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In situ crosslinkable hyaluronan hydrogels for tissue engineering

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Abstract

We describe the development of an injectable, cell-containing hydrogel that supports cell proliferation and growth to permit in vivo engineering of new tissues. Two thiolated hyaluronan (HA) derivatives were coupled to four α,β-unsaturated ester and amide derivatives of poly(ethylene glycol) (PEG) 3400. The relative chemical reactivity with cysteine decreased in the order PEG-diacrylate (PEGDA) $\gg$ PEG-dimethacrylate > PEG-diacrylamide > PEG-dimethacrylamide. The 3-thiopropanoyl hydrazide derivative (HA-DTPH) was more reactive than the 4-thiobutanoyl hydrazide, HA-DTBH. The crosslinking of HA-DTPH with PEGDA in a molar ratio of 2:1 occurred in approximately 9 min, suitable for an in situ crosslinking applications. The in vitro cytocompatibility and in vivo biocompatibility were evaluated using T31 human tracheal scar fibroblasts, which were suspended in medium in HA-DTPH prior to addition of the PEGDA solution. The majority of cells survived crosslinking and the cell density increased tenfold during the 4-week culture period in vitro. Cell-loaded hydrogels were also implanted subcutaneously in the flanks of nude mice, and after immunohistochemistry showed that the encapsulated cells retained the fibroblast phenotype and secreted extracellular matrix in vivo. These results confirm the potential utility of the HA-DTPH-PEGDA hydrogel as an in situ crosslinkable, injectable material for tissue engineering.

Keywords: Glycosaminoglycan; Cell encapsulation; In vivo biocompatibility; Polyethylene glycol; Diacrylates; Cell-compatible crosslinking

1. Introduction

Hydrogels are crosslinked hydrophilic polymer networks, and may absorb more than 1000 × their dry weight in water, giving them physical characteristics similar to soft tissues. These biocompatible materials can be ideal for clinical applications [1], since adverse reactions are minimized [2–4]. Swelling and hydration occur without dissolution of the polymer, since the process of crosslinking creates an insoluble network. In addition, hydrogels are highly permeable, which facilitates exchange of oxygen, nutrients, and other water-soluble metabolites. Over the past three decades, chemically and physically diverse hydrogels have become standard materials for drug delivery, contact lenses, corneal implants, and scaffolds for the regeneration of new skin, encapsulation of cells, and regeneration of tendons, and cartilage [5–8].

A major limitation of most scaffold materials used for tissue engineering is the need for surgical implantation. For many clinical uses, injectable in situ crosslinkable hydrogels would be strongly preferred for three main reasons. First, an injectable material could be formed into any desired shape at the site of injury. Because the initial materials could be sols or moldable putties, the systems may be positioned in complex shapes and then subsequently crosslinked to conform to the required dimensions. Second, the crosslinkable polymer mixture would adhere to the tissue during gel formation, and the resulting mechanical interlocking that would arise from surface microroughness would strengthen the tissue-hydrogel interface. Third, introduction of an in situ crosslinkable hydrogel could be accomplished by injection or laparoscopic methods, thereby minimizing the invasiveness of the procedure. During the last decade, many potential applications have been examined to-date for natural and synthetic polymeric systems [9–26].

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Development of an injectable hydrogel for tissue repair or tissue regeneration also presents considerable challenges. The gelation conditions for in vivo use are limited to a narrow range of physiologically acceptable temperatures, and the crosslinking must occur with no byproducts in a sensitive aqueous environment. Reagents must be nontoxic reagents and tolerant of moist, oxygen-rich environments. Furthermore, gelation must occur at a sufficiently rapid rate for clinical use in an outpatient or operating suite setting, yet sufficiently slow that complete mixing occurs prior to gelation. Hydrogels formed from photopolymerization of α,β-unsaturated esters and amides of PEG meet most of these requirements, and are consequently commonly used in tissue engineering [4,12,17,18,20–23,27–31]. However, there are limitations: the reactions may be too exothermic, particularly during radical polymerization, and in most cases photoinitiators must be used. Even though transdermal photopolymerization had been developed as a clinical approach, in many cases photopolymerization has not proven suitable for injectable in vivo use, despite the development of a transdermal photoactivation methodology [32].

Hyaluronan (HA) is a major constituent of the extracellular matrix (ECM), and is the only non-sulfated glycosaminoglycan (GAG) [33]. This polyanionic GAG is biocompatible, and biodegradable, and performs important biological functions, such as stabilizing and organizing the ECM [34,35], regulating cell adhesion and motility [35,36], and mediating cell proliferation and differentiation [37]. Thus, HA and its derivatives are now widely used in medicine [38–49]. Recently, new crosslinking strategies were developed in order to prepare HA-based hydrogels, including the disulfide crosslinking of thiolated HA derivatives [50]. The resulting isulfide-crosslinked materials were cytocompatible, and murine L-929 fibroblasts, which were encapsulated in situ during air-induced hydrogel formation, remained viable and proliferated in vitro. However, the disulfide crosslinking reaction was too slow for injectable cell delivery.

