Published in final edited form as:

Biomacromolecules. 2013 April 8; 14(4): 1199–1207. doi:10.1021/bm400169d.

Synthesis of Photodegradable Macromers for Conjugation and Release of Bioactive Molecules

Donald R. Griffin^{1,+}, Jessica L. Schlosser^{1,+}, Sandra F. Lam¹, Thi H. Nguyen², Heather D. Maynard^{2,3}, and Andrea M. Kasko^{1,3,*}

¹Department of Bioengineering, University of California, Los Angeles, 410 Westwood Plaza, 5121 Eng V, Los Angeles, CA 90095 United States

²Department of Chemistry and Biochemistry, University of California, Los Angeles, 607 Charles E. Young Drive East, Los Angeles, California 90095-1569 United States

³California Nanosystems Institute, 570 Westwood Plaza, Los Angeles, CA 90095 United States

Abstract

Hydrogel scaffolds are used in biomedicine to study cell differentiation and tissue evolution, where it is critical to control the delivery of chemical cues both spatially and temporally. While large molecules can be physically entrapped in a hydrogel, moderate molecular weight therapeutics must be tethered to the hydrogel network through a labile linkage to allow controlled release. We synthesized and characterized a library of polymerizable *ortho*-nitrobenzyl (*o*-NB) macromers with different functionalities at the benzylic position (alcohol, amine, BOC-amine, halide, acrylate, carboxylic acid, activated disulfide, N-hydroxysuccinyl ester, biotin). This library of polymerizable macromers containing *o*-NB groups should allow direct conjugation of nearly any type of therapeutic agent and its subsequent controlled photorelease from a hydrogel network. As proof-of-concept, we incorporated the *N*-hydroxysuccinyl ester macromer into hydrogels, and then reacted phenylalanine with the NHS ester. Upon exposure to light ($\lambda=365$ nm, 10 mW/cm², 10 min) 81.3% of the phenylalanine was released from the gel. Utilizing the photodegradable macromer incorporating an activated disulfide, we conjugated a cell-adhesive peptide (GCGYGRGDSPG), a protein that exhibits enzymatic activity (bovine serum albumin (BSA)), and a growth factor (transforming growth factor- β 1 (TGF- β 1)) into hydrogels, controlled their release with light ($\lambda=365$ nm, 10 mW/cm², 0–20 min), and verified the bioactivity of the photoreleased molecules. The photoreleasable peptide allows real-time control over cell adhesion. BSA maintains full enzymatic activity upon sequestration and release from the hydrogel. Photoreleased TGF- β 1 is able to induce chondrogenic differentiation of human mesenchymal stem cells comparable to native TGF- β 1. Through this approach, we have demonstrated that photodegradable tethers can be used to sequester peptides and proteins into hydrogel depots and release them in an externally controlled, predictable manner without compromising biological function.

*Corresponding author: Department of Bioengineering, University of California, Los Angeles, 410 Westwood Plaza, 5121 Eng V, Los Angeles, CA 90095 United States, Tel. +1 310 794 6341, fax +1 310 794 5956, akasko@ucla.edu.

⁺contributed equally to this work

Supporting information available. Experimental details for the synthesis of all macromers, Schemes S1 and S2. This material is available free of charge via the internet at <http://pubs.acs.org>.

Keywords

photoreleasable therapeutics; degradation; hydrogel; photolysis

Introduction

Hydrogels are important biomaterials used in tissue engineering and regenerative medicine, providing physical support for cells. Additionally, soluble cues such as proteins or other biomolecules can be sequestered within and released from hydrogels.¹ Three general techniques exist for controlling the delivery of biomolecules from hydrogels – physical entrapment, covalent tethering, and affinity-based sequestration. The method used to control a biomolecule's release from a hydrogel is dictated, at least in part, by its size (molecular weight). Large molecules such as proteins can be physically entrapped within the mesh of the hydrogel, which impedes their diffusion. Lower molecular weight species are typically covalently conjugated to the network through degradable linkages (usually ones sensitive to hydrolytic or enzymatic degradation) because their diffusion is not significantly retarded by the hydrogel. For example, therapeutic agents such as dexamethasone² or statins³ can be released via hydrolysis to induce the differentiation of mesenchymal stem cells (MSCs) into osteoblasts. Growth factors such as vascular endothelial growth factor (VEGF) can be released via enzymatic degradation of an MMP-sensitive tether to induce angiogenesis⁴. Alternatively, affinity interactions (such as ion interactions) can be used to sequester and release biomolecules from hydrogels. Affinity interactions are more transient than covalent bonds, but if sufficiently strong they can retard the diffusion of species out of the hydrogel. All three methods typically result in a sustained release profile. While this is desirable in many therapeutic settings, the ability to externally control the release of the therapeutic may allow the administration of a more complex dosing profile.

