Synthesis and Characterization of Novel Thiol-Reactive Poly(ethylene glycol) Cross-Linkers for Extracellular-Matrix-Mimetic Biomaterials

Janssen L. Vanderhooft,[†,§] Brenda K. Mann,[†] and Glenn D. Prestwich*[,†,‡,§]

Department of Bioengineering, Department of Medicinal Chemistry, and Center for Therapeutic Biomaterials, The University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, Utah 84108-1257

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Synthetic extracellular matrix hydrogels can be used for three-dimensional cell culture, wound repair, and tissue engineering. Using the bifunctional electrophile poly(ethylene glycol) diacrylate (PEGDA), thiol-modified glycosaminoglycans and polypeptides can be cross-linked into biocompatible materials in the presence of cells or tissues. However, the rate of in situ cross-linking with PEGDA under physiological conditions may occur too slowly for clinical applications requiring a fast-curing preparation. To explore a wider range of cross-linking time courses, five homo-bifunctional PEG derivatives were synthesized and examined as cross-linking agents for thiol-modified derivatives of hyaluronan (HA). Thiol reaction rate constants were measured over a pH range of 7.4 to 8.6. The order of reactivity for the functional groups used was determined to be maleimide > iodoacetate > bromoacetate > iodoacetamide > acrylate ≫ bromoacetamide, with rates increasing exponentially with increasing pH. The range of gelation times at physiological pH varied from less than 1 min to over 2 h. Addition of the cross-linkers to cell culture medium showed minimal cytotoxicity toward primary human dermal fibroblasts at concentrations anticipated during in situ cross-linking. Moreover, hydrogels prepared from thiol-modified gelatin and thiol-modified HA were biocompatible and supported attachment and proliferation of fibroblasts and hepatocytes.

Introduction

Cross-linked hydrogels are hydrophilic polymer networks that absorb more than 1000 times their dry weight in water, and the similarity of their physical properties to soft tissue has led to applications in tissue engineering.[1−3] The method of cross-linking—which may be physical, chemical, or photochemical—greatly affects the material properties of hydrogels, in particular the rate at which the shear strength increases. The rate at which the shear modulus increases becomes very important when the material is subjected to mechanical stresses, as in cardiovascular applications, or gravitational force, as in sinus surgery when the patient is moved upright.

Recently, hydrogels that mimic the extracellular matrix have been prepared and employed for scar-free healing, tissue engineering, and three-dimensional (3-D) cell culture.[4,5] These in situ cross-linkable synthetic extracellular matrix (sECM) hydrogels are based on chemically modified derivatives of hyaluronan (HA),[6] a nonsulfated glycosaminoglycan (GAG) component of the extracellular matrix (ECM) in all tissues, which promotes both cell mobility and proliferation while supporting tissue morphology.[7] Thus, thiol-modified HA (3,3′-dithio-*bis*-(propanoic dihydrazide) (HA-DTPH)) or thiol-modified carboxymethyl HA (CMHA-S) hydrogels cross-linked in situ with poly(ethylene glycol) (PEG)-based cross-linkers (Figure 1) have shown potential as cell-seeded injectable tissue engineering materials.[4,8−10] PEG has been widely used for the preparation of biologically relevant conjugates due to its wide array of useful properties such as solubility, non-biodegradability, and ease of excretion from living organisms.[11] Factors affecting hydrogel gelation rate include temperature, HA molecular weight, HA and cross-linker concentration, and cross-linker chemistry.[9] With suitable cross-linker chemistry, the rate of gelation can be greatly increased without affecting hydrogel density and cytocompatibility. Cytocompatiblity of these hydrogels has been maintained, and cell attachment and spreading enabled by covalently attaching Cys-modified RGD peptides,[12] functional fibronectin domains,[13] or thiol-modified gelatin (Gtn-DTPH)[14,15] during the cross-linking process. Protein−PEG graft copolymer hydrogels that include biomimetic adhesion signals and degradation domains provide cell-instructive microenvironments suitable for tissue engineering.[16−18] Increased local concentration of cell attachment sites without increased general attachment sites that may impair cell migration may be achieved using tetravalent cross-linkers to form peptide clusters.[19]

A limited number of cytocompatible cross-linkers with suitable reaction kinetics are available for the preparation of these hydrogels. Homo-bifunctional PEG cross-linkers modified with terminal *bis*-methacrylate, *bis*-acrylamide, and *bis*-methacrylamide functional groups were found to cross-link too slowly for clinical use, while the *bis*-acrylate (PEG diacrylate or PEGDA) formed a gel within 10 min.[9] Some surgical applications, such as cardiac surgery where the heart is in constant motion or as a tissue sealant during any surgical procedure, require faster cross-linking. The general reactivity trend of thiol-reactive groups is such that the faster reacting groups, such as acrylates, result in esters that are more prone to base-catalyzed hydrolysis than the slower reacting groups, such as acrylamides.[20] These limitations can be overcome through the synthesis of additional bifunctional thiol-reactive PEG derivatives that are cytocompatible, such as PEG divinyl sulfone.[21]

* Author to whom correspondence should be addressed. E-mail: gprestwich@pharm.utah.edu.
† Department of Bioengineering.
‡ Department of Medicinal Chemistry.
§ Center for Therapeutic Biomaterials.