Herein, we describe a new methodology to obtain chemically novel hydrogels in which thiol-modified GAGs can be crosslinked in situ in a fashion suitable for both cell encapsulation and in vivo injection, with subsequent tissue production. First, thiolated GAGs were synthesized [50,51] and then hydrogels were fabricated based on the conjugate addition of thiols to α,β-unsaturated esters and amides of PEG. The resulting injectable hydrogels were evaluated in vitro with cultured human tracheal scar fibroblasts and by growth of new fibrous tissue in nude mice in vivo from in situ crosslinkable hydrogels seeded with human fibroblasts.

2. Materials and methods

2.1. Materials

Fermentation-derived hyaluronan (HA, sodium salt, M_w 1.5 MDa) was provided by Clear Solutions Biotechnology, Inc. (Stony Brook, NY). 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDCI), PEG acrylate (M_w 375) and PEG (M_w 3400 Da) were purchased from Aldrich Chemical Co. (Milwaukee, WI). PEG diamine (M_w 3400 Da, percent of substitution: 100% (^{1}H NMR) and 95% (HPLC)) was purchased from Shearwater Polymers (Huntsville, AL). Dulbecco's phosphate buffered saline (DPBS), cysteine, and bovine testicular hyaluronidase (HAse, 330 U/mg) were obtained from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) was purchased from Diagnostic Chemical Ltd. (Oxford, CT). 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB) was purchased from Acros (Houston, TX). Both 3,3′-dithiobis (propanoic dihydrazide) (DTP) and 4,4′-dithiobis(butanoic dihydrazide) (DTB) were synthesized as described [50,52].

2.2. Analytical instrumentation

Proton NMR spectral data were obtained using a Varian INOVA 400 at 400 MHz and gel permeation chromatography (GPC) analysis was performed using a Waters 515 HPLC pump, differential refractometer, tunable absorbance detector and Ultrahydrogel 250 or 1000 columns (7.8 mm i.d. × 130 cm) (Milford, MA). The eluent was 200 mM phosphate buffer (pH 6.5): MeOH = 80:20 (v/v) and the flow rate was either 0.3 or 0.5 ml/min. The system was calibrated with standard HA samples provided by Dr. U. Wik (Pharmacia, Uppsala, Sweden). Fluorescence images of viable cells were recorded using laser scanning confocal microscopy (LSM 510 Carl Zeiss Microimaging, Inc., Thornwood, NY). Cell proliferation was determined by MTS assay at 550 nm, which was recorded on an OPTImax microplate reader (Molecular Devices, Sunnyvale, CA).

2.3. Synthesis of thiolated HA

Low molecular weight (LMW) HA (M_w 246 kDa, M_n 120 kDa, polydispersity index 1.97) was used after the degradation of a 1.0% (w/v) solution of HA (M_w 1500 kDa) in dilute HCl pH 0.5 for 24 h at 37°C 150 rpm [50]. Thiolated HA derivatives (HA-DTPH and HA-DTBH) were synthesized and characterized as described [50,53]. Using a modified Ellman method [54,55], the number of thiols per 100 disaccharide units was 42% for HA-DTPH and 44% for HA-DTBH. The materials used for hydrogels had the following size parameters: HA-DTPH, M_w 158 kDa, M_n 78 kDa, polydispersity

index 2.03; HA-DTBH, M_w 187 kDa, M_n 88 kDa, polydispersity 2.11.

2.4. Synthesis of homobifunctional PEG electrophiles

PEG-diacrylate (PEGDA), PEG-dimethacrylate (PEGDM), PEG-diacrylamide (PEGDAA) and PEG-dimethacrylamide (PEGDMA) were synthesized [31] from PEG (M_w 3400 kDa, Aldrich) or PEG-diamine (M_w 3400, Shearwater Polymers). Briefly, PEG (or PEG-diamine) M_w 3400 (10 g, 5.88 mmol of functional group) was azeotropically dried by distillation with toluene under argon. The anhydrous solution was cooled under argon, and dry dichloromethane (Aldrich) (ca. 50 ml) was added until the solution become clear. Triethylamine (1.23 ml, 8.82 mmol, Aldrich) was added dropwise with stirring, followed by the dropwise addition of 0.72 ml of acryloyl chloride (8.82 mmol, Aldrich) or 0.85 ml of methacryloyl chloride (8.82 mmol, Aldrich). The reaction was stirred in the dark, overnight under argon. The solutions were then filtered and the product was precipitated in diethyl ether, collected by filtration, and dried under vacuum. Next, 10 g of the product were dissolved in 10 ml of distilled water, and 5 g of NaCl was added, and the pH was adjusted to 6.0. The derivatives were then extracted 3 × with dichloromethane, precipitated in diethyl ether, and collected by filtration and dried in vacuo.

PEGDA: yield 75%. ^{1}H-NMR ($DCCl_3$): 3.6 ppm (303.5 H, PEG), 4.3 ppm (t, 4H, $–CH_2–CH_2–O–CO–CH=CH_2$), 5.8 ppm (dd, 2 H, $CH_2=CH–COO$), 6.1 and 6.4 ppm (dd, 4H, $CH_2=CH–COO–$). Degree of substitution, 95%.

PEGDM: yield 60%. ^{1}H-NMR ($DCCl_3$): 2.3 ppm (s, 6H, $CH_2=C(CH_3)–COO–$), 3.6 ppm (303.5 H, PEG), 4.3 ppm (t, 4H, $–CH_2–CH_2–O–CO–C(CH_3)=CH_2$), 5.8 and 6.1 ppm (d, 4H, $CH_2=C(CH_3)–COO–$). Degree of substitution, 91%.

