Development of photocrosslinkable hyaluronic acid-polyethylene glycol-peptide composite hydrogels for soft tissue engineering

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Abstract: Hyaluronic acid (HA; also called hyaluronan) is a naturally derived, nonimmunogenic, nonadhesive glycosaminoglycan that has important roles in several wound-healing processes. In previous work, we created photocrosslinkable glycidyl methacrylate–HA (GMHA) hydrogel biomaterials that were cytocompatible, biologically active, and had a decreased rate of hyaluronidase degradation compared with native HA. The goal of the studies presented herein was to explore peptide conjugation techniques to further adjust the material and biological properties of the GMHA hydrogels. We conjugated GMHA with acrylated forms of polyethylene glycol (PEG) and PEG–peptides to yield GMHA-PEG-peptide composite hydrogels. By varying the reactant concentrations, we created stable hydrogels with high peptide conjugation efficiencies (up to 80%), controllable peptide concentrations (in the range of 1–6 μmol peptide per milliliter of hydrogel), and defined physicochemical properties (e.g., swelling ratio, enzymatic degradation rate). These composite hydrogels may prove to be a promising scaffolding biomaterial for a variety of soft tissue engineering applications. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 70A: 74–82, 2004

Key words: hyaluronic acid; polyethylene glycol; adhesive peptides; hydrogels; tissue engineering

INTRODUCTION

Hyaluronic acid (HA), a naturally occurring glycosaminoglycan, offers many unique advantages as a building block for biomaterials.[1] For example, HA is nonimmunogenic, enzymatically degradable, and relatively nonadhesive to cells and proteins.[2,3] Physiologically, HA has a role in several processes, including angiogenesis, extracellular matrix homeostasis, wound healing, and the mediation of long-term inflammation.[2] Furthermore, HA is particularly suitable for biomaterial fabrication because it can be produced in large quantities through bacterial fermentation,[3] thus avoiding the risk of the animal-derived pathogens that are possibly associated with naturally derived materials.

Drawing on these advantages, we have recently modified HA with glycidyl methacrylate, yielding a photocrosslinkable conjugate, GMHA.[4] The resulting polymers are cytocompatible, biologically active, and can be photocrosslinked to form GMHA hydrogels with tunable material properties (e.g., degradation rate, crosslink density). When implanted subcutaneously in rats, the GMHA hydrogels were associated with similar levels of vascularization at the implant edge as fibrin positive controls. However, *in vitro* studies of fibroblast and endothelial cell adhesion indicated that the GMHA hydrogels alone do not support cell adhesion and spreading (unpublished data). These *in vitro* results are in agreement with published studies on similar crosslinked HA hydrogels,[5] and given HA's highly hydrated and nonadhesive nature, these *in vitro* results are not unexpected.

The specific goal of this study was to modify GMHA with peptide sequences, ultimately leading to a cell-adhesive GMHA construct for soft tissue engineering applications. Work with other nonadhesive

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hydrogels such as polyethylene glycol (PEG),[6,7] and alginate[8] has demonstrated that covalently bound peptide sequences can be used to specifically tune cellular adhesion, migration, and proliferation. Fortunately, GMHA has several possible sites for modification, including carboxyl, hydroxyl, and acetamido groups found naturally on HA in addition to the conjugated photoreactive methacrylate groups.

Other researchers have also created crosslinked HA–peptide conjugates. For example, Glass et al.[9] reported a periodate oxidation method to conjugate RGD onto HA hydrogel matrices. The RGD–HA matrices were associated with higher levels of osteosarcoma cell adhesion and proliferation. However, periodate oxidation of HA opens ring structures in the glucuronic acid moieties of the polymer backbone; such changes may result in decreased biological activity or otherwise altered physicochemical properties.[10] Very recently, the Hubbell laboratory published an extensive study of the mechanical properties of HA-based hydrogels for cartilage repair.[5] The hydrogels were reinforced with low-molecular-weight PEG diacrylate and functionalized with acrylated PEG–RGD conjugates created through Michael-type addition chemistry. Similar work with HA–RGD conjugate hydrogels was also recently published by Shu et al.[11]; in this study, a dihydrazide-based method was developed to crosslink low-molecular-weight HA in the presence of RGD–PEG diacrylate. These examples of crosslinked HA–RGD conjugates demonstrate the growing interest in HA scaffolding materials for tissue engineering applications.

In this work, we created flexible photopolymerizable GMHA–peptide conjugates for general soft tissue engineering applications (e.g., nerve regeneration, wound healing). To conserve cost, the model peptide hexaglycine (Gly-Gly-Gly-Gly-Gly-Gly) was used to develop three methods for GMHA conjugation. The most efficient method yielded a GMHA-PEG-peptide composite hydrogel, which was then characterized in terms of its swelling properties and enzymatic degradation rate.