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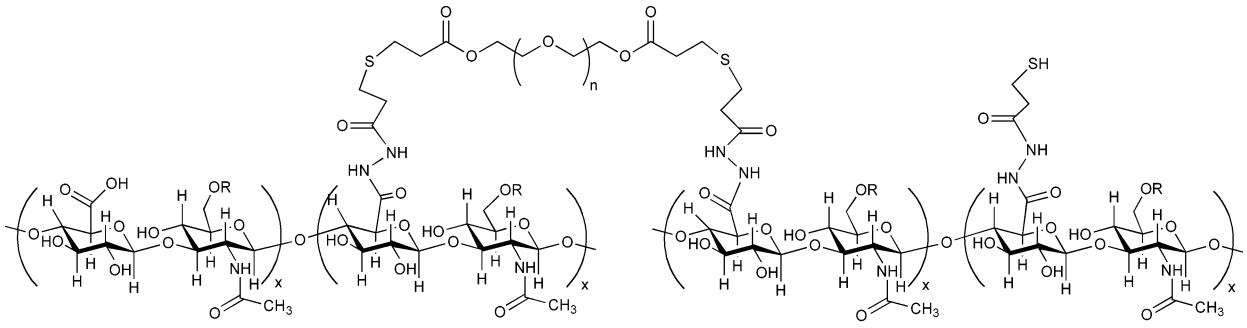

CMHA-SX; R = H or CH_2CO_2H

Figure 1. PEGDA cross-linked CMHA-S, a chemically modified HA derivative.

Herein we describe the synthesis and characterization of five alternative PEG-based cross-linkers for creating sECM hydrogels. Specifically, we determined rate constants for the new cross-linkers for reaction with thiols as a function of pH. We also investigated the cytotoxicity of these cross-linkers in vitro using human dermal fibroblasts (HDFs), and we document the suitability of gelatin-containing synthetic ECM hydrogels using HDFs and human HepG2-C3A hepatocytes.

Materials and Methods

General Synthetic Procedures. All materials and solvents were obtained from Sigma-Aldrich or Fisher-Acros unless otherwise indicated. NMR and functional kinetic assays were used to verify all products. Proton NMR data were obtained using a Varian INOVA 400 at 400 MHz. The materials synthesized were used directly in kinetic experiments and filter-sterilized for use in cytocompatibility experiments.

PEG *Bis*-bromoacetate (PEGDBrAc). PEGDBrAc was prepared by a modification of the method by Elbert.[20] Thus, 12.8 g of PEG (3400 g/mol) was lyophilized overnight and dissolved in anhydrous dichloromethane. Triethylamine (1.0 mL, 1 equiv relative to hydroxyl groups) was added dropwise, followed by dropwise addition of 1.3 mL (2 equiv) of bromoacetyl chloride under nitrogen. The reaction was stirred overnight in the dark. The product was purified by precipitation in diethyl ether. Then the product was dissolved in water, and the pH of the solution was adjusted to 6. The product was then extracted three times with 20 mL of dichloromethane and precipitated by addition of diethyl ether and stored at −20 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.3−3.7 (305 H, −O−CH_2−CH_2−O−), δ 4.15 (s, 4 H, −CH_2−Br), δ 4.2 (t, 4 H, −CH_2−COO−).

PEG *Bis*-iodoacetate (PEGDIAc). A sample of 6.0 g of **PEGDBrAc** was dissolved in acetone with mild heating (about 35 °C). The solution was cooled before stirring overnight with 6.0 g of NaI (>10 equiv) to exchange iodine for bromine via the Finkelstein reaction. The insoluble NaBr product was filtered out of the solution before **PEGDIAc** was precipitated with diethyl ether. Then the product was dissolved in water, and the pH of the solution was adjusted to 6. The product was then extracted three times with 20 mL of dichloromethane and precipitated with diethyl ether cooled on dry ice. The product was then stored at −20 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.3−3.7 (305 H, −O−CH_2−CH_2−O−), δ 3.8 (s, 4 H, −CH_2−I), δ 4.15 (t, 4 H, −CH_2−COO−).

PEG *Bis*-bromoacetamide (PEGDBrAm) and PEG *Bis*-iodoacetamide (PEGDIAm). PEGDBrAm and **PEGDIAm** were prepared similarly to their acetate analogues, using 2.5 g of polyoxyethylene *bis*-amine (3350 g/mol) as a starting material and the same equivalents of reagents to amine groups as was used for alcohol groups. **PEGDIAm** was synthesized via the Finkelstein reaction using 1.27 g of **PEGDBrAm**. ^{1}H NMR **PEGDBrAm** (400 MHz, DMSO-d_6): δ 3.03 (m, 2 H, −CH_2−NH−COO−), δ 3.1 (m, 4 H, −CH_2−NH−COO−), δ 3.3−3.7 (305 H, −O−CH_2−CH_2−O−), δ 4.02 (s, 4 H, −CH_2−Br), δ 4.02 (t, 4 H, −O−CH_2−CH_2−NH−COO−). ^{1}H NMR **PEGDIAm** (400 MHz, DMSO-d_6): δ 2.98 (m, 2 H, −CH_2−NH−COO−), δ 3.06 (m, 4 H, −CH_2−NH−COO−), δ 3.3−3.7 (305 H, −O−CH_2−CH_2−O−), δ 3.58 (s, 4 H, −CH_2−I), δ 4.02 (t, 4 H, −O−CH_2−CH_2−NH−COO−).

PEG *Bis*-maleimide (PEGDMal). Preparation of **PEGDMal** was modified from methods described by Kalgutkar et al.[22] and Liu et al.[23] Thus, 2.0 g of polyoxyethylene *bis*-amine (3350 g/mol) was dissolved in 6 mL of glacial acetic acid and stirred with 155 mg of maleic anhydride overnight to produce the PEG−maleamic acid. After precipitation in diethyl ether and drying under vacuum, the maleamic acid was dissolved in 5 mL of acetic anhydride with 60 mg of sodium acetate and heated on a boiling water bath for 2 h to close the ring and form the maleimide. The reaction was quenched with 15 mL of water, extracted three times with 15 mL of dichloromethane, and precipitated in diethyl ether. The product was dissolved in water, and the pH of the solution was adjusted to 6. The product was then extracted four times with 20 mL of dichloromethane and precipitated in diethyl ether cooled on dry ice. The product was then stored at −20 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.1 (m, 4 H, −O−CH_2−CH_2−N−), δ 3.3−3.7 (305 H, −O−CH_2−CH_2−O−), δ 4.0 (t, 4 H, −O−CH_2−CH_2−N−), δ 7.0 (s, 4 H, −CH═CH−).