PEGDAA: yield 75%. ^{1}H-NMR ($DCCl_3$): 3.6 ppm (304.4H, PEG), 5.6 ppm (dd, 2H, $CH_2=CH–CON–$), 6.1 and 6.3 ppm (dd, 4 H, $CH_2=CH–COO–$). Degree of substitution, 100%.

PEGDMA: yield 71%. ^{1}H-NMR ($DCCl_3$): 2 (s, 6 H, $CH_2=C(CH_3)–CON–$), 3.6 ppm (304.4H, PEG), 5.3 and 5.8 ppm (d, 4H, $CH_2=C(CH_3)–CON–$). Degree of substitution, 100%.

2.5. Conjugate addition

The relative reactivity of the conjugate addition of α,β-unsaturated esters and amides of PEG to thiols was first evaluated using cysteine as a model. Cysteine (2.5 mg) and PEGDA, PEGMA, PEGDAA, or PEGDMA were dissolved in 5 ml of 0.1 M PBS, pH 7.4 (double bond:thiol = 2:1). The consumption of thiol groups was monitored using DTNB [54] or NTSB [56]. The conjugate addition of thiols with different reactivity (i.e., different pKa values) was evaluated using the 375 Da monofunctional PEG-acrylate as a model compound. Thus, HA–DTPH or HA–DTBH (10 mg) was dissolved in 5 ml of 0.1 M PBS, pH 7.4, and then PEG-acrylate was added (double bond:thiol = 10:1). The consumption of free thiols was monitored using DTNB [54,56].

2.6. Hydrogel preparation

Thiolated HA was dissolved in DPBS to give a 1.25% (w/v) solution, and the pH was adjusted to 7.4. Each of the four PEG derivatives (PEGDA, PEGDM, PEGDAA, and PEGDMA) was dissolved in DPBS to give stock solutions of three different concentrations. Then, 1 ml of the stock reactive PEG solution was added in one portion to 4 ml of the thiolated HA (in ratios of thiols:double bonds = 1:1, 2:1, and 3:1) and mixed for 30 s. Gel formation occurred within 10 min (PEGDA) to several days (PEGDMA). All hydrogels were prepared under a blanket of nitrogen gas to minimize air oxidation and disulfide formation. The solution-gel transition was determined by a flow test utilizing the test tube inversion method [57].

2.7. Determination of crosslinking efficiency

Crosslinking was evaluated for both thiolated HA derivatives, with PEGDA as the homobifunctional crosslinker. After 1 h, a mixture of each thiolated HA (HA-DTPH and HA-DTBH) and PEGDA had completely gelled. The resulting hydrogels were then incubated in medium (pH 4.5 or 1.0) to quench the crosslinking addition, and the crosslinking efficiency was determined by measuring the remaining free PEG electophile and the remaining free thiols and performing the calculations indicated below.

First, the quantity of free PEGDA in the hydrogel was determined by GPC with monitoring of the eluent at 233 nm. Briefly, the hydrogel (0.1 ml) was ground into small particles and suspended in 2 ml of 0.1 M acetate buffer (pH 4.5). After stirring for 4 h at RT, the amount of residual PEG derivatives was monitored using a standard calibration curve. No free thiolated HA was detected by GPC at 210 nm.

Next, the number of free thiols in the hydrogel was determined using either the DTNB or NTSB assay [54,56]. Briefly, a 0.05-ml fragment of hydrogel was suspended in 0.5 ml of 0.1 N HCl solution. After 48 h at RT with agitation at 150 rpm, the hydrogel had dissociated. Next, 2.0 ml of either NTSB or DTNB reagent was added to each gel, and the number of free thiols in the hydrogel was obtained spectrophotometrically at 412 nm. Thiolated HA solutions alone were used as reference materials, and the disulfide formation

during hydrogel preparation (1 h) under nitrogen protection was negligible.

The extent of effective crosslinking (i.e., double-end anchorage), unreacted pendant double bond groups during the coupling reaction (i.e., single-end anchorage) was calculated from the total PEGDA used (**A**), the unreacted PEGDA (**B**), the total number of thiols (**C**) and the number of free thiols in hydrogel (**D**). Single-end anchorage equals the theoretical consumed thiols (2 (**A**–**B**)) minus the actually consumed thiols (**C**–**D**). Subtraction of single-end anchorage from the experimentally measured consumed thiols (**C**–**D**) reveals the extent of double-end anchorage.

2.8. Swelling ratios

Hydrogels were placed in DPBS buffer at 37°C for 48 h, and the medium was changed frequently. The swelling ratio (Q) was defined as a ratio of the weight of swollen gel to the weight of dry gel. The weight of the dry gels was determined by washing the hydrogel with distilled water 5 × and then drying the gel under vacuum (1 mm Hg) at RT for 3 days.