MATERIALS AND METHODS

All materials were acquired from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

GMHA conjugates

We synthesized GMHA conjugates using previously developed methods.[4] Briefly, we prepared GMHA with 11% methacrylation by reacting a 1% (w/v) solution of fermentation-derived HA [$\sim 2 \times 10^6$ molecular weight (MW); Clear Solutions Biotech, Stony Brook, NY] in deionized water with 20-fold molar excess of glycidyl methacrylate in the presence of excess triethylamine and tetrabutyl ammonium bromide. Specifically, 1.0 g of HA was dissolved in 100 mL of deionized water; 3.6 mL of triethylamine, 3.6 mL of glycidyl methacrylate, and 3.6 g of tetrabutyl ammonium bromide were added separately and thoroughly mixed before the next component was added. The reaction was mixed overnight at room temperature and then for 1 h at 60°C. Finally, the GMHA was precipitated in acetone and dissolved in deionized water twice to remove excess reactants and then lyophilized. ^{1}H NMR spectroscopy was used to verify the methacrylation reaction on HA and the near-complete removal of excess reactants from the product. GMHA conjugates were dissolved in D_2O and the spectra were recorded using a Varian Inova-500 spectrophotometer. Percent methacrylation was determined from the ratio of the relative peak integrations of the methacrylate protons (peaks at ~5.6 and ~6.1 ppm) and HA's methyl protons (1.9 ppm).[4]

EDC-mediated GMHA–peptide conjugation

We conjugated the model peptide hexaglycine onto uncrosslinked GMHA using reactions mediated by *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC)[8,10] [shown schematically in Fig. 1(a)]. All steps were performed at room temperature. The number of GMHA's carboxylate groups was calculated from the polymer mass and the average molecular weight of the disaccharide repeat (each repeat contains one carboxylate group). Solutions of 1% (w/v) GMHA were prepared in buffered pH 4.5–7.5 solutions that contained 0.1*M* morpholinoethanesulfonic acid and 0.3*M* NaCl. 1-Hydroxybenzotriazole hydrate (HOBt; American Peptide, Sunnyvale, CA) or *N*-hydroxysuccinimide (NHS) was added at a 0.25, 0.5, or 1 molar ratio to GMHA's carboxylate groups and mixed for 5 min. Next, EDC (0.1, 0.5, 2, or 4 molar ratio to GMHA's carboxylate groups) was added and mixed for 5 min. Finally, hexaglycine (1.0–2.0 molar ratio to GMHA's carboxylate groups) was added and mixed for 2 h. The reaction was transferred to 7000 MW cut-off dialysis tubing (Pierce, Rockford, IL), dialyzed overnight against deionized water (20 times the volume of the reaction), and then lyophilized. The extent of hexaglycine conjugation was determined with a ninhydrin assay as described below.

Photocrosslinked GMHA-PEG-peptide hydrogels

We also used an acrylated PEG–peptide method[6] to conjugate hexaglycine to GMHA during the crosslinking reaction [shown schematically in Fig. 1(b,c)]. To synthesize the acrylated PEG–hexaglycine, 62 μmol of hexaglycine (2.24 mL of a 10 mg/mL stock solution of hexaglycine in 1*M* HCl) was added to 15 mL of 50 m*M* sodium bicarbonate buffer, pH 8.5. The pH was readjusted to 8.5 with approximately 2.2 mL of 1*M* NaOH. Next, 62 μmol (0.2 g) dry acryloyl-PEG-NHS (MW 3400; Nektar Therapeutics, Huntsville, AL) was added to the hexaglycine solution and mixed immediately to