***N*-Acetyl Cysteine Methyl Ester** (**AcCysOMe**) was prepared for kinetic studies according to Joseph et al.[24] and Conchillo et al.[25] Thus, 3.0 g of *N*-acetyl cysteine was dissolved in 50 mL of methanol containing 10 drops of concentrated (37%) HCl and refluxed for 4 h. The reaction solution was cooled to room temperature before elimination of the solvent under vacuum, leaving a viscous residue that crystallized overnight at room temperature. The residue was extracted three times with 40 mL of ethyl acetate, leaving some residue behind. The ethyl acetate extract was washed three times with 40 mL of brine before being dried with magnesium sulfate. The organic solvent was then removed under vacuum, leaving a viscous liquid that crystallized under stronger vacuum. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.04 (s, 3 H, −COO−CH_3), δ 2.98 (dq, 2 H, α-CH−CH_2−SH), δ 3.76 (s, −NH−CO−CH_3), δ 4.86 (dt, 1 H, α-CH−CH_2−SH), δ 6.43 (w, 1 H, −CH_2−SH).

CMHA-S and Gtn-DTPH. Thiol-modified carboxymethyl hyaluronic acid (CMHA-S) and thiol-modified gelatin (Gtn-DTPH) were synthesized in the Center for Therapeutic Biomaterials at the University of Utah as previously described.[8,14]

Kinetics−Thiol Consumption. Thiol reaction kinetics of each cross-linker were monitored using a modified 2-nitro-5-thiosulfobenzoate (NTSB) colorimetric assay.[26] An NTSB stock solution was prepared by dissolving 100 mg of 5,5′-dithio-*bis*-2-nitrobenzoic acid (DTNB, Ellman's reagent) in 1 M Na_2SO_3, adjusting the pH to 7.5 and bubbling oxygen through the solution in a 38 °C water bath until the color changed from a bright orange to a pale yellow, indicating cleavage of

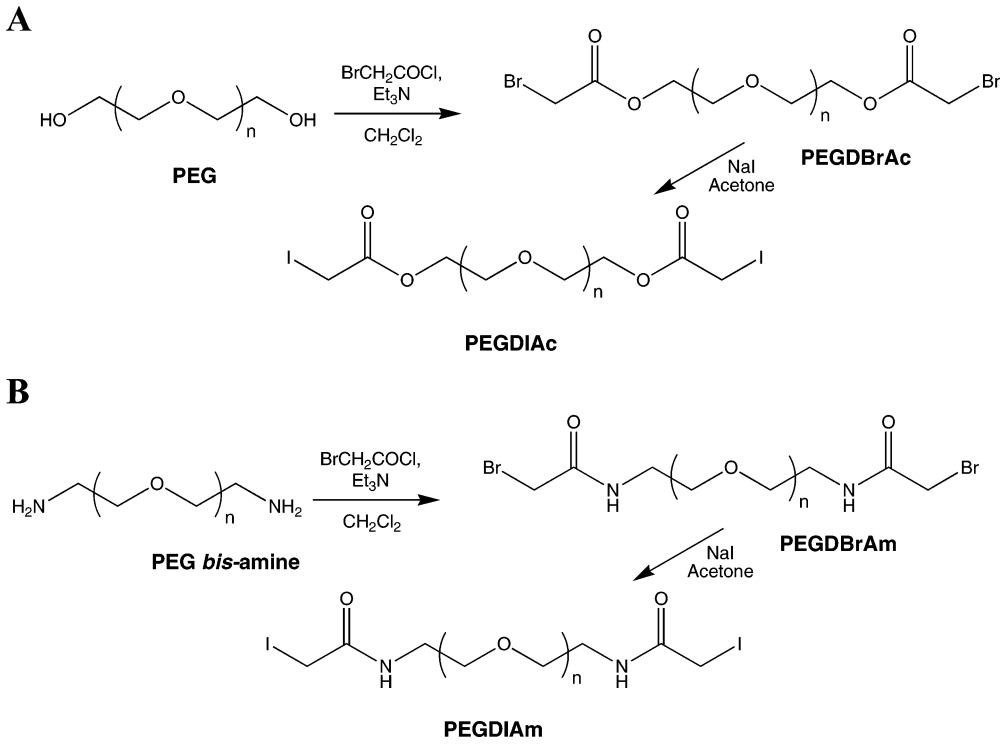

Figure 2. (A) PEG-*bis*-haloacetate synthesis. (B) PEG-*bis*-haloacetamide synthesis.

the disulfide bond to form NTSB. This stock solution was stored at −30 °C until needed. The NTSB assay solution was prepared by diluting 1 mL of stock solution with 100 mL of buffer at pH 9.5 containing 100 mM Na_2SO_3 and 5 mM EDTA. Triplicate 1 mL aliquots of each bifunctional PEG derivative (70 mg/mL), including **PEGDA** for comparison, were allowed to react at room temperature (21 ± 1 °C) with 4 mL of **AcCysOMe** (5.16 mM). An electrophile/thiol ratio of 2:1 was used in 10× phosphate-buffered saline (PBS) at pH 7.4, 7.8, 8.2, and 8.6. At each time point, 100 μL of the reaction solution was removed and quenched with 3 mL of the NTSB assay solution. This assay mixture was then stored in the dark to prevent photodegradation of the colorimetric species, 2-nitro-5-thiobenzoate (NTB), before absorbance at 412 nm was measured. The resulting spectrophotometric data showed the consumption kinetics of thiol groups by the PEG electrophiles. Apparent first-order rate constants k_1 were calculated by finding the slope of the $\ln(A_{412})$ versus time plot.[27]