While hydrolysis and enzymolysis are both effective methods for sustained release of therapeutic agents, the release rate cannot be adjusted or arrested after the hydrogel is fabricated, and release is not spatially controlled. As an alternative to hydrolytic and enzymatic degradation for controlled (sustained) release, we have developed and optimized photodegradation as a mechanism for controlled drug release. Photodegradation offers precise external temporal and spatial control over drug release. Photodegradable groups have been used in the presence of live cells to uncage neurotransmitters⁵, to pattern physical voids within a hydrogel^{6–9}, and to spatially pattern functional groups on and within^{10–13} hydrogels.

We previously reported coupling a photosensitive polymerizable *ortho*-nitrobenzyl (*o*-NB) group to fluorescein (model drug) to generate a model photoreleasable therapeutic agent.¹⁴ We copolymerized this macromer into hydrogel depots and quantified the release of fluorescein as a function of light exposure at multiple wavelengths (365–436 nm), intensities (5–20 mW/cm²) and durations (0–20 minutes), and correlated the release profiles to a predictive model. Although these results were promising, the conjugation was performed in organic solvent, which would be unsuitable for many biomolecules, and the site we chose for conjugation left the *ortho*-nitroso ketone fragment attached to the model therapeutic.

Furthermore, each new therapeutic agent of interest would require independent synthesis. We next reported a series of *o*-NB linkers with different rates of photodegradation to allow the multistaged release of cells¹⁵ and model therapeutics¹⁶. Although these reports resolved some of the issues noted above, the variety of functional groups that could be incorporated was still limited.

Bioconjugation techniques take advantage of functional groups commonly found on biomolecules such as amines, carboxylic acids, alcohols and thiols. In order to allow conjugation of a wider variety of molecules, we are interested in *o*-NB macromers with different reactive groups at the benzylic position (release site) that allow easy incorporation under mild conditions. Here we report the synthesis of photodegradable *o*-NB macromers with a variety of functional groups at the benzylic position. This will allow for covalent conjugation of a wider variety of biomolecules and therapeutics to the *o*-NB linker, and their subsequent delivery from a hydrogel, without having to resynthesize the macromer each time. We demonstrate that amino acids, peptides, and proteins can be quantitatively sequestered into hydrogels using a photodegradable tether and subsequently released in an externally controlled, predictable manner without compromising biological function.

Experimental Section

Release Experiments

Phenylalanine release—Stock solutions of PEG526-methacrylate-PDG NHS (10 mg/mL in DMSO), tetramethylethylenediamine (TEMED, 10% by vol. in Phosphate Buffered Saline (PBS), pH 7.4, 1 mM), and ammonium persulfate (APS, 10 wt%, in PBS) were prepared prior to addition. PEG 10000 DA hydrogel disks were fabricated by dissolving PEG 10000 diacrylate (0.10 g, 9.9 μ mol) in PBS (0.35 mL) and DMSO (0.4 mL), followed by addition of PEG526-methacrylate-4-(4-(1-((4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxabutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (1.0 mg, 1.9 μ mol, 0.1 mL stock). To initiate polymerization APS (100 μ L) and TEMED (25 μ L) were added sequentially, followed by immediate placement of solution between two glass slides separated by a glass slide (1 mm). The resulting hydrogels were cured for 90 minutes, cut into 5 mm discs, and leached with 1:1 DMSO/PBS. All hydrogels were placed in a 3 mL loading solution of L-Phenylalanine (10 mg/mL in 1:1 DMSO/PBS) overnight. The hydrogels were then washed with the 50% DMSO/PBS solution. All gels were placed in individual wells of a 48-well plate and placed with 500 μ L of the DMSO solution. Half the gels ($N=3$) were exposed ($\lambda=365$ nm, 10 mW/cm², 10 min) while the remaining 3 remained unexposed. All gels were allowed to leach on a shaker plate overnight, then tested for the presence of L-Phe at 257 nm via standard UV/Vis protocol. A standard curve of L-Phe was prepared prior to testing.