2.9. Enzymatic degradation of hydrogels

Hydrogel discs (0.5 ml) were prepared from HA-DTPH and PEGDA as described above by crosslinking at the bottom of a 6-mm diameter vial. HAse solutions (0, 50, 150, and 250 U/ml) were prepared in 30 mM citric acid, 150 mM Na_2HPO_4, 150 mM NaCl (pH 6.3); 5 ml of enzyme solution was added to each vial that contained the hydrogel, and vials were incubated at 37°C with orbital agitation at 150 rpm. The degradation of the gel was determined from the release of glucuronic acid into the supernatant as measured by the carbazole assay [58].

2.10. Cell culture in vitro

2.10.1. Seeding of T31 fibroblasts into HA-DTPH-PEGDA hydrogels

An HA-DTPH solution (1.25% w/v) was prepared by dissolving HA-DTPH in complete DMEM/F-12 medium supplemented with 10% new-born calf serum, 2 mM L-glutamine and 100 U/ml antibiotic–antimycotic (GIBCO BRL, Life Technologies, Grand Island, NY), adjusted to pH 7.4 with 1.0 M NaOH, and sterilized by filtration through a 0.45 μm syringe filter. Next, a 4.5% w/v PEGDA solution was prepared by dissolving PEGDA in DPBS buffer, and then sterilized by filtration through a 0.45 μm syringe filter. T31 human tracheal scar fibroblasts, which were isolated from human tracheal scars, were kindly provided by Ms. W. Li (Head and Neck Surgery Laboratory, UUtah Hospital) [59]. T31 cells were cultured in triple flasks (175 cm^2) (Fisher, Springfield, NJ) until 90% confluence, and then trypsinized with 0.25% sterile trypsin in 0.05% EDTA, and suspended in a freshly prepared HA-DTPH solution at concentration of 10^6 cells/ml. To 4 ml of the cell suspension was added 1 ml of the PEGDA stock solution (double bonds:thiol = approximately 1:2), and the mixture was vortexed gently. Next, 300 μl of the fibroblast seeded HA-DTPH-PEGDA mixture was transferred by pipette into FALCON™ cell culture inserts ([35] 3103, Becton Dickinson) and placed in 12-well plates. The seeded hydrogels were used to determine in vitro cell viability, proliferation, and for transplantation in vivo into nude mice for fibrous tissue generation.

2.10.2. Cell viability and proliferation in vitro

One hour after transferring HA-DTPH-PEGDA encapsulated cells to the plate, 1.5 ml of complete medium was added into each well and plates were incubated at 37°C and 5% CO_2. The medium was changed every three days without damaging the hydrogel. Viability of T31 cells was observed with live-dead staining methods at day 6 and day 28 of culture in vitro. At each time point, four fibroblast-seeded HA-DTPH-PEGDA hydrogels were rinsed twice with DPBS buffer, stained for 3 min with fluorescein diacetate (F-DA, 0.02 mg/ml) (Molecular Probes, Eugene, OR) and propidium iodide (PI, 0.2 μg/ml) (Sigma) at RT for 3 min, rinsed twice with DPBS buffer, stored on ice, and observed by confocal microscopy.

Cell proliferation was measured at day 0, 3, 6, 14, and 28. At each time point, four fibroblast-seeded HA-DTPH-PEGDA hydrogels were transferred into each well of a 12-well plate, and rinsed twice with DPBS buffer. Next, 900 μl of DMEM/F-12 medium with 5% newborn calf serum and 180 μl of CellTiter 96 Proliferation Kit (Promega, Madison, WI) were added into each well of a 12-well plate. After 2 h at 37°C and 5%°CO_2 with orbital shaking, 125 μl aliquots of the solution were added to a 96-well plate, and absorbance (λ= 550 nm) was measured using an OPTIMax microplate reader (Molecular Devices). Cell numbers were calculated using a standard curve.

2.11. In vivo hydrogel evaluation

Animal experiments were carried out according to NIH guidelines for the care and use of laboratory animals (NIH publication #85-23 rev. 1985). Male nude mice ($n = 12$) (Simonsen Laboratories Inc., Gilroy, CA), 4–6 weeks old, were reared in the Animal Resources Center at UUtah. Under anesthesia, four fibroblast-seeded HA-DTPH-PEGDA hydrogels were implanted bilaterally into subcutaneous pockets surgically prepared in the backs of nude mice. These served as the experimental group, totaling 24 implants in 6 nude mice, following an approved IACUC protocol. Six additional nude mice received 24 non-cell-loaded

HA-DTPH-PEGDA hydrogels as the control group. At each time point (2, 4, and 8 weeks after implantation), four nude mice (two experimental and two control) were sacrificed and the specimens were dissected for macrographical and immunohistochemical (anti-fibronectin) evaluation.

Tissues were removed from the mouse, fixed in 10% buffered formalin for 24 h, embedded in paraffin, cut into 5-μm sections, and mounted onto slides. As required, the slides were deparaffinized and rehydrated, washed with Tris-buffered saline (TBS), and finally incubated in 3% hydrogen peroxide for 5 min. Samples were then treated with Proteinase-K enzyme for 5 min, rinsed again with TBS for 5 min, and incubated for 10 min with anti-fibronectin antibody (1:800, Dako-Cytomation California Inc., Carpinteria, CA). The samples were then treated with the DAKO-LSAB kit (Dako): biotin, 10 min; TBS rinse and StreptAvidin, 10 min. The slides were then rinsed with TBS and treated for 5 min with DAB substrate solution (Research Diagnostics), rinsed with water, and cover-slipped with hematoxylin (Dako).

