesis of ACRL-PEG-Peptide. GRGDS peptide was dissolved in anhydrous DMF containing 4 molar excess of TEA. ACRL-PEG-NHS was also dissolved in anhydrous DMF and immediately after, mixed with 1.1 molar excess of peptide. After incubating for 3 hr at room temperature, ACRL-PEG-GRGDS was precipitated twice in cold anhydrous ether and dried in a vacuum oven overnight at room temperature. The peptide coupling reaction and molecular mass of the product was monitored by MALDI-TOF MS.

Hydrogel Preparation. 2%, 5%, 7%, and 10% by mass fraction of HAGM macromers were mixed in deionized water and combined with ACRL-PEG-GRGDS to a final concentration of 4 µmol/mL. The hydrogels were made by exposing the aqueous solution of HAGM to UV-light in the presence of the photoinitiator Irgacure 2959 (0.1% by mass fraction). Cylindrical samples of 15 mm in diameter and 1 mm in height were cured under a UV source (365 nm, 300 µW/cm2) for 10 min to obtain bioactive hydrogels. The HAGM liquid cross-linked into materials ranging from soft to stiff and brittle solids, depending on the degree of methacrylation and mass fraction of macromers used.

Swelling Experiments. Cylindrical gel specimens of known dry mass were equilibrated with polymer solutions containing poly(vinyl pyrrolidone) (PVP, Mn = 29 kDa). The PVP concentration was varied in the range from 2 to 20% (w/w). In order to prevent penetration of PVP molecules into the swollen network, the gel was separated from the surrounding solution by a semipermeable membrane (dialysis bag). After equilibration (6-10 days) the mass of the gel was measured. The elastic (shear) modulus was obtained from uniaxial compression measurements made on isotropically deswollen gel cylinders using a TA.XT2I HR Texture Analyser (Stable Micro Systems, UK). Gel samples were rapidly transferred from the dialysis bag into this apparatus, which measures the deformation (± 0.001 mm) as a function of an applied force (± 0.01 N).

In-vitro biocompability and cell response testing of hyaluronan gels. After photopolymerization, the gels were placed in the bottom of a 24-well plate. The gels were subsequently washed two times in sterile PBS, sterilized once in 70% ethanol and washed twice with sterile PBS, and finally conditioned in cell culture medium. Mouse muscle fibroblast C2C12 cell lines were seeded onto the hydrogel disks at a density of 50,000 cells/gel. The cells were cultured for 1, 3, and 5 d, to assess cell attachment and cytotoxicity. Cell attachment, morphology and spreading of were examined using phase-contrast light microscopy (Zeiss Axiovert).

Results and Discussion

Methacrylation of Hyaluronic Acid. The methacrylation of hyaluronic acid is shown in **Figure 1**. Glycidyl methacrylate targets both the pendant hydroxyl group on the N-acetylglucosamine ring through opening of the epoxide group and the carboxylate group on the glucuronic acid ring through transesterification. Quantification of the degree of methacrylation was performed using ^{1}H NMR spectroscopy using the doublet centered at 5.8 ppm. By using a 100-fold excess of glycidyl methacrylate on a per monomer basis and 50% PBS/50% DMF as the solvent, a 90% degree of methacrylation was achieved, while a 50-fold excess of glycidyl methacrylate in pure PBS resulted in only 14% methacrylation. By tuning the solvent ratio and molar ratio of glycidyl methacrylate to hyaluronan monomer, it is possible to systematically vary the methacrylation. In this work, we were able to synthesize hyaluronan with degrees of methacrylation 14%, 21%, 32%, 60%, and 90%.

Hydrogels of methacrylated HA were prepared by photopolymerization using Irgacure 2959 as the initiator. Solutions ranging from 5% to 10% HA were injected into molds and cured under a UV lamp for 10 minutes. The mechanical properties of the hydrogels depended strongly on the degree of methacrylation; the 14% samples were soft to the touch while the 90% samples were hard and brittle. The mechanical properties were measured as a function of the degree of methacrylation and the swelling of the hydrogels, and the results are shown in Figure 3. There was a monotonic increase in the Young's modulus as a function of degree of methacrylation with the lowest modulus in the highly swollen 14% methacrylation hydrogel being 17 kPa.

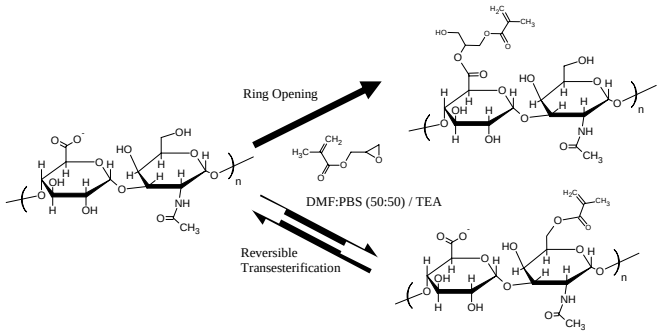

Figure 1. Methacrylation of hyaluronic acid.