Fabrication of Hydrogels Containing Cell Adhesive Peptide—Stock solutions of PEG526-methacrylate-4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-yl)disulfanyl)ethoxy)butanoyl)oxy))butanoate (10 mg/mL in DMSO), TEMED (10% by vol. in Phosphate Buffered Saline (PBS), pH 7.4, 1 mM), and APS (0.22 M, in PBS) were prepared prior to addition. PEG 10000 DA hydrogel disks were fabricated by dissolving PEG 10000 diacrylate (0.10 g, 9.9 μ mol) in PBS (0.35 mL) and DMSO (0.4 mL), followed

by addition of PEG526-methacrylate-4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-yl)disulfanyl)ethoxy)butanoyl)oxy))butanoate (1.0 mg, 1.9 μmol , 0.1 mL stock). To initiate polymerization APS (100 μL) and TEMED (25 μL) were added sequentially, followed by immediate placement of solution between two glass slides separated by rubber spacers (0.33 mm). The resulting hydrogels were cured for 90 minutes, cut into 5 mm discs, and leached with 1:1 DMSO/PBS, ethanol and PBS. The hydrogels were divided into sets (10 gels/set, N=3) and each set was placed in a 1 mL loading solution of buffered aqueous GCGYGRGDSPG (0.1 mM in PBS, 3 equivalents total) overnight. The loading solution was tested for the presence of released pyridine-2-thione (8080 $\text{M}^{-1}\text{cm}^{-1}$) at 1 hour and 24 hours after exposure to check the progress of the disulfide exchange by the standard UV-Vis protocol.¹⁷ The hydrogels were then washed with PBS and either seeded with cells (30,000 cells per well), exposed ($\lambda=365\text{ nm}$, 10 mW/cm^2 , 20 min) and seeded with cells, or exposed to fluorescein-NHS (5 mol. equiv. in 1:1 DMSO/PBS) for 2 hours, before washing repeatedly with 1:1 DMSO/PBS to remove unconjugated fluorescein.

Fluorescence Calibration Curve—Fluorescein-NHS (4.8 mg, 10 μmol) was dissolved in DMSO (5.07 mL), isoleucine (6.6 mg, 51 μmol) was dissolved in PBS (5.07 mL), and the two solutions were combined and stirred overnight. This stock solution (1 mM) was diluted serially and tested on a Beckman Coulter DTX 880 Multimode Detector ($\lambda_{\text{ex}} = 485\text{ nm}$; $\lambda_{\text{em}} = 535\text{ nm}$) to create a calibration curve.

Cell-adhesive hydrogel exposure and release measurement—Each hydrogel was placed individually in the well of a 48-well plate, exposed for a specified time to light (N=3, 365 nm, 10 mW/cm^2) at 21°C. Following exposure each hydrogel was leached with a 1:1 DMSO/PBS mixture (1 mL) overnight before testing on a Beckman Coulter DTX 880 Multimode Detector ($\lambda_{\text{ex}} = 485\text{ nm}$; $\lambda_{\text{em}} = 535\text{ nm}$).

Mesh size calculation—To calculate the mesh size of the polymerized hydrogels, a separate hydrogel was polymerized between glass slides separated by a larger spacer (1.66 mm) using identical polymerization and leaching conditions to those stated above. The complex modulus was measured using a TA Instruments Q800 DMA. The hydrogel mass was measured before and after lyophilization, and combined with the density of PEG 10K¹⁸ to determine the swelling ratio (Q). The molecular weight between cross-links (M_c) was then calculated using a modified equation from the literature (Equation 1)¹⁹ and used to find the crosslinked network characteristic length of the hydrogel (ξ) (Equation 2).

$$\frac{1}{M_c} = \frac{2}{M_n} + \frac{E}{2(1+\nu)} \frac{1}{\rho_p RT (\nu_2)^{\frac{1}{3}}} \quad \text{Eq. 1}$$

$$\xi = \nu_2^{-\frac{1}{3}} C_n^{\frac{1}{2}} \ln^{\frac{1}{2}} \quad \text{Eq. 2}$$

BSA loading and diffusion—10 wt% PEG 10KDA hydrogels (d=5 mm, h=1 mm) were placed in individual wells on a 48 well plate and each well was loaded with 250 μL of

fluorescein tagged BSA (1 mg/ml in PBS) for 16 hours. After equilibration, all solution was taken out of each well, tested on a Beckman Coulter DTX 880 Multimode Detector, $\lambda_{\text{ex}} = 485 \text{ nm}$; $\lambda_{\text{em}} = 535 \text{ nm}$ and replaced with fresh PBS every 5 minutes until diffusion of fluorescein out of the gel was no longer detected.