3. Results and discussion

3.1. Relative rates of conjugate addition reactions

The conjugate addition of thiols to α,β-unsaturated esters and amides of PEG was selected for in situ gelation. The reaction is rapid, not exothermic, and both the thiolated components and unsaturated crosslinkers were readily prepared. Moreover, byproducts are minimized since the reaction is highly thiol-selective; competing reactions of hydroxyl, carboxylate, phosphate, and amine nucleophiles occurred several orders of magnitude more slowly in an aqueous environment at RT for the pH values selected [53,60,61].

To meet the requirements for fabrication of an injectable hydrogel, a thorough understanding of the reactivity of thiolated HA with a variety of potential electrophilic crosslinkers was needed. We previously described the preparation of HA-DTPH and HA-DTBH and demonstrated that the corresponding solutions and hydrogels were biocompatible [50]. In this study, four α,β-unsaturated ester and amide derivatives of PEG were synthesized and used to crosslink thiolated HA (Fig. 1).

First, we investigated the relative rates of thiol conjugate addition in an aqueous medium to each of the four electrophilic PEG derivatives using cysteine as a model compound. The kinetics of the conjugate addition of cysteine to each of the four electrophiles are shown in Fig. 2a. PEGDA exhibited the highest rate of reaction with cysteine, proceeding to >90% completion in 2 min. The methyl group of PEGDM decreased the electrophilicity of the double bond and increased steric hindrance, with only 73% completion after 24 h. Both amides, PEGDAA and PEGDMA, were substantially less reactive with Cys than their corresponding esters [31], with 40% reaction and 25% reaction, respectively, after 24 h.

The reactivity of the thiol also influenced its rate of addition since the thiolate is the reactive form [53,60,61]. The pK_a values were 8.87 for HA-DTPH and 9.01 for HA-DTBH [50]. Thus, the conjugate addition of HA-DTPH to a 375 Da monofunctional PEG-acrylate was slightly faster than to the less reactive HA-DTBH (Fig. 2b). After 150 min, reaction with HA-DTPH reached 92% completion, while HA-DTBH reached only 82% completion.

The foregoing results demonstrate that the addition of PEGDA was considerably faster than methacrylate, acrylamide and methacrylamide derivatives of PEG. PEGDA, which is currently used in tissue engineering for photopolymerization, was further evaluated in cell-seeded hydrogels. PEG divinylsulfones, another highly

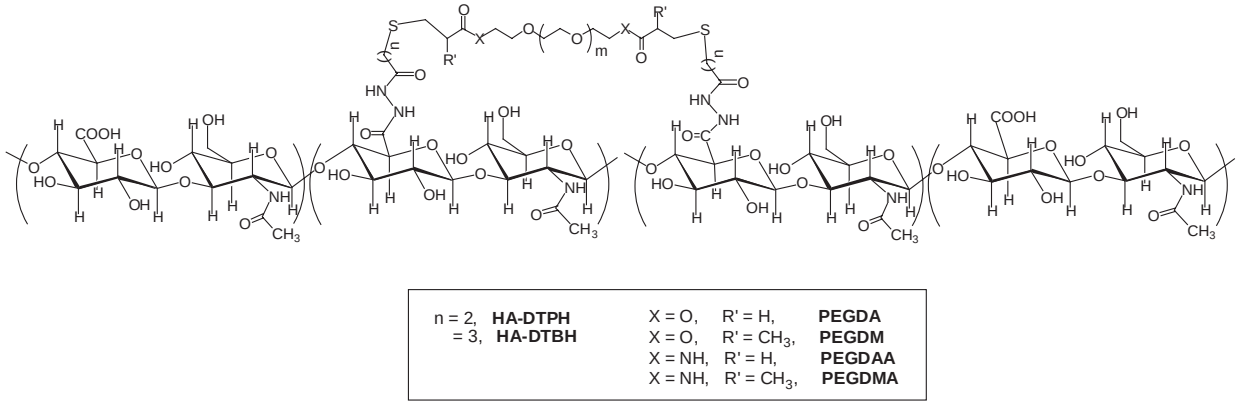

Fig. 1. Structures of α,β-unsaturated esters and amides of PEG crosslinked with thiolated HA.

thiol-reactive electrophile with potential utility for biomaterial construction [62], were not examined because of the reduced thiol selectivity and the increased cytotoxicity of residual reagent [63].

3.2. Fabrication and characterization of in situ crosslinked hydrogels

We next examined the rates of gelation of HA-DTPH with the four PEG electrophiles. Consistent with the model system, the gelation times of mixtures of thiolated HA with α,β-unsaturated esters and amides of PEG were strongly correlated with the nature of the electrophile (data not shown). We observed that using a 1.25% (w/v) solution of thiolated HA with each PEG electrophile in a thiol:double bond ratios of 3:1, 2:1, and 1:1, the gelation times could be varied from 5 min with PEGDA to tens of hours with the less reactive species. For potential clinical applications, PEGDA was chosen as the crosslinker.