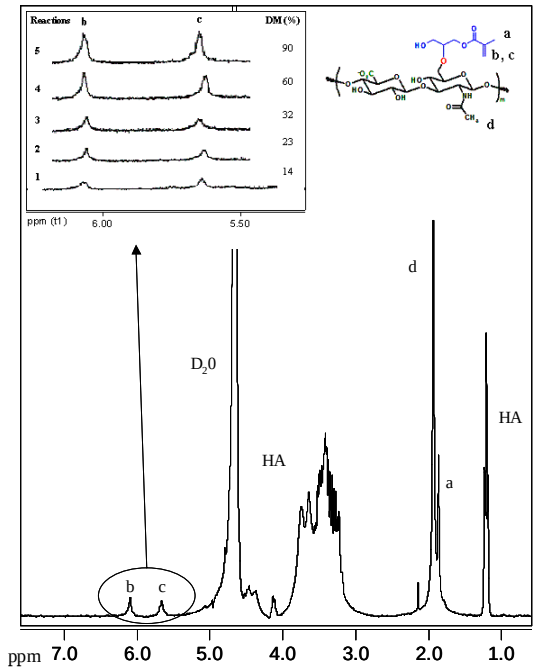

Figure 2. ^{1}H NMR spectrum of methacrylated HA.

For the 90% methacrylation hydrogels, the highest modulus was 120 kPa in the most solvent-depleted material. As a function of the mass fraction of the hydrogel, the shear modulus varied with polymer concentration with an exponent ranging from 0.39-0.49, slightly greater than the value of 1/3 that is expected from the predictions of rubber elasticity theory.

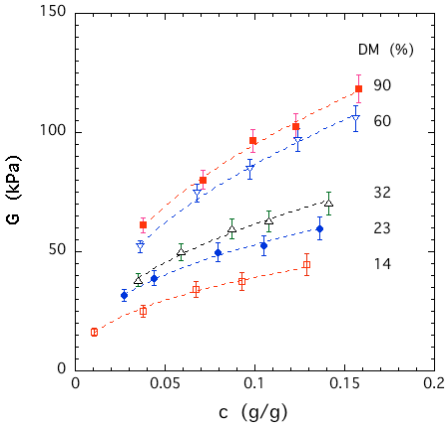

Figure 3. Young's modulus as a function of polymer concentration and degree of methacrylation.

The biocompatibility of HA hydrogels was assessed by culturing C2C12 cells on the gels. Methacrylated HA was photocrosslinked with 4 mM GRGDS peptide that had been linked to a vinyl-terminated PEG molecular. Cells proliferated well on the samples, indicating there were no residual cytotoxic components in the hydrogels.

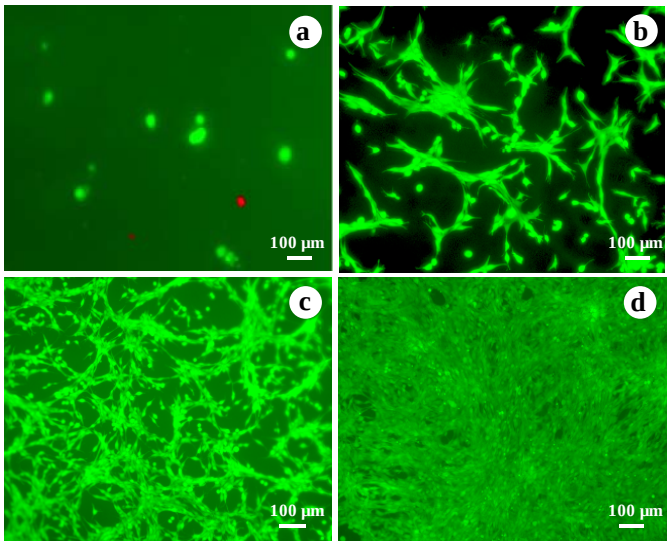

Figure 4. Cells were not adherent in the absence of the GRGDS peptide (a) but showed excellent adhesion and proliferation with 4 nM GRGDS peptide incorporated in the gel (b-d) over a 5 d time course.

Conclusions

By varying the reaction conditions, it is possible to prepare hyaluronic acid with methacrylate groups on up to 90% of the monomers. The mechanical properties of the resulting hydrogels ranged from soft and flexible to hard and brittle. Cell culture results indicate these materials are biocompatible and potentially suitable for tissue engineering applications.

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