Hydrogel synthesis for protein conjugation after polymerization (Linker w/PEG 526MA)—Hydrogels were made with PEG526-methacrylate-4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-ylidisulfanyl)ethoxy)butanoyl)oxy))butanoate identical to the samples made for RGD incorporation.

Protein infusion into PEG526-methacrylate-4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-ylidisulfanyl)ethoxy)butanoyl)oxy))butanoate containing hydrogels—Following polymerization and leaching the hydrogels were infused with a BSA solution (1 mM). Hydrogels with PEG526-methacrylate-4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-ylidisulfanyl)ethoxy)butanoyl)oxy))butanoate were also infused with PBS only and glutathione (1 mM) solutions to act as negative and positive controls, respectively. The pyridine-2-thione release ($8080 \text{ M}^{-1}\text{cm}^{-1}$) was monitored at 342 nm for 48 hours using UV/Vis spectroscopy. No change in absorbance was seen relative to control hydrogels during this period.

Hydrogel synthesis for protein conjugation after polymerization (Linker w/PEG 10KMA, 10 wt%)—PEG 10K methacrylate 4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-ylidisulfanyl)ethoxy)butanamido)ethyl)phenoxy)butanoate/PEG 10KMA (4:96 mol%, 0.15 g) was dissolved in PBS (1.275 mL). Solutions of APS (150 μL , 10 w/v%) and TEMED (75 μL , 10 v/v%) were added sequentially, and the hydrogels polymerized between two glass slides (thickness = 0.5 mm) for one hour. The hydrogels were then cut into 5 mm discs using a biopsy punch. The discs were washed with PBS six times to remove unreacted material ($5 \times 30 \text{ min}$ and $1 \times \text{overnight}$ washes) and stored at 5°C until use.

Protein conjugation after polymerization (Linker w/PEG 10KMA, 10 wt%)—Following polymerization and leaching the hydrogels were infused with a BSA solution (1 mM). Two sets of hydrogels were also infused with PBS only and glutathione (1 mM) solutions to act as negative and positive controls, respectively. The pyridine-2-thione release ($8080 \text{ M}^{-1}\text{cm}^{-1}$) was monitored at 342 nm for 24 hours using UV/Vis spectroscopy and compared to the expected exchange based on complete incorporation of the *o*-NB linker during polymerization.

Pre-polymerization exchange with BSA and subsequent hydrogel synthesis (10 wt% PEG)—Stock solutions of PEG 10KMA 4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-ylidisulfanyl)ethoxy)butanamido)ethyl)phenoxy)butanoate/PEG 10DKMA (4:96 mol%, 224 mg in 950 μL) and BSA (1 mM) were predissolved in PBS. 475 μL of each stock solution were combined to initiate exchange, while 475 μL of each solution were also combined with PBS (475 μL) to act as negative controls of exchange. After 4 hours, aliquots (100 μL) of all three solutions (two negatives, one experimental) were diluted (1:10) with PBS and tested for ($8080 \text{ M}^{-1}\text{cm}^{-1}$) absorbance at 342 nm by UV/Vis spectroscopy. APS (75 μL , 10 w/v%) and TEMED (75 μL , 10 v/v%) were added sequentially to the

experimental solution. The solution was polymerized between two glass slides (thickness = 0.5 mm) for one hour and washed with PBS (5 × 30 minutes, 1 × overnight).

Pre-polymerization exchange with TGF-β1 and subsequent hydrogel

synthesis (10 wt% PEG)—Stock solutions of PEG 10KMA 4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-yl)disulfanyl)ethoxy)butanamido)ethyl)phenoxy)butanoate/PEG 10KDMA (4:96 mol%, 224 mg in 950 μL) and TGF-β1 (1 μg/mL) were predissolved in PBS. 100 μL of each stock solution were combined to initiate exchange and were tested for (8080 M⁻¹cm⁻¹) absorbance at 342 nm by UV/Vis spectroscopy at t=0 and t=4 hrs. PEG 10,000 diacrylate (90 mg in 750 μL) was dissolved in PBS and combined with the exchanged TGF-β1 solution. APS (25 μL, 50 w/v%) and TEMED (25 μL, 50 v/v%) were added sequentially to the experimental solution. The solution was polymerized between two glass slides (thickness = 1 mm) for 12 hours then washed with ultra pure water (4 × 30 minutes), ethanol (for sterilization) (1 × 1 hour), 50:50 ethanol:PBS (2 × 30 min), and PBS (2 × 30 min).