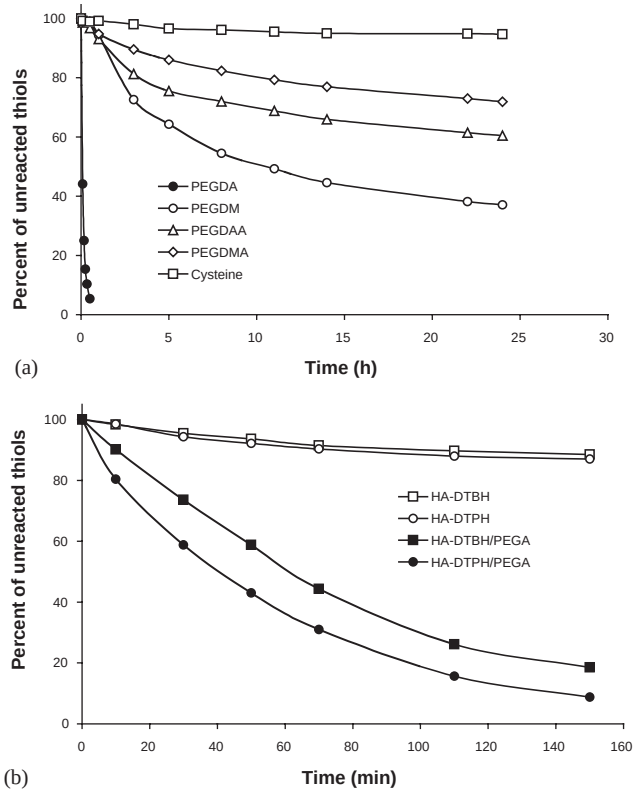

Fig. 2. Determination of reaction kinetics by monitoring unreacted thiol groups. Panel (a): conjugate addition of cysteine to PEGDA, PEGDM, PEGDAA, and PEGDMA. The rapid addition of PEGDA occurs in minutes, while other conjugate additions occur with a time scale in hours. Panel (b): filled symbols, conjugate addition of HA-DTPH, HA-DTBH to PEG-acrylate (monofunctional); open symbols, controls with no PEG-acrylate added.

Thus, 1 ml of PEGDA solution was added to 4 ml of a 1.25% w/v solution of each thiolated HA in DPBS, pH 7.4 to give thiol:double bond ratios of 3:1, 2:1, and 1:1, with a final concentration of thiolated HA in the hydrogel of 1.0% (w/v). All experiments were performed under N_2 to minimize disulfide formation. Gelation of thiolated HA/PEGDA solution was sufficiently rapid for injectable in vivo use. The crosslinking efficiency of PEGDA to HA-DTPH and HA-DTBH (Table 1) confirms that the 4-thiobutanoylhydrazide derivative reacts more slowly and less efficiently than the more reactive 3-thiopropanoylhydrazide derivative [50]. No residual PEGDA was detected by GPC with the molar ratio of thiols to double bonds 2:1 and 3:1, with quantitative double-end anchorage observed for the 3:1 ratio. This important result suggests that no unreacted PEGDA or resulting acrylic acid (from hydrolysis) will be present in the final hydrogel. In most other photopolymerized hydrogels, a significant amount of unreacted oligomer (ca. 5–20%) and single-end anchorage takes place [64–67].

The crosslinking densities, equilibrium swelling ratios, and gelation times for the gels obtained by reaction of HA-DTPH and HA-DTBH with PEGDA are shown in Table 2. Consistent with the crosslinking efficiency in Table 1, the crosslinking density—i.e., the effective concentration of crosslinks in 1 ml hydrogel—was higher for HA-DTPH-PEGDA hydrogels than for HA-DTBH-PEGDA hydrogels (Table 2). For instance, with an equimolar ratio of thiols to double bonds, the crosslinking density for HA-DTPH-PEGDA was 8.1 mmol/ml, while for HA-DTBH-PEGDA. the value was only 5.1 mmol/ml.

Table 1
Crosslinking efficiency of PEGDA to HA-DTPH and HA-DTBH

	Molar ratio of thiols to double bonds	Crosslinking efficiency (%) of PEGDA		
		Double-end anchorage	Single-end anchorage	Unreacted
HA-DTPH:PEGDA	1:1	76.2	9.7	14.1
	2:1	93.7	6.3	0
	3:1	100.0	0	0
HA-DTBH:PEGDA	1:1	48.3	19.3	32.4
	2:1	60.0	12.7	27.3
	3:1	73.8	8.3	17.9

Table 2
Crosslinking density, equilibrium swelling ratio (Q) and gelation time for gels prepared using PEGDA (M_w 3400) with HA-DTPH and HA-DTBH

	Molar ratio of Thiols to double bonds	Crosslinking density (mmol/ml)[a]	Swelling ratio (Q)	Gelation time (min)
HA-DTPH:PEGDA	1:1	8.1	39.41±0.34	5
	2:1	5.0	46.15±0.38	9
	3:1	3.5	61.06±0.89	19
HA-DTBH:PEGDA	1:1	5.1	58.14±0.94	11
	2:1	3.2	69.33±2.94	19
	3:1	2.6	84.62±1.98	31

[a] Crosslinking density was defined as the number of effective crosslinking sites in 1 ml of hydrogel.