Kinetics—Gelation. A functional kinetic assay was also employed by cross-linking CMHA-S with each PEG cross-linker, including **PEGDA** for comparison, and measuring the gel transition time using the test tube inversion method.[28] Briefly, 4.5 mg of cross-linker was dissolved in 100 μL of 1× PBS pH 7.4 and mixed with 400 μL of 1.5% CMHA-S in a test tube, giving a final cross-linker concentration of 0.9% (w/v). The tube was then inverted at regular intervals until the solution ceased to flow. Gelation time was determined to be the point at which the material would no longer flow under the force of gravity.

Cell Maintenance. Normal HDFs were obtained from Cambrex. Hep-G2-C3A cells were obtained from the American Type Culture Collection. HDFs were maintained on Dulbecco's Modified Eagle Medium (Gibco) containing 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Gibco). Hep-G2-C3A cells were maintained on Eagle's minimal medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37 °C/5% CO_2. HDFs were used at passage number 8, and Hep-G2-C3A cells were used at passage number 30.

Cytotoxicity. HDFs were seeded in 24-well plates at 10^5 cells/mL and then cultured on tissue culture polystyrene until they were 90% confluent. The medium was then changed to include 1, 10, or 25 mg/mL (0.1, 1.0, or 2.5% (w/v)) of each cross-linker, and the plates were incubated for 4 h. The medium was removed, and the plates were frozen at −80 °C. After thawing, the relative cell number in each well was determined using the Cy-QUANT fluorometric assay (Molecular Probes).

Cell Culture on Hydrogel Surfaces. Hydrogels for surface cell culture were prepared by dissolving CMHA-S and Gtn-DTPH separately in 1× PBS at 1% (w/v) and then adjusting the pH to 7.4. The solutions were then mixed in a 1:1 volumetric ratio and filter-sterilized (0.45 μm filter). This polymer solution was then dispensed into 96-well tissue culture plates. Each of the cross-linkers was dissolved at 2% (w/v) in 1× PBS and filter-sterilized (0.45 μm filter). Cross-linker solution was then added to the wells in a 4:1 polymer to cross-linker volumetric ratio, and the resulting solutions were mixed, giving a final cross-linker concentration of 0.4% (w/v). These solutions were allowed to gel at room temperature for 2 h, after which they were covered with excess PBS and allowed to reach equilibrium swelling overnight at 4 °C. The PBS was then replaced with cell culture media, and the gels were incubated at 37 °C for 4 h before seeding with cells.

HDFs and Hep-G2-C3A cells were seeded on the surface of the hydrogels at 10^4 cells/well. The plates were incubated at 37 °C/5% CO_2 with regular media changes. At 3, 7, and 17 days, the metabolic activity was quantified using the MTS assay. MTS, or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt is the active agent for detecting metabolic activity using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).

Statistical Analysis. Kinetic and cytotoxicity experiments were performed in triplicate. Cell culture experiments were performed with five replicates. The significance of cell number reduction was tested using a lower-tailed *t*-test, and $p < 0.05$ was considered significant. Error bars in the resulting figures for both kinetic and cell culture assays were calculated as the standard error of the mean.

Results

PEGDA has been used extensively in biomaterials and tissue engineering both as a base material and as a cross-linker in forming hydrogels. Alternative PEG-based cross-linkers, how-

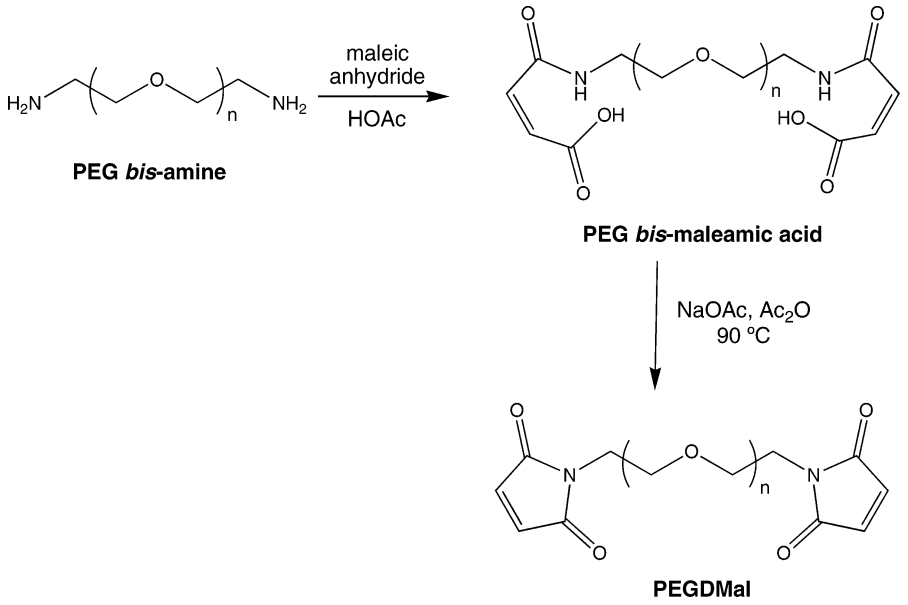

Figure 3. PEG-*bis*-maleimide synthesis.