Hydrogel exposure and release measurement—Each hydrogel was placed individually in the well of a 48-well plate, exposed for a specified time to light (N=3, 365 nm, 10 mW/cm²) at 21°C. Following exposure each hydrogel was leached with PBS (0.25 mL) overnight before testing each solution by micro-BCA analysis (Pierce).

BSA activity test—Assessment of BSA esterase activity was performed following a literature procedure²⁰. Briefly, the concentration of the released BSA solution (N = 3) was quantified by BCA. Subsequently a solution of native BSA was created of equal concentration. These solutions were combined, separately, with solutions of *p*-nitrophenyl acetate. Following incubation, the change in absorbance for each solution was measured by UV/Vis at 348 nm and compared.

hMSC culture—Human mesenchymal stem cells (hMSCs, including RFP and GFP expressing hMSCs) were provided by the Texas A&M Health Science Center College of Medicine. hMSCs were cultured in αMEM with 2 mM L-glutamine(Hyclone) supplemented with 16.5% fetal bovine serum (FBS, Atlanta Biologicals) and 100 μg/mL Penicillin-Streptomycin (Hyclone) at 37°C in a 5% CO₂ environment. Growth media was exchanged every 2–3 days.

Cell differentiation—The hMSCs were cultured in monolayer at a density of 5 × 10³ cells/cm² in 24 well plates for 8 hours at 37°C in 5% CO₂. TGF-β1 (Peprotech) was diluted to concentrations of 10 ng/mL in serum-free medium and applied to hMSCs for the positive control. Medium containing released TGF-β1 from the exposed hydrogels was applied to hMSCs to verify its bioactivity in comparison to the positive control. For the negative control, the hMSCs received fresh serum-free medium that did not contain any TGF-β1. Cells were cultured for three days with no medium changes and then fixed overnight at 4°C in 10% buffered formaldehyde and rinsed twice with PBS. The cells were permeabilized using 0.1% Triton X-100 in PBS for 5 min at RT and rinsed twice. Blocking solution (1% BSA in PBS) was applied for 30 minutes, and the cells were subsequently rinsed 3x with PBS. The cells were incubated with toluidine blue (1:400 in blocking solution) at RT for 1 h

and rinsed 3x with PBS. Phase contrast images (Zeiss AxioObserver Inverted Fluorescent Microscope) of the (stained) hMSCs were taken.

Histology—Cells were stained with toluidine blue (Acros Organics) to visualize sulfated glycosaminoglycan (GAG) deposition. Following standard protocol²¹, a 5 mg/ml solution of toluidine blue was used to stain the cells for 15 minutes and then washed 3 times with PBS for 5 minutes each.

GAG measurement—After culturing the cells for 3 days, GAG content was quantitatively measured spectrophotometrically using the dimethylmethylene blue (DMMB) (Polysciences, Inc.) assay with slight modifications²². Briefly, cells were digested with 1 mL papain solution (Acros Organics) for 16 hours at 60°C. The cell solution was then passed through a syringe filter and a DMMB solution was applied to the sample. Absorbance was measured at 650 nm, and compared to a chondroitin sulfate solution standard (Sigma-Aldrich).

TGF- β 1 Quantification—The PBS leach solutions surrounding the hydrogels were diluted 1:100 with PBS, then tested for TGF- β presence using a sandwich ELISA (TGF- β Emax ImmunoAssay System, Promega).

Statistics—Data are presented as mean $\pm$ standard deviation with three samples averaged for each data point.

Results and Discussion

The main building block for the photodegradable macromers in this report is 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid, the synthesis of which has been previously reported.^{6,14,23} This *o*-NB group contains both a carboxylic acid and a benzylic alcohol, allowing for separate functionalization of these two moieties. In order to obtain a functional group reactive in the radical polymerizations typically used to fabricate poly(ethylene glycol) hydrogels, we first esterified the carboxylic acid group using tosylated PEG 526 methacrylate and potassium fluoride in DMF²⁴ (Scheme 1). Unlike carbodiimide couplings or acid chloride mediated esterifications, this nucleophilic substitution leaves the benzylic alcohol unaffected. While the yield of this reaction is modest (52%), this is in part due to the difficulty of isolating the product, which is a viscous oil.