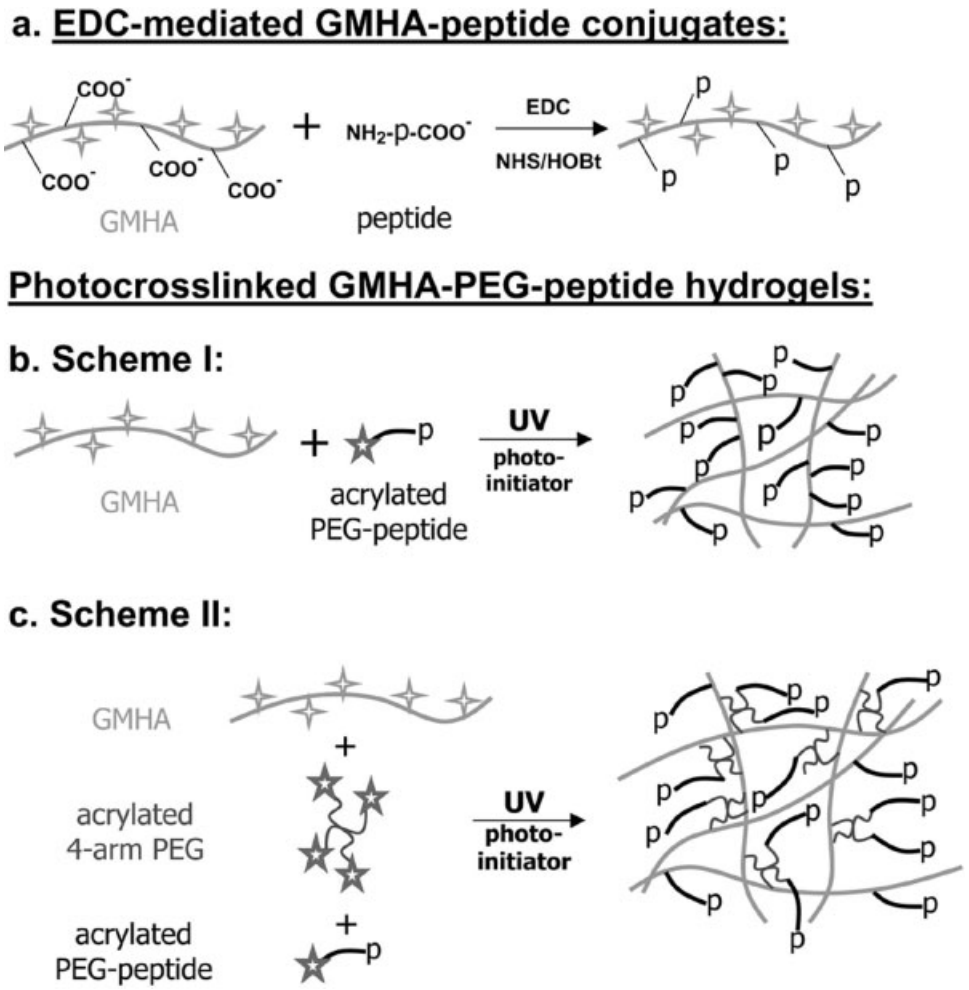

Figure 1. Schematic of peptide conjugation chemistries. Three approaches were evaluated as means of covalently binding peptides (a) to uncrosslinked GMHA and (b,c) during the hydrogel crosslinking reaction. (a) GMHA, drawn schematically to denote the carboxylate groups (—COO^-) and photocrosslinkable methacrylate groups (four-point stars), is conjugated with peptides through a reaction mediated by EDC and an ester-activating agent (either NHS or HOBt). To incorporate peptides during the hydrogel photocrosslinking, acrylated PEG–peptides (acrylate groups denoted by five-point stars) are mixed with (b; Scheme I) GMHA alone or (c; Scheme II) GMHA and acrylated 4-arm PEG.

dissolve. The reaction was mixed for 2 h at room temperature, transferred to 1000 MW cut-off dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA), and dialyzed overnight against deionized water (20 times the volume of the reaction solution). The product was lyophilized and stored with desiccant at −20°C. The PEG–peptide conjugation was verified by determining the mass of the product with matrix-assisted laser desorption/ionization mass spectrometry.

The crosslinked GMHA-PEG-peptide hydrogels were synthesized by two separate reaction schemes. In the first scheme [Scheme I; shown schematically in Fig. 1(b)], we exposed a mixture of GMHA (1% w/v or approximately 2.3 μmol photoreactive sites per milliliter in phosphate-buffered saline) and acrylated PEG–hexaglycine (approximately 1.0–30.0 μmol/mL) to UV light (365 nm, ~22 mW/cm^2, 60-s exposure) in the presence of the photoinitiator Irgacure 2959 (1% w/v; Ciba Specialty Chemicals, Basel, Switzerland) and *N*-vinyl pyrrolidinone (0.3% v/v). The second scheme [Scheme II; Fig. 1(c)] is similar to Scheme I, except that we also added acrylated 4-arm PEG (MW 10,000; SunBio, Walnut Creek, CA) to supplement the total number of photoreactive sites available within the hydrogel. The concentrations of acrylated 4-arm PEG investigated were 0.5, 3.5, and 4.6% (w/v), which correspond to approximately 2.1, 13.4, and 17.5 μmol photoreactive sites per milliliter.

Ninhydrin assay

To verify the moles of peptide bound to uncrosslinked GMHA (EDC-mediated GMHA conjugation) and crosslinked GMHA-PEG-peptide hydrogels, we used the ninhydrin assay[12,13] to measure the free amino acid in hydrolyzed samples. Crosslinked hydrogel samples first were washed by swelling for 10 min in deionized water, deswelling for 10 min in 70% ethanol (v/v in deionized water), and then reswelling for 10 min in deionized water. Hydrogel samples

and peptide standards were then hydrolyzed at 1 mg/mL in 13.5*M* NaOH in an autoclave (at least 40 min at 121°–125°C). Next, 12.1*M* HCl was carefully added drop-wise to neutralize the base. The volume was measured and the sample concentration was recorded. The hydrolyzed solution was diluted to 50 μg/mL in 0.1*M* citric acid buffer, pH 4.7. The pH was measured and readjusted to 4.5–5.5, if necessary.

To prepare the ninhydrin reagent, stock solutions of stannous chloride (100 mg/mL in ethylene glycol) and 4*N* acetate buffer (pH 5.5–8.0) are required. The 4*N* acetate buffer was made by dissolving 54.4 g of sodium acetate in 100 mL of acetic acid and then adjusting the total volume to 500 mL with deionized water. The ninhydrin reagent was freshly prepared by dissolving 0.2 g of ninhydrin in 7.5 mL of ethylene glycol and 2.5 mL of 4*N* acetate buffer. Then, 250 μL of stannous chloride solution was added with mixing. The ninhydrin reagent was only used while it was pale red in color.

In a 96-well plate, 80 μL of the hydrolyzed samples or standards and 80 μL of the ninhydrin reagent were added per well in triplicate. The well plate was covered with sealing tape (Corning Costar, Acton, MA) and incubated at 95°–100°C in a water bath for 10 min. The absorbance of each well was read at 595 nm using a microplate reader (Biotek Instruments, Winooski, VT) and the amino acid content of each sample was calculated from a standard curve.

Swelling ratio

To investigate the relative degrees of crosslinking in the hydrogels, we determined the swelling ratio of each sample using published methods.[4] The swelling ratio is inversely related to the extent of crosslinking and was calculated by dividing the gel mass after swelling (M_s) by the dry gel mass (M_d). A PerkinElmer (Wellesley, MA) thermogravimetric analyzer (TGA) was used to measure M_s and M_d. The hydrogels were preswollen in phosphate-buffered saline overnight and a small piece (10–20 mg) was placed in the TGA weighing pan. The initial mass was recorded as M_s, and the sample was heated slowly to 100°C until a constant mass was achieved. The final mass obtained was recorded as M_d.