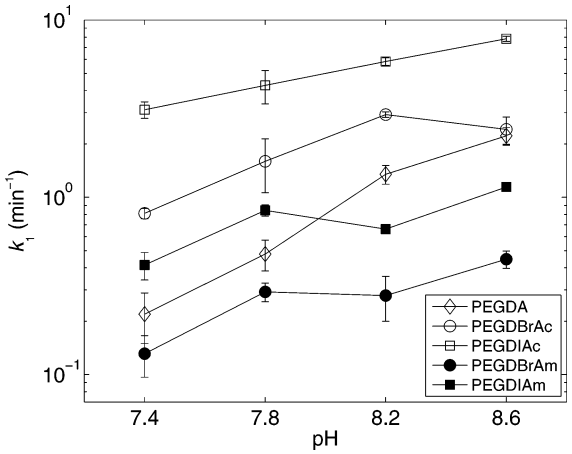

Figure 4. Rate constants for thiol consumption by PEG cross-linkers, including **PEGDA** as a control.

ever, may provide flexibility when making biomaterials in the method of cross-linking, time of cross-linking, and stability of the cross-links formed. Here, five different PEG-based cross-linkers were synthesized and the time to cross-link a thiol-modified hyaluronic acid and cytocompatibility were compared to **PEGDA**.

Synthesis. As illustrated in Figure 2, **PEGDBrAc** was prepared by acylation of PEG 3400 with bromoacetyl chloride. The corresponding **PEGDIAc** was prepared from **PEGDBrAc** by a Finkelstein reaction in which the bromo substituent was displaced by iodide in an S_N2 reaction driven to completion by the precipitation of sodium bromide from acetone solution. **PEGDBrAm** and **PEGDIAm** were prepared analogously using PEG 3350 *bis*-amine as the starting material.

PEGDMal was prepared from PEG 3350 *bis*-amine in a two-step process (Figure 3). First, reaction of the *bis*-amine in glacial acetic acid with maleic anhydride provided the intermediate PEG−maleamic acid. Heating this intermediate with sodium acetate in acetic anhydride at 90 °C for 2 h led to cyclization of both maleimide rings to give **PEGDMal**.

The characteristic ^{1}H NMR chemical shifts and multiplicities of α-carbon protons allowed the reactions to be followed quantitatively. Acylation or amidation led to downfield shifts, while replacement of Br with I led to upfield shifts. Signals from −O*H* and −NH_2 protons were not detected in the purified product, indicating 100% conversion for all cross-linkers. Furthermore, iodinated cross-linkers also showed no signals from the −CH_2−Br protons from which they were derived. To purify the cross-linkers with bulky end groups, e.g., iodoacetate, iodoacetamide, and maleimide, diethyl ether had to be cooled on dry ice to recrystallize the product. Use of the nonsolvent at room temperature resulted in the formation of oils. Unfortunately, this low-temperature technique in a low humidity environment afforded fine powdery products susceptible to static cling and air movement.

Table 1. Gel Transition Time

cross-linker	gelation time (min)
PEGDA	36
PEGDMal	<0.5
PEGDIAc	3
PEGDBrAc	14
PEGDIAm	33
PEGDBrAm	~120

Reaction Kinetics. Thiol reaction kinetics for each cross-linker were determined by reacting the cross-linkers with **AcCysOMe** and monitoring thiol consumption using a modified NTSB assay. Apparent first-order rate constants k_1 for thiol consumption by PEG cross-linkers at varying pH values were determined by finding the initial slope of the plot of the natural logarithm of A_{412} versus time. These results are shown in Figure 4 for all cross-linkers except **PEGDMal**, for which the reaction was complete in less than 15 s at all pH values. Following **PEGDMal**, **PEGDIAc** was found to be the most reactive, while **PEGDBrAm** was the least reactive.

The cross-linkers were also used to cross-link CMHA-S, a novel thiol-modified derivative of HA that has shown superior in vitro and in vivo results to HA-DTPH in cell culture[5] and tissue engineering models.[4,10] The gelation time was monitored using a test tube inversion assay. This assay determined the time necessary for the material to no longer flow under force of gravity, which was found to be more reliable for visual observation than the onset of gelation. The same reactivity trend for the cross-linkers was observed in this assay, shown in Table 1, as was found above for thiol consumption.

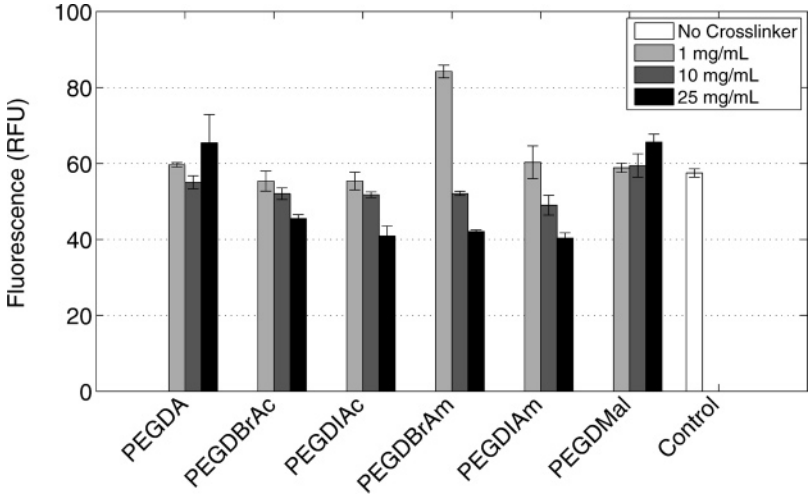

Figure 5. Cell count after 4 h exposure to PEG cross-linker.