An important advantage of these HA-derived hydrogels for tissue engineering is their high water content (97.5–98.8%), as determined from the swollen gels (Table 2). That is, the HA hydrogels will have high permeability for oxygen, nutrients, and other water-soluble metabolites. In contrast, the photopolymerization hydrogel based on PEGDM and poly(propylene fumarate) had a water content of only ca. 20–30% [18], and decreased viability of osteoblast cells in injectable, photopolymerized RGD-modified PEGDA hydrogel following in vitro culture has been attributed to the inadequate permeability of the hydrogel [24].

By altering the parameters of hydrogel preparation, the rate of gelation could be controlled from 5 to 31 min (Table 2), which is suitable for many of the clinical uses for which injectable, in situ crosslinkable materials. Thus, HA-DTPH-PEGDA hydrogels with a molar ratio of thiols to double bond of 2:1 were selected for further evaluation for in situ cell encapsulation and injectable cell delivery, since the 9 min gelation time was optimal for manipulation in the operating suite, and unreacted acrylate groups could be kept to a minimum. Using only 0.5 equiv of electrophile relative to thiol ensures that no unreacted and potentially cytotoxic electrophiles remain in the hydrogel. Moreover, the residual thiol groups allow for additional oxidative crosslinking of the hydrogels into more robust dried films [50] or solid materials, if desired.

The ester hydrolysis of PEGDA and PEGDM is very slow and no evidence of degradation has been reported over the course of several weeks for PEGDM-based hydrogels [17,28,67]. However, as for tissue engineering, the scaffold should degrade as the cells migrate and proliferate. Indeed, as we have demonstrated with biointeractive wound dressings [49], the degradation of the HA hydrogel and its integration into the newly formed ECM appears to be an important contributor to the utility of HA-derived materials for tissue repair.

To mimic in vivo conditions, the digestion of HA-DTPH/-PEGDA hydrogel by HAse was measured and found to be dependent on enzyme concentration (Fig. 3). After 24 h at 37°C with gentle agitation, 27% of the hydrogel was digested at the highest HAse concentration (250 U/ml) employed. No significant degradation occurred in the absence of added HAse. The hydrogel was completely digested in 250 U/ml HAse solution after 3 days, if fresh enzyme solution was added each day.

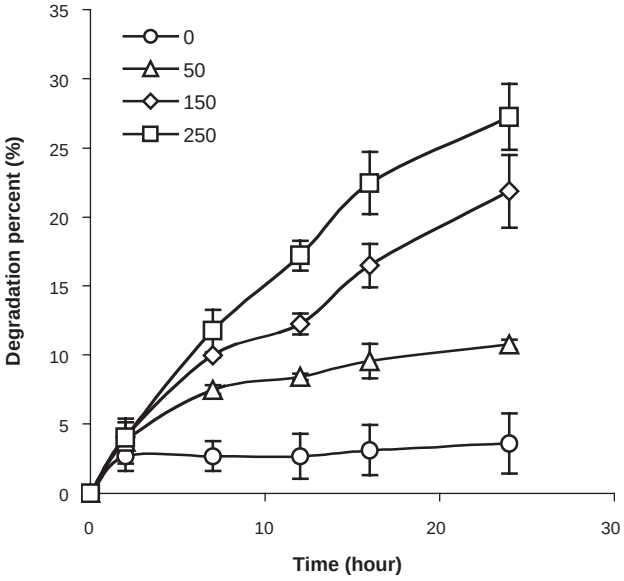

Fig. 3. Digestion of HA-DTPH-PEGDA hydrogels with HAse.

3.3. *In vivo and in vitro evaluation in situ crosslinkable hydrogels*

HA-DTPH-PEGDA hydrogels were prepared in which human T31 tracheal scar fibroblasts had been added as a suspension in cell culture medium into the HA-DTPH solution prior to initiating gelation by addition of the PEGDA solution. The density of living cells in the gel, observed by in situ fluorescence staining, greatly increased after 28 days in vitro relative to 6 days in culture (data not shown). Only a few dead (PI-stained) cells were observed.

T31 fibroblast viability was conclusively demonstrated by the cell proliferation study (Fig. 4). The number of fibroblasts in the HA-DTPH-PEGDA

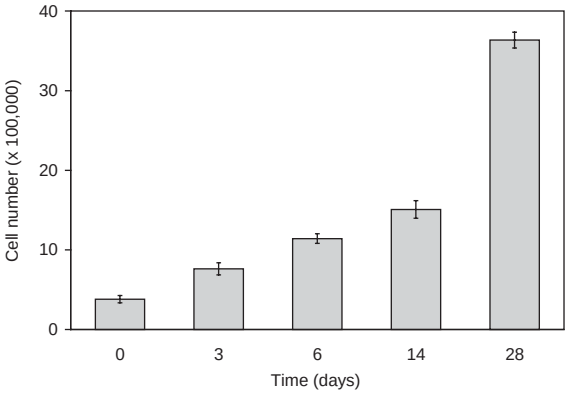

Fig. 4. Proliferation of T31 fibroblasts in HA-DTPH-PEGDA hydrogel.

hydrogel increased almost tenfold after 28 days of culture in vitro. The collagen typing of these cultured fibroblasts showed that even after 28 days of in vitro culture, the cells retained the same phenotype as characterized by collagen type I production (data not shown). Taken together, HA-DTPH-PEGDA hydrogels support cell growth and proliferation in vitro.