In vitro degradation by hyaluronidase

We determined the enzymatic degradation rate of GMHA–PEG hydrogels using previously developed methods.[4] Solutions of GMHA (1%, w/v) and acrylated 4-arm PEG (0.3, 0.6, 1.2, or 4.6%, w/v) were photocrosslinked in 8-mm-diameter, 2-mm-deep rubber perfusion chamber molds (Sigma-Aldrich). The hydrogels were carefully transferred to 24-well plates and soaked in citrate buffer (0.15*M* NaCl, 0.15*M* Na_2HPO_4-7 H_2O, 0.03*M* citric acid, pH 5.3) overnight. The gels were removed and the gel masses were recorded. Bovine testicular hyaluronidase in citrate buffer (1.0 mL of either 5 or 50 U/mL) was added to each gel and then incubated at 37°C with mild mixing on a platform shaker. Every 2 h, the solution was removed, the gels were blotted, and the masses were carefully determined. GMHA–PEG hydrogels in citrate buffer without hyaluronidase were used as negative controls.

Statistical analysis

We performed Student *t* tests to determine the statistical significance of the differences between results. Linear regressions were calculated using the Regression Data Analysis package in Microsoft Excel (Redmond, WA). A significance level of $p < 0.05$ was used as the cutoff.

RESULTS

Three approaches (shown schematically in Fig. 1) were examined as means of covalently binding peptides to GMHA. To conserve cost, hexaglycine was used as a model peptide and each approach was optimized for maximal conjugation efficiency (i.e., the percent of the input hexaglycine that is conjugated).

EDC-mediated GMHA–peptide conjugation

To add hexaglycine onto uncrosslinked GMHA, an EDC-mediated reaction was used to covalently link the carboxylates of GMHA with the primary amines of the peptides. We investigated five reaction variables: reaction pH, the choice and moles of ester-activating agent (e.g., NHS or HOBt) and the moles of EDC and hexaglycine. The moles of NHS, HOBt, EDC, and hexaglycine used in the reactions were expressed as the molar ratio of the component to the moles of GMHA's carboxylate groups (designated, for example, as EDC/GMHA $-COO^-$). The ninhydrin assay was used to measure the moles of hexaglycine conjugated for at least two separate hydrolyzed samples for each condition.

First, we examined the effect of reaction pH on EDC-mediated reactions in the presence of NHS or HOBt [Fig. 2(a)]. Reactions were performed with 0.5 EDC/GMHA $-COO^-$, 0.25 NHS/GMHA $-COO^-$, or 0.25 HOBt/GMHA $-COO^-$, 1 hexaglycine/GMHA $-COO^-$, and pH values of 4.5, 5.5, 6.5, and 7.5. Reactions that were performed at pH 5.5 had the greatest conjugation efficiencies (NHS: 2.6 ± 0.5%, $n = 6$; HOBt: 3.0 ± 0.7%, $n = 3$) and were statistically different than the negative control (no EDC, NHS, or HOBt: 0.4 ± 0.3%, $n = 3$). Reactions with NHS at pH 4.5 (1.5 ± 0.5%, $n = 5$) and pH 6.5 (1.8 ± 0.8%, $n = 6$) were also statistically different than the negative control.

Next, the effect of varying EDC/GMHA $-COO^-$ was investigated [Fig. 2(b)]. Reactions were completed with 0.25 HOBt/GMHA $-COO^-$, 1 hexaglycine/

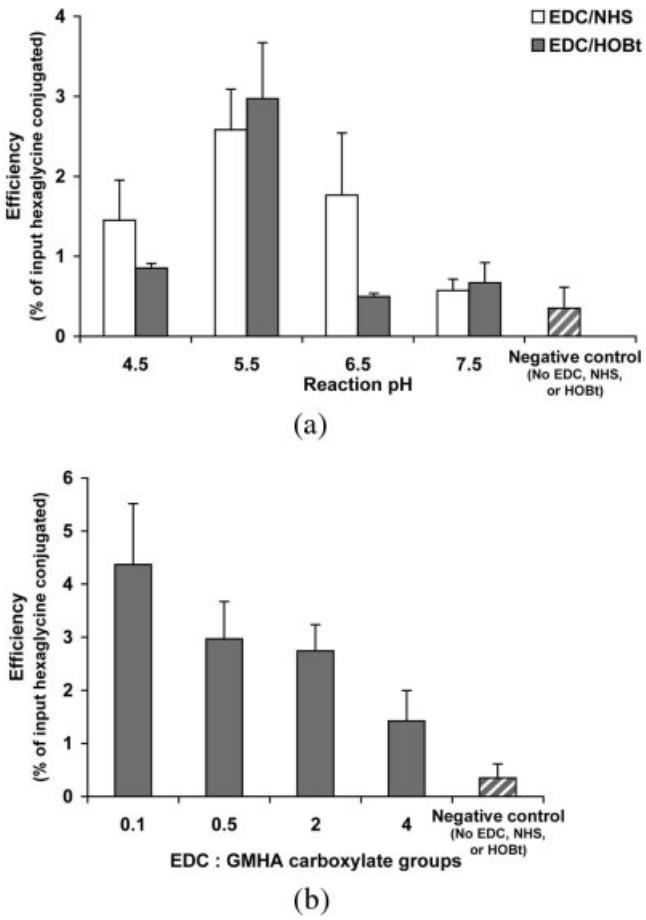

Figure 2. Efficiency of EDC-mediated GMHA–hexaglycine conjugations. Shown are the results of three reaction variables: reaction pH, ester-activating agent (NHS, open bars; HOBt, shaded bars), and the molar ratio of EDC to GMHA's carboxylate groups. (a) Reactions at pH 5.5 had the greatest conjugation efficiencies (i.e., the percent of the input hexaglycine that is conjugated) and were statistically greater than the negative control (no EDC, NHS, or HOBt; hatched bar). (b) The effect of the molar ratio of EDC to GMHA's carboxylate groups was investigated for reactions in the presence of HOBt. All efficiencies were statistically different from the negative control. Each bar represents the average plus the standard deviation.