Cytotoxicity. Cytotoxicity of the cross-linkers was determined by incubating the cross-linkers (0.1, 1.0, or 2.5% (w/v) in media) for 4 h with HDFs grown on tissue culture plates. The cross-linker concentrations were chosen to bracket concentrations typically used to cross-link CMHA-S and HA-DTPH (0.8−0.9% (w/v)).[4,5,9,10,12,15,21] A period of 4 h was chosen to double the maximum gelation time observed above to examine extreme cases of cross-linker exposure. A Cy-QUANT fluorometric assay was then used to determine relative cell numbers. For all cross-linkers used at 0.1% (w/v), there was no significant decrease in cell number compared to unexposed controls (lower-tailed *t*-test, $p > 0.10$ for all samples) (Figure 5). As the concentration of the cross-linker in the medium increased, the cells began to show sensitivity to four of the cross-linkers (**PEGDIAc**, **PEGDBrAc**, **PEGDIAm**, and **PEGDBrAm**), particularly at 2.5% (w/v). While it is unlikely that cells would be exposed to the cross-linkers for such an extended period of time, given that the longest cross-linking time observed (for **PEGDBrAm**) was 2 h, it should be noted that cytotoxicity should always be determined for target cell types at the concentration and exposure time expected for a given application.

Cell culture. To assess the cytocompatibility of hydrogels made using the different cross-linkers, thin hydrogels composed of CMHA-S, Gtn-DTPH, and a cross-linker were created in 96-well tissue culture plates. HDFs or Hep-G2-C3A cells were then seeded on top of the hydrogels and incubated for up to 17 days. The metabolic activities of the cells on the different gels were determined at 3, 7, and 17 days using an MTS assay and are shown in Figures 6 and 7. The MTS assay, in this case, can generally be considered an indicator of cell numbers.

For HDFs, similar cell numbers were found at 3 days on all gels except those made with **PEGDBrAm**. At 7 days, cell numbers were lower on both bromine-containing gels. The cells appeared to have recovered, however, on both of these gels by 17 days. For Hep-G2-C3A cells, similar cell numbers were found at 3 days on all gel types. At 7 days, cell numbers were again lower on both bromine-containing gels, as well as on **PEGDMal** gels. By 17 days, cell numbers were once again similar on all gel types.

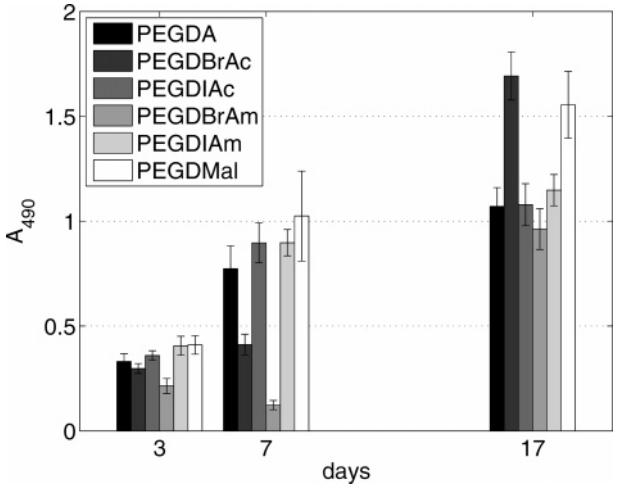

Figure 6. Cell viability (MTS assays) for HDFs grown on materials cross-linked with newly synthesized cross-linkers compared with cells grown on materials cross-linked with **PEGDA**.

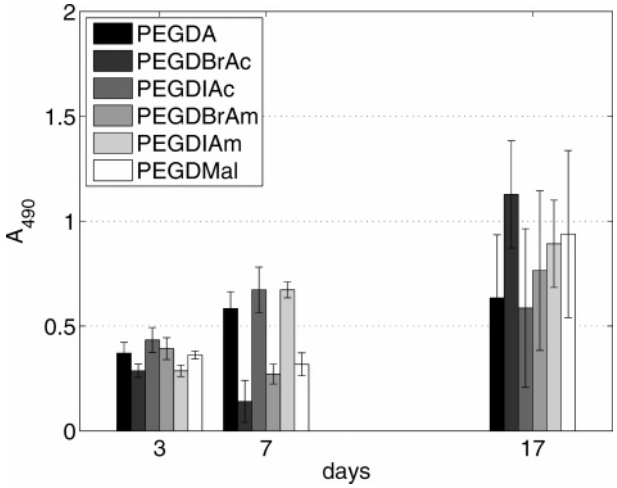

Figure 7. Cell viability (MTS assays) for Hep-G2-C3A cells grown on materials cross-linked with newly synthesized cross-linkers compared with cells grown on materials cross-linked with **PEGDA**.

Discussion

The objective of this work was to investigate the feasibility of using alternative PEG-based cross-linkers to **PEGDA** that would exhibit a faster rate of cross-linking and yet remain cytocompatible during in situ cross-linking. Thus, the cross-linkers **PEGDBrAc**, **PEGDIAc**, **PEGDBrAm**, **PEGDIAm**, and **PEGDMal** were synthesized and characterized in terms of their kinetics in cross-linking thiol-containing compounds and their cytocompatibility related to one another and to PEGDA. All

syntheses performed here have two or fewer reaction steps, making them straightforward to synthesize with less potential for impurities. However, further optimization of the recrystallization procedure may lead to a larger crystal size, making the materials easier to handle. This was particularly problematic for the *bis*-maleimide, *bis*-iodoacetate, and *bis*-iodoacetamide derivatives.