We next evaluated the compatibility and durability of the cell-seeded HA-DTPH-PEGDA hydrogel in vivo. Thus, fibroblast-seeded gels were implanted subcutaneously in nude mice and implants were evaluated at 2, 4, and 8 weeks post-implantation. Control gels lacking fibroblasts were also evaluated in vivo. No evidence of biological incompatibility—e.g., necrosis or damage to surrounding tissues—was observed (data not shown). After removal from the mice, the explants appeared more opalescent and elastic with increasing implantation time, suggesting higher cell density (data not shown). The gross examination was confirmed by histology (Fig. 5). The fibronectin staining of the proliferating cells demonstrated that the cells have retained their original phenotype and were actively secreting new ECM. Recent results have demonstrated that this in situ crosslinkable hydrogel can indeed form biocompatible gels when injected subcutaneously in mice prior to gelation. These data will be reported in due course.

4. Conclusions

We described the preparation and evaluation of novel in situ crosslinkable hydrogels as biointegrative materials for tissue engineering. The reaction of four electrophilic derivatives of PEG 3400 with cysteine and with two thiol-containing HA derivatives was measured. An optimal system, HA-DTPH-PEGDA in a molar ratio of 2:1, had a gelation time of 9 min and was selected for in situ cell encapsulation and to measure growth and proliferation of human tracheal scar fibroblasts. When cultured in vitro, HA-DTPH-PEGDA hydrogels that were pre-seeded with fibroblasts showed high cell viability and a tenfold increase in cell density over

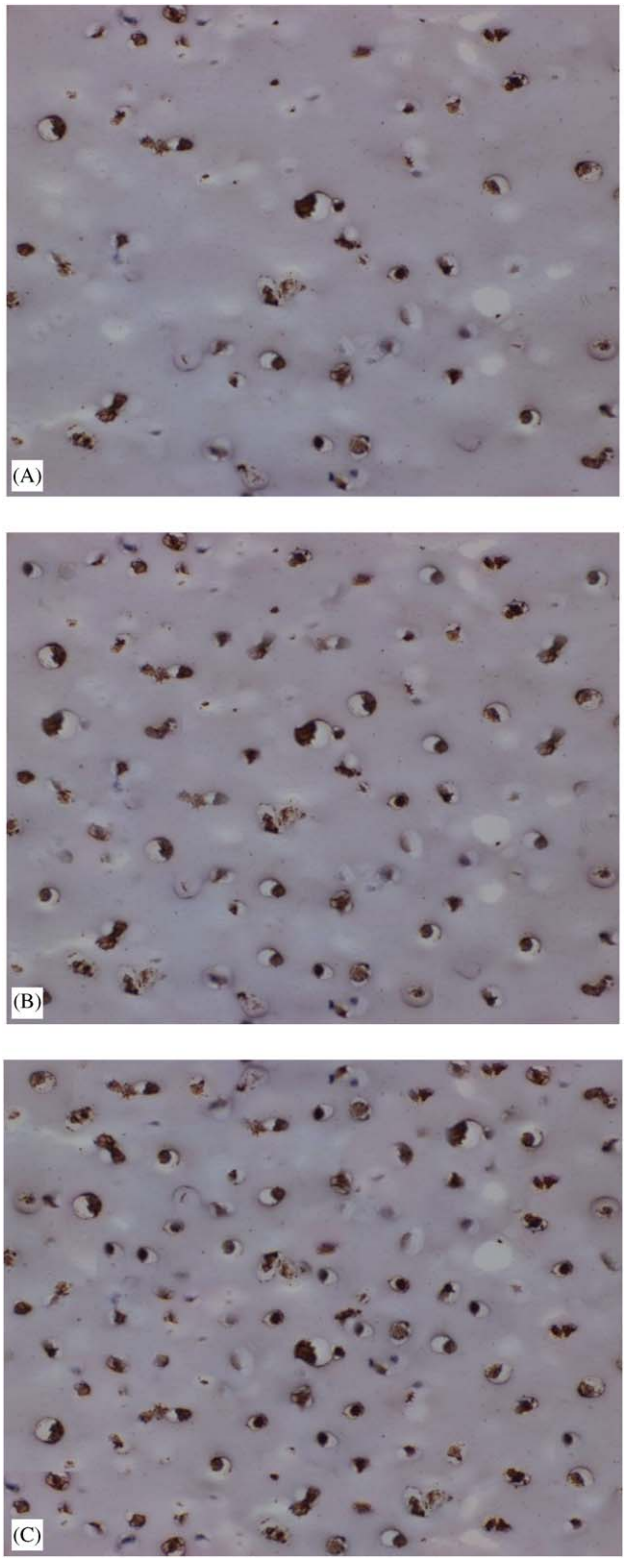

Fig. 5. Histological examination of the explants after incubation in nude mice for 2 weeks (Panel A), 4 weeks (Panel B), and 8 weeks (Panel C), immunohistochemistry (fibronectin). Original magnification × 200.

a 4-week incubation period. Both in vitro and in vivo, the cells maintained a healthy fibroblast phenotype. Taken together, these results clearly demonstrate significant potential for the use of in situ crosslinkable HA-DTPH-PEGDA hydrogels for clinical applications of cell encapsulation and tissue engineering.

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