GMHA $–COO^-$, and at pH 5.5. All efficiencies were statistically different from the negative control. For EDC/GMHA $–COO^-$ values of 0.l, 0.5, 2, and 4, the respective efficiencies are 4.4 ± 1.2% (n = 4), 3.0 ± 0.7% (n = 3), 2.7 ± 0.5% (n = 4), and 1.4 ± 0.6% (n = 4). For reactions with 0.1 EDC/GMHA $–COO^-$, the average conjugation efficiency is greater, but not statistically distinct from reactions performed with a 0.5 EDC/GMHA $–COO^-$. Similarly, for reactions with 0.5 EDC/GMHA $–COO^-$, the conjugation efficiency is greater, but not statistically different than reactions performed at 2.0 EDC/GMHA $–COO^-$. It may seem unexpected that conjugation efficiency would decrease with increasing EDC concentrations. However, it is important to note that 0.25 HOBt/GMHA $–COO^-$ was used in each case. When the concentration of EDC was roughly equal to the concentration of HOBt, the conjugation efficiencies obtained were higher than when EDC was in excess of HOBt. This trend reinforces published work,[10] which suggests the importance of ester-activating agents in EDC-mediated HA modification reactions.

The remaining variables (i.e., moles of ester activating agent and hexaglycine) were investigated at pH 5.5 and with a constant 0.5 EDC/GMHA $–COO^-$. We found that with 1 hexaglycine/GMHA $–COO^-$ and NHS/GMHA $–COO^-$ or HOBt/GMHA $–COO^-$ varying from 0.25 to 1, the conjugation efficiencies were not statistically different and were all in the range of 1–3% (data not shown). Likewise, with 0.25 HOBt/GMHA $–COO^-$ and hexaglycine/GMHA $–COO^-$ from 0.5 to 2, the differences in conjugation efficiencies were not statistically significant and were in the range of 1–3% (data not shown).

Photocrosslinked GMHA-PEG-peptide hydrogels

To incorporate the model peptide hexaglycine into GMHA hydrogels during the crosslinking reaction, two general reaction schemes were developed. For each scheme, we sought a chemistry that would yield stable (i.e., insoluble) hydrogels and conjugated peptide yields in the range of 1–5 μmol/mL (based on the range found to be optimal for cell adhesion and migration in similar hydrogel systems[7,14,15]).

In Scheme I, the effect of varying acrylated PEG–hexaglycine concentration was evaluated in gelation solutions of 1% (w/v) GMHA, 1% Irgacure 2959, and 0.3% (v/v) *N*-vinyl pyrrolidinone. For acrylated PEG–hexaglycine input concentrations above 1.4 μmol/mL, stable hydrogels did not form. We presume that in these cases, the number of photocrosslinkable sites available on GMHA alone is not sufficient to support crosslinking as well as peptide conjugation. In previous work,[4] we have found that 11% methacrylation is the highest extent of modification achievable under the chosen reaction conditions. Therefore, we sought another method of increasing the number of available photocrosslinkable sites within the gelation solution.

In Scheme II, we supplemented the total number of available photoreactive groups by adding acrylated 4-arm PEG to the gelation solutions from Scheme I. The hydrogel samples were washed, hydrolyzed, and then the ninhydrin assay was used to determine the mass of conjugated hexaglycine. From this information, the conjugation efficiency (i.e., the percent of the input hexaglycine that is conjugated to the hydrogel) and yield (i.e., the concentration of peptide conjugated to the hydrogel) were calculated. In Figure 3, a single data set is shown for three concentrations of total