The reaction kinetics of three of the new cross-linkers (**PEGDMal**, **PEGDIAc**, and **PEGDBrAc**) were faster than **PEGDA**, one was similar (**PEGDIAm**), and one was slower (**PEGDBrAm**). The overall reactivity trend for the PEG derivatives studied is **PEGDMal** > **PEGDIAc** > **PEGDBrAc** > **PEGDIAm** > **PEGDA** ≫ **PEGDBrAm**, with reactivity increasing with increasing pH due to the thiolate anion being the reactive sulfur species. This observed reactivity trend agrees with that found in the literature,[29] in that the maleimide reacts much faster than the acetates and the acetamides. This reactivity trend was also observed during the functional gelation assay. While only one cross-linker concentration was used in the gelation assay, a concentration typically used for producing such sECMs,[4,5,9,10,12,15,21] to demonstrate the reactivity trend, it should be noted that a different cross-linker concentration may be used to change the gelation time or degree of cross-linking to tailor the material to a particular application as shown by Ghosh et al. for **PEGDA**.[21] This method, however, would also increase the total cross-linking density and therefore alter the terminal mechanical properties of the gel.

However, understanding the reactivity trend itself may provide the initial information for choosing a cross-linker for a given application. For example, a gel may be needed that can be applied directly to a beating heart following cardiac surgery to prevent postsurgical adhesions. The faster cross-linkers may be needed in such a case as they would afford a faster increase in viscosity than **PEGDA** upon application of the HA gel and reduce the potential for the pregel solution to be sheared off the pericardial surface prior to complete gelation and mucoadhesion. In addition, cross-linkers that result in a thioether−imide linkage that is more hydrolytically labile than a thioether−ester linkage may have some advantages. When placed directly in the cell culture medium of fibroblasts, low concentrations (0.1% (w/v)) of the cross-linkers did not appear to be cytotoxic. In fact, **PEGDBrAm** at that concentration appeared to stimulate cell proliferation. While the reaction kinetics of this cross-linker render it unlikely to be of practical use in an in situ clinical application, it may find utility in other applications where slower reaction times would be beneficial, such as in large-scale production of hydrogel-based biomaterials. The highest concentration of cross-linker (2.5% (w/v)) did exhibit signs of cytotoxicity, aside from **PEGDA** and **PEGDMal**, although the conditions examined here (particularly the 4 h exposure time) are likely exaggerated for most applications. As previously mentioned, 0.9% (w/v) has typically been used for cross-linking CMHA-S and HA-DTPH. Additionally, the maximum gelation time to a solid for the cross-linkers here at 0.9% (w/v) was 2 h, with most of the gelation times at about 30 min or less. These results do indicate, however, the need to test potential new cross-linkers for cytotoxicity if cells and/or tissue are likely to be exposed to them. Future work will examine viability and behavior of cells encapsulated in hydrogels cross-linked with the various cross-linkers. For **PEGDA** and **PEGDMal**, no correlation was observed between cross-linker concentration and cytotoxicity. **PEGDMal** is known to react 1000 times faster with thiols than with amines,[29] making it reactive with far fewer biological functional groups exposed on the surfaces of cells.

For two different cell types cultured on the surfaces of hydrogels made using the different cross-linkers, the bromine-containing cross-linked materials appeared to have some short-term toxic effects. At 7 days, both cell types showed a decrease in activity when grown on gels cured with the cross-linkers containing bromine, namely, **PEGDBrAc** and **PEGDBrAm**. The cells grown on these materials did recover by day 17, when all materials showed cytocompatibility comparable to the **PEGDA** control. It is unclear at this point what may have influenced the cells on the gels made with bromine-containing cross-linkers compared to the other gels given the fact that the cytotoxicity study did not demonstrate such specific potential toxicity to those two cross-linkers. Given the fact that all of the cross-linkers made here demonstrated complete conversion, it is unlikely that there were any significant differences in mechanical properties of the resultant hydrogels that may have affected cell growth. However, we are currently characterizing mechanical properties of these materials. Also, as previously mentioned, future work will examine cells encapsulated within hydrogels made using these cross-linkers, and such studies may shed light on other aspects of cell behavior affected by the cross-linkers themselves.

In conjunction with **PEGDA**, the other PEG-based cross-linkers synthesized and characterized here provide a spectrum of compounds that may be useful in cross-linking thiol-containing compounds, with different reaction rates to choose from. Further, the results of this study show the potential of **PEGDMal** as an alternative cytocompatible cross-linker for use in applications that require a very fast gelation time. Further investigation regarding the biocompatibility of these cross-linkers will be performed, including in vitro cell culture within gels and in vivo studies of gels made using the new cross-linkers. Additionally, degradation of gels cross-linked with these compounds will be examined to assess both hydrolytic and enzymatic stability for varying degrees of cross-linking, which may also influence the choice of a particular cross-linker and its concentration for a given application.

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Supporting Information Available. NMR spectra for **PEGDBrAc**, **PEGDIAc**, **PEGDBrAm**, **PEGDIAm**, and **PEGDMal**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Lee, K.; Mooney, D. Hydrogels for tissue engineering. *Chem. Rev.* **2001**, *101*, 1869−1879.
(2) Nguyen, K. T.; West, J. L. Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials* **2002**, *23*, 4307−4314.
(3) Kirker, K. R.; Prestwich, G. D. Physical properties of glycosaminoglycan hydrogels. *J. Polym. Sci. Part B: Polym. Phys.* **2004**, *42*, 4344−4356.
(4) Prestwich, G. D.; Shu, X. Z.; Liu, Y.; Cai, S.; Walsh, J. F.; Hughes, C. W.; Kirker, K. R.; Orlandi, R. R.; Park, A. H.; Thibeault, S. L.; Smith, M. E. Injectable synthetic extracellular matrices for tissue engineering and repair. *Adv. Exp. Med. Biol.* **2006**, *585*, 125−133.
(5) Prestwich, G. D.; Liu, Y.; Yu, B.; Shu, X. Z.; Scott, A. 3-D Culture in synthetic extracellular matrices: New tissue models for drug toxicology and cancer drug discovery. *Adv. Enzyme Regul.*, published on line Feb. 28, 2007.