The benzylic alcohol can be reacted with succinic anhydride to produce a carboxylic acid (Scheme 2). The carboxylic acid is easily esterified with N-hydroxysuccinimide (NHS) or with 2-(pyridin-2-yl)disulfany)ethanol via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) coupling (Scheme 2). The yield of this reaction was uncharacteristically low, as a significant amount of product was lost during purification via gradient chromatography. The NHS ester should allow for direct conjugation of proteins to the photodegradable group through any free amines²⁵, while the activated pyridyldisulfide reacts with free thiols via disulfide exchange¹⁷.

In order to functionalize the *o*-NB linker with an amine at the benzylic position, we first converted the benzyl alcohol of 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic

acid to a bromide using phosphorous tribromide. We then reacted the benzyl bromide with ammonium hydroxide to yield the benzyl amine, which we then protected with *tert*-butyl carbonate. We coupled PEG-526 methacrylate to the carboxylic acid to yield a macromer containing a protected amine (Scheme 3). Deprotection under standard acidic conditions (trifluoroacetic acid) simultaneously cleaves ester linkages in the macromer, and deprotection using tetrabutylammonium fluoride was also unsuccessful. However, the *t*-BOC can be selectively removed using bismuth (III) trichloride in a mixture of acetonitrile and water, with all other functionalities remaining intact.²⁶

4-(4-(1-Bromoethyl)-2-methoxy-5-nitrophenoxy)butanoic acid can be converted to the acid chloride using thionyl chloride or phosphorous pentachloride and used to esterify PEG-526 methacrylate, however, some halogen exchange occurs in the process, producing a mixture of benzyl bromide and benzyl chloride macromers (Supporting information Scheme S2).

The final macromer we synthesized contained both an acrylate and a methacrylate functionality; free thiols (such as those found on cysteine) react rapidly with acrylates through a base catalyzed Michael addition, while reaction with the methacrylate is slow.²⁷ 4-(4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid is acrylated, and the carboxylic acid is subsequently converted to an ester by EDC coupling to PEG-526 methacrylate (Scheme 4).

Chart 1 summarizes the reactivity of each of the *o*-NB macromers in this report. This modular library of *o*-NB linkers allows conjugation to a wide variety of functional groups found on biomolecules and therapeutic agents. Depending on the linker chosen, a small molecular fragment may remain attached to the therapeutic agent after photorelease. For the *o*-NB linkers with alcohol, alkyl halide or amine at the benzylic position, depending on how the therapeutic agent is conjugated, it may be released in its unaltered state. Conjugation of a therapeutic agent to *o*-NB linkers with either the carboxylic acid, NHS ester, or pyridyl disulfide results in an additional small molecular fragment attached to the therapeutic agent (i.e. succinic acid) which may or may not affect the therapeutic activity of the drug.

In order to demonstrate the utility of these linkers for releasing therapeutic agents we first copolymerized PEG 10K diacrylate and the NHS-functionalized macromer, PEG526-methacrylate-4-(4-(1-((4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxabutanoyl)oxy) ethyl)-2-methoxy-5-nitrophenoxy)butanoate (abbreviated PEG-526MA-*o*-NB-NHS), using ammonium persulfate (APS) and N,N,N',N'-tetramethylethylene diamine (TEMED) as the redox initiating system. The resultant hydrogels were leached to remove any unreacted macromer or initiator, and then incubated with a solution of L-phenylalanine. The free amine should react with the NHS ester to produce an amide linkage and release *N*-hydroxysuccinimide, analogous to the standard bioconjugation technique that utilizes amines in proteins to react with NHS-functionalized molecules. The in-gel reaction was allowed to proceed overnight before any unreacted phenylalanine was leached from the gels through successive washing. One set of gels was then exposed to light ($\lambda=365$ nm, 10 mW/cm², 10 min), and the amount of phenylalanine released was quantified via UV-Vis spectroscopy. Assuming a) 100% reactive incorporation of PEG-526MA-*o*-NB-NHS into the hydrogel, b) none of the NHS esters hydrolyzed during polymerization or exchange, and

c) all of the *o*-NB groups photolyzed, 81.3% of the succinyl amide of phenylalanine was released from the gel. Although these results indicate that PEG-526MA-*o*-NB-NHS can be used to conjugate molecules containing free amines into the gel, there is no easy way to quantify the amount of amino acid or other amine-containing molecule into the gel prior to release.