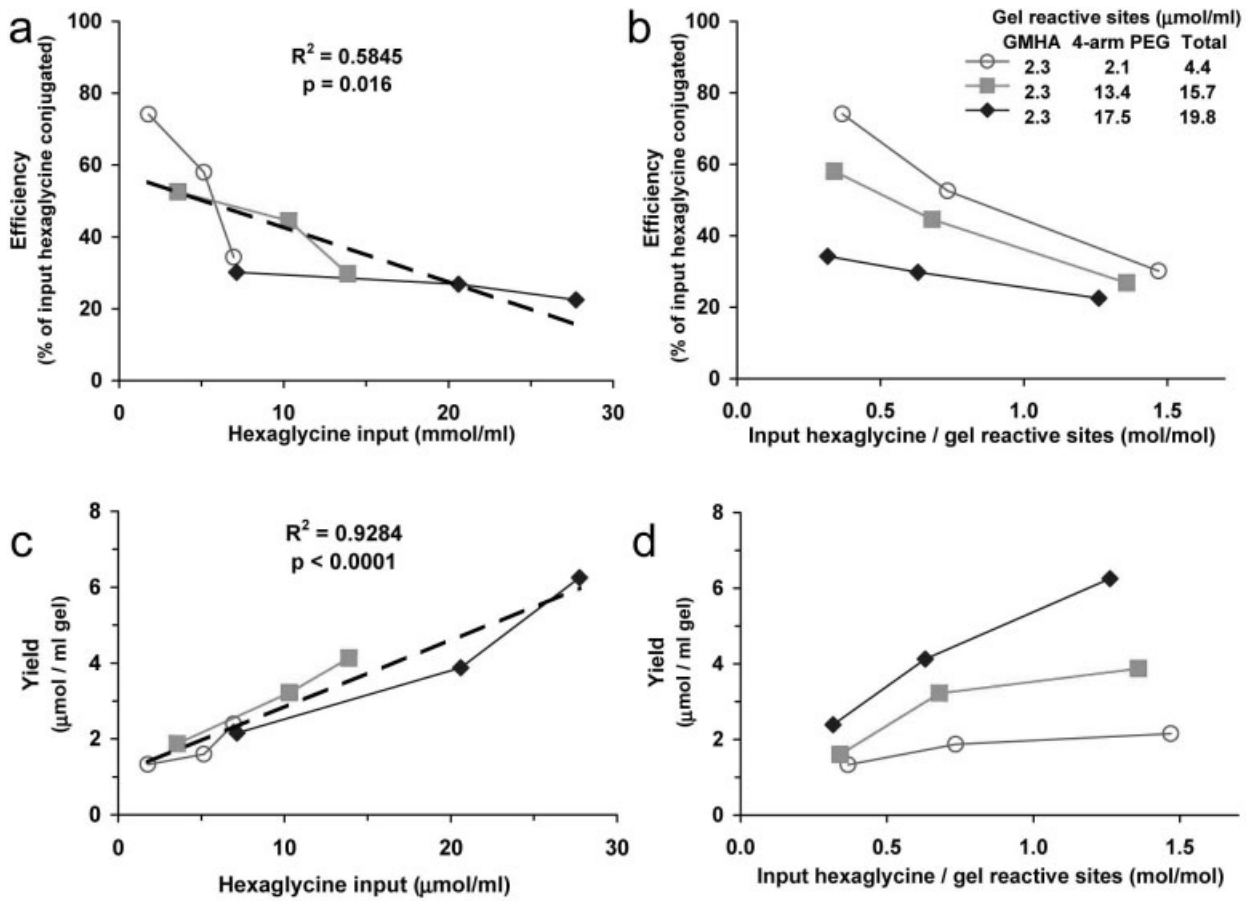

Figure 3. Efficiency and yield of photocrosslinked GMHA-PEG-peptide conjugations. Hydrogels were synthesized with GMHA and varying amounts of acrylated 4-arm PEG and PEG–hexaglycine. The mass of hexaglycine conjugated was determined and used to calculate conjugation (a,b) efficiency (i.e., the percent of the input hexaglycine that is conjugated to the hydrogel) and (c,d) yield (i.e., the concentration of peptide conjugated to the hydrogel). A single data set is plotted versus (a,c) micromole of hexaglycine input per milliliter of gelation solution and (b,d) molar ratio of input hexaglycine to hydrogel reactive sites. Linear regression analysis indicates an inverse correlation between efficiency and peptide input ($p = 0.016$) and a direct correlation between yield and peptide input ($p < 0.0001$). (The dotted line denotes the linear fit through all data points.)

hydrogel reactive sites (4.4, 15.7, and 19.8 μmol/mL), which are plotted versus the hexaglycine concentration in the gelation solution [Fig. 3(a,c)] and the molar ratio of input hexaglycine to hydrogel reactive sites [Fig. 3(b,d)]. Efficiency was found to vary inversely with the moles of hexaglycine ($p = 0.016$), the total number of reactive sites, and the ratio of hexaglycine to hydrogel reactive sites. Yield, however, increased with increasing hexaglycine put into the reaction ($p < 0.0001$), the total number of reactive sites, and the ratio of hexaglycine to hydrogel reactive sites.

Swelling ratio

To evaluate the effect of acrylated 4-arm PEG and acrylated PEG–hexaglycine concentrations on the relative extents of crosslinking, we determined the swelling ratio of the GMHA-PEG-hexaglycine hydrogels (Fig. 4). For gels with 4.4 μmol/mL hydrogel reactive sites and 0.4, 0.7, and 1.5 molar ratios of hexaglycine to hydrogel reactive sites, the swelling ratios were 35.9 ± 4.1 ($n = 2$), 29.6 ± 5.8 ($n = 3$), and 27.6 ± 3.4 ($n = 3$), respectively. Gels with 15.7 μmol/mL hydrogel reactive sites and 0.3, 0.6, and 1.2 molar ratios of hexaglycine to hydrogel reactive sites, had swelling ratios of 19.3 ± 1.7 ($n = 2$), 16.3 ± 0.1 ($n = 2$), and 17.8 ± 1.7 ($n = 2$), respectively. For gels with 19.8 μmol/mL hydrogel reactive sites and 0.3, 0.6, and 1.3 molar ratios of hexaglycine to hydrogel reactive sites, the swelling ratios were 16.2 ± 0.1 ($n = 2$), 14.9 ± 0.1 ($n = 2$), and 15.7 ± 0.9 ($n = 2$), respectively.