(6) Shu, X. Z.; Prestwich, G. D. Therapeutic biomaterials from chemically modified hyaluronan. In *Chemistry and Biology of Hyaluronan*; Garg, H. G., Hales, C. A., Eds.; Elsevier Press: Amsterdam, 2004; pp 475−504.
(7) Knudson, C. B.; Knudson, W. Cartilage proteoglycans. *Semin. Cell Dev. Biol.* **2001**, *12*, 69−78.
(8) Shu, X. Z.; Liu, Y.; Luo, Y.; Roberts, M. C.; Prestwich, G. D. Disulfide cross-linked hyaluronan hydrogels. *Biomacromolecules* **2002**, *3*, 1304−1311.
(9) Shu, X. Z.; Liu, Y.; Palumbo, S.; Luo, Y.; Prestwich, G. D. In situ crosslinkable hyaluronan hydrogels for tissue engineering. *Biomaterials* **2004**, *25*, 1339−1348.
(10) Duflo, S.; Thibeault, S. L.; Li, W.; Shu, X. Z.; Prestwich, G. D. Vocal fold tissue repair in vivo using a synthetic extracellular matrix. *Tissue Eng.* **2006**, *12*, 3201−3207.
(11) Zalipsky, S. Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconjugate Chem.* **1995**, *6*, 150−165.
(12) Shu, X. Z.; Ghosh, K.; Liu, Y.; Palumbo, S.; Luo, Y.; Clark, R. A.; Prestwich, G. D. Attachment and spreading of fibroblasts on an RGD peptide-modified injectable hyaluronan hydrogel. *J. Biomed. Mater. Res., Part A* **2004**, *68*, 365−375.
(13) Ghosh, K.; Ren, X.-D.; Shu, X. Z.; Prestwich, G. D.; Clark, R. A. F. Fibronectin functional domains coupled to hyaluronan stimulate primary human dermal fibroblast responses critical for wound healing. *Tissue Eng.* **2006**, *12*, 601−613.
(14) Shu, X. Z.; Liu, Y.; Palumbo, F.; Prestwich, G. D. Disulfide-crosslinked hyaluronan−gelatin hydrogel films: A covalent mimic of the extracellular matrix for in vitro cell growth. *Biomaterials* **2003**, *24*, 3825−34.
(15) Shu, X. Z.; Ahmad, S.; Liu, Y.; Prestwich, G. D. Synthesis and evaluation of injectable, in situ crosslinkable synthetic extracellular matrices (sECMs) for tissue engineering. *J. Biomed. Mater. Res., Part A* **2006**, *79*, 902−912.
(16) Halstenberg, S.; Panitch, A.; Rizzi, S.; Hubbell, J. A. Biologically engineered protein-graft-poly(ethylene glycol) hydrogels: A cell adhesive and plasmin-degradable biosynthetic material for tissue repair. *Biomacromolecules* **2002**, *3*, 710−723.
(17) Sakiyama-Elbert, S.; Hubbell, J. A. Functional biomaterials: Design of novel biomaterials. *Annu. Rev. Mater. Res.* **2001**, *31*, 183−201.
(18) Lutolf, M. P.; Hubbell, J. A. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat. Biotechnol.* **2005**, *23*, 47−55.
(19) Lutolf, M. P.; Hubbell, J. A. Synthesis and physiochemical characterization of end-linked poly(ethylene glycol)-*co*-peptide hydrogels formed by Michael-type addition. *Biomacromolecules* **2003**, *4*, 713−722.
(20) Elbert, D. L.; Hubbell, J. A. Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering. *Biomacromolecules* **2001**, *2*, 430−441.
(21) Ghosh, K.; Shu, X. Z.; Mou, R.; Lombardi, J.; Prestwich, G. D.; Rafailovich, M. H.; Clark, R. A. F. Rheological characterization of in situ crosslinkable hyaluronan hydrogels. *Biomacromolecules* **2005**, *6*, 2857−2865.
(22) Kalgutkar, A. S.; Crews, B. C.; Marnett, L. J. Design, synthesis, and biochemical evaluation of N-substituted maleimides as inhibitors of prostaglandin endoperoxide synthases. *J. Med. Chem.* **1996**, *39*, 1692−1703.
(23) Liu, X.-h.; Wang, H.-k.; Herron, J. N.; Prestwich, G. D. Photopatterning of antibodies on biosensors. *Bioconjugate Chem.* **2000**, *11*, 755−761.
(24) Joseph, J. T.; Elmore, J. D.; Wong, J. L. Comparative sulfhydryl reaction pathways of chlorooxirane and chloroacetaldehyde. *J. Org. Chem.* **1990**, *55*, 471−474.
(25) Conchillo, A.; Camps, F.; Messeguer, A. 3,4-Epoxyprecocenes as models of cytotoxic epoxides: Synthesis of the *cis* adducts occurring in the glutathione metabolic pathway. *J. Org. Chem.* **1990**, *55*, 1728−1735.
(26) Thannhauser, T. W.; Konishi, Y.; Scheraga, H. A. Analysis for disulfide bonds in peptides and proteins. *Methods Enzymol.* **1987**, *143*, 115−119.
(27) Morpugo, M.; Veronese, F. M.; Kachensky, D.; Harris, J. M. Preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjugate Chem.* **1996**, *7*, 363−368.
(28) Jeong, B.; Bae, Y. H.; Kim, S. W. Thermoreversible gelation of PEG-PLGA-PEG triblock copolymer aqueous solutions. *Macromolecules* **1999**, *32*, 7064−7069.
(29) Hermanson, G. T. 2.2 Maleimides. In *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1996; p 148.

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