When the total number of hydrogel reactive sites was held constant and the moles of hexaglycine added to the reaction were increased, the differences in swelling ratios were not statistically different. However, the swelling ratios for hydrogels with 4.4 μmol/mL reactive sites were statistically greater than those for hy-

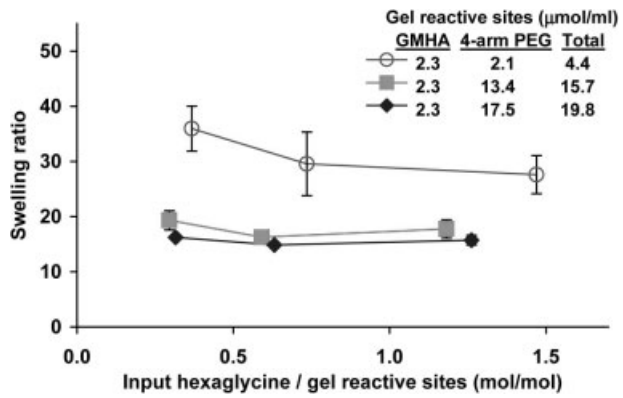

Figure 4. Swelling ratio of GMHA-PEG-peptide hydrogels. The swelling ratio is inversely related to the hydrogel crosslink density. The swelling ratios for hydrogels with 4.4 μmol/mL reactive sites are statistically greater than the hydrogels with either 15.7 or 19.8 μmol/mL reactive sites. The swelling ratios for hydrogels with 15.7 μmol reactive sites per milliliter are not statistically distinct from the hydrogels with 19.8 μmol reactive sites per milliliter. Within each set of hydrogels with equal numbers of gel reactive sites, the differences in swelling ratios are not statistically significant. Each point represents the average ± standard deviation.

drogels with either 15.7 or 19.8 μmol/mL reactive sites. Also, the difference in swelling ratios for the hydrogels with 15.7 μmol/mL reactive sites was not statistically different from those for the hydrogels with 19.8 μmol/mL reactive sites.

In vitro degradation by hyaluronidase

GMHA degrades in the presence of hyaluronidase,[4] but PEG does not. Thus, we determined the maximum concentration of acrylated 4-arm PEG beyond which the hydrogels are no longer degradable by hyaluronidase. The initial linear slope of gel mass versus time data [Fig. 5(a)] was used to calculate the *in vitro* degradation rates [Fig. 5(b)] of the GMHA–PEG hydrogels in 5 and 50 U/mL hyaluronidase.

In the presence of 5 U/mL hyaluronidase, the degradation rates for GMHA hydrogels (7.12 ± 1.80%/h, $n = 4$) and GMHA–PEG hydrogels with 1.0 μmol/mL reactive sites contributed by 4-arm PEG (3.76 ± 0.66%/h, $n = 4$) are statistically distinct and also greater than the degradation rates for the negative control (GMHA–PEG hydrogels incubated in citrate buffer alone; 0.98 ± 0.38%/h; $n = 3$) as well as the GMHA–PEG hydrogels with 2.3 μmol/mL or greater photoreactive sites contributed by 4-arm PEG. However, hydrogels with 2.3 μmol/mL (0.63 ± 0.15%/h; $n = 4$), 4.6 μmol/mL (0.83 ± 0.45%/h; $n = 4$), and 17.5 μmol/mL (0.94 ± 0.08%/h; $n = 2$) photoreactive sites contributed by 4-arm PEG were statistically indistinguishable from the negative control.

In the presence of 50 U/mL hyaluronidase, the degradation rates for GMHA hydrogels (30.22 ± 0.62%/h; $n = 2$) and GMHA–PEG hydrogels with 1.0 μmol/mL (32.02 ± 0.44%/h; $n = 4$) and 2.3 μmol/mL (32.22 ± 4.12%/h; $n = 3$) reactive sites contributed by 4-arm PEG are statistically similar, but greater than the degradation rates for the GMHA–PEG hydrogels with 4.6 μmol/mL (2.49 ± 0.30%/h; $n = 3$) and 17.5 μmol/mL (0.94 ± 0.08%/h; $n = 3$) 4-arm PEG photoreactive sites and also the negative control. In summary, in the presence of either 5 or 50 U/mL hyaluronidase, hydrogels with up to 1 μmol/mL 4-arm PEG reactive sites degraded, whereas those hydrogels with greater than 4.6 μmol/mL reactive sites contributed by 4-arm PEG were not degradable.

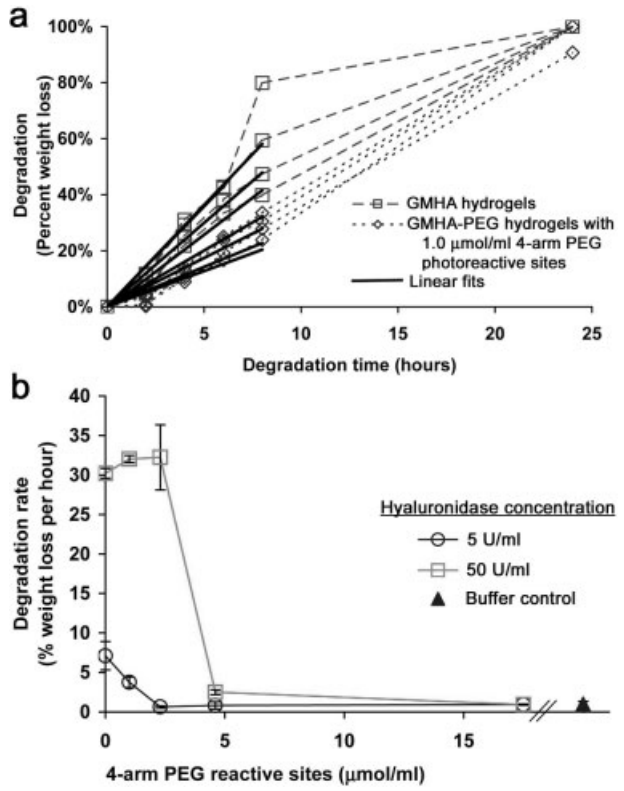

Figure 5. Degradation rate of GMHA–PEG hydrogels. (a) Representative raw data are shown for GMHA hydrogels and GMHA–PEG hydrogels with 1.0 μmol/mL 4-arm PEG reactive sites. Initial linear slopes were calculated from the linear fits of percent weight loss plotted versus degradation time in the presence of 5 U/mL hyaluronidase. (b) Degradation rates were calculated from the averaged linear slopes for each hydrogel type. For both hyaluronidase concentrations (5 and 50 U/mL), hydrogels with up to 1.0 μmol/mL 4-arm PEG photoreactive sites degraded, whereas gels with greater than 4.6 μmol/mL 4-arm PEG reactive sites were not degradable. Each point represents the average ± standard deviation.

DISCUSSION

The specific goals of this work were to modify GMHA hydrogels with peptide sequences and to determine the effects of the peptide conjugation on the hydrogels' physicochemical properties. To conjugate peptides onto GMHA, we investigated three complementary techniques: an EDC-mediated reaction on uncrosslinked GMHA and two photoactivated conjugations that occur during crosslinking (Fig. 1). To conserve cost, the conjugation chemistries were performed with a model peptide, hexaglycine, which we specifically chose for its simple chemical structure, as we intend for this work to serve as a stepping-stone to conjugations with more functional peptides (e.g., RGD).

First, we investigated an EDC-mediated reaction between the carboxylates of uncrosslinked GMHA and hexaglycine's primary amine (Fig. 2). This seemed to be a sensible approach because EDC-mediated reactions are often used for conjugating carboxylates and amines.[16] However, EDC reactions are highly dependent on pH and an excess of amine (e.g., peptide) is required for high levels of modification.[10,16] Furthermore, EDC-mediated reactions with HA have been found to be particularly challenging, because they readily convert HA's carboxylate to an unreactive *N*-acylurea.[10,17,18] We examined a variety of reaction conditions and found that the EDC-mediated GMHA–peptide conjugations resulted in very low reaction efficiencies (<10%).

Next, we investigated two methods of incorporating peptides during photocrosslinking. We hypothesized that a photoactivated reaction would allow higher conjugation frequencies as long as there are sufficient numbers of methacrylate or acrylate groups available for crosslinking as well as conjugation with acrylated PEG–peptides. Therefore, we sought to develop a chemistry that would yield stable hydrogels with conjugated peptide yields in the range of 1–5 μmol/mL. This range of peptide densities is based on those found to be optimal for cell adhesion and migration in nonadhesive PEG hydrogel systems.[7,14,15]

In Scheme I, acrylated PEG–peptides were mixed in a GMHA solution with a photoinitiator (Irgacure 2959) and a reaction accelerant (*N*-vinyl pyrrolidinone), and exposed to UV light. The acrylated PEG–peptides are incorporated into the hydrogel as the GMHA chains crosslink together. However, the solutions did not form stable hydrogels, possibly because there was an insufficient number of GMHA methacrylates available to contribute to both crosslinking and peptide modification.

In Scheme II, we supplemented the total number of available photoreactive reactive sites by adding acrylated 4-arm PEG to the Scheme I gelation solutions. By varying the concentrations of acrylated 4-arm PEG and acrylated PEG–peptides, we were able to synthesize hydrogels with a range of conjugation efficiencies, yields, swelling ratios, and enzymatic degradation rates.

To establish a working range of reactant concentrations, we first focused on conjugation efficiency [Fig. 3(a,b)] and yield [Fig. 3(c,d)]. We expected that for a constant peptide input concentration, greater numbers of hydrogel reaction sites available would allow higher conjugation efficiencies. But, although the Scheme II reactions more readily formed stable hydrogel–peptide conjugates than the Scheme I reactions, increased acrylated 4-arm PEG concentration did not always allow higher conjugation efficiencies. We hypothesize that in these cases, the peptide conjugation reaction could have been limited by diffusion because efficiency decreased as the gelation solutions became more concentrated. Regardless, the Scheme II GMHA-PEG-peptide composites had significantly higher conjugation efficiencies (20–80%, compared with <10% for the EDC-mediated reactions) and resulted in stable hydrogels with yields within the target range of 1–5 μmol/mL. Statistically significant trends were established between conjugation efficiency and hexaglycine concentration (inverse correlation; $p = 0.016$) and also between conjugation yield and hexaglycine concentration (direct correlation; $p < 0.0001$). In other words, for a constant number of acrylate sites available in the gel bulk, increasing the peptide concentration allowed more peptide to be conjugated, but this increased yield was met at a cost of decreased efficiency.

In addition to peptide density, the hydrogel structure can greatly impact cellular behavior.[15,19,20] Therefore, we measured the hydrogel swelling ratios (Fig. 4) as one means of determining the structural effects of varying acrylated 4-arm PEG and PEG–peptide concentrations. For a constant number of hydrogel reactive sites, increasing the concentration of acrylated PEG–peptide does not have a significant effect on swelling ratio. However, the hydrogels with the lowest number of reactive sites (4.4 μmol/mL) have statistically greater swelling ratios than the hydrogels with greater moles of hydrogel reactive sites. This makes sense because fewer reactive sites will result in a lower crosslink density, which will be reflected by a relatively higher swelling ratio.

In conclusion, we report a simple method to conjugate peptide sequences to photocrosslinkable GMHA hydrogels. The properties of the GMHA-PEG-peptide composites can be adjusted by varying acrylated 4-arm PEG and PEG–peptide concentration. These stable hydrogels had high conjugation efficiencies and yields, as well as defined swelling ratios and degradation rates. We believe that these GMHA-PEG-peptide composites are promising bio-

materials for a variety of scaffolding applications in soft tissue engineering.

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