Since many proteins either contain free thiols or are easily functionalized with a thiol group, and peptides are easily synthesized with cysteine residues, we next investigated the photodegradable macromer containing an activated disulfide linkage, poly(ethylene glycol) (PEG)-526-methacrylate-4-(2-methoxy-5-nitro-4-(1-((4-oxo-4-(2-(pyridin-2-yl)disulfanyl)ethoxy)butanoyl)oxy)butanoate (abbreviated as PEG-526MA-*o*-NB-SSpyr). The pyridine disulfide moiety undergoes disulfide exchange with free thiols¹⁷, releasing pyridine-2-thione, which is quantified via absorbance spectroscopy (Scheme 5). This approach allows conjugation of thiol-containing biomolecules to the photodegradable macromer either before (Scheme 5a) or after (Scheme 5b) formation of the hydrogel. Not only can the amount of incorporated biomolecule be easily quantified (by measuring pyridine-2-thione release) but biomolecules sensitive to hydrogel formation conditions can be introduced post-fabrication.

In order to demonstrate the utility of this linker for sequestering and releasing peptides we copolymerized PEG 10K diacrylate and PEG-526MA-*o*-NB-SSpyr using APS and TEMED. Hydrogels containing 1 mM activated disulfide were incubated with a solution of the cell-adhesive peptide GCGYGRGDSPG. In solution, disulfide exchange is complete within five minutes at pH 6–8, however, release of pyridine-2-thione is somewhat slower from the hydrogel (likely due to sterics²⁸), so gels were allowed to react overnight at 4 °C. Based on pyridine-2-thione release, the gels were found to incorporate 0.34 mM RGD via exchange. Although this concentration is lower than the concentration of the pyridine disulfide groups available within the gel, the RGD concentration is sufficient to promote cell adhesion. In order to quantify release of RGD and determine the exposure time required to fully release the adhesive peptide, a set of hydrogels were incubated with NHS-FITC, which reacts with the N-terminus of the peptide. The unreacted FITC was washed from the hydrogels, which were subsequently exposed to 365 nm light ($I_0=10 \text{ mW/cm}^2$). The amount of released peptide was quantified via fluorescence. Complete release occurs in less than ten minutes (Figure 1a), indicating that these exposure conditions are sufficient to release all of the cell-adhesive peptide from the gels.

In order to test the activity of the peptide and confirm its release from the gel, fibroblasts were seeded onto gels containing the photoreleasable RGD peptide, and onto gels that had been exposed to light ($\lambda=365 \text{ nm}$, $I_0=10 \text{ mW/cm}^2$, $t=20 \text{ min}$) and washed multiple times to remove the photoreleased peptide. Cells adhere to gels containing the RGD, and begin to spread within 60 minutes, while cells seeded onto gels from which the peptide was photoreleased round up (Figure 1b) and are washed away (data not shown). Photodegradation can therefore be used as a tool to control cell adhesion to these biomaterials.

Low molecular weight compounds diffuse freely into and out of hydrogels; however, the diffusion of larger species is retarded by the gel, and, above a certain molecular weight, prevented. The diffusion coefficient for a molecule in the gel, D_g , relative to its diffusion coefficient in free solution, D_0 , is a function of the radius of that molecule, R_s , the mesh size of the hydrogel (ξ), and the polymer volume fraction in the gel (v_2) ((Equation (3); Y is the ratio of critical volume needed for translational movement of the molecule to average free volume per liquid molecule, usually approximated to equal one). We characterized the physical properties of the hydrogel ($E^* = 32.75$ kPa, $Q=20$), to determine the effect of the gel structure ($\xi=143.5$ Å) on the diffusion of larger biomolecules in the gel¹⁹, and determine the approximate size of biomolecules that could be effectively introduced into and released from the hydrogel. For this hydrogel system, where $\xi=143.5$ Å and $v_2=0.05$, D_g/D_0 decreases from 0.88 to 0.62 when R_s increases from 10 Å to 50 Å, a relevant size range for macromolecular species such as proteins. Practically, this means that any macromolecular agent loaded into or released from these hydrogel depots requires extended equilibration time (on the order of a few hours) to account for retarded diffusion through the gel.

$$\frac{D_g}{D_0} = \left(1 - \frac{R_s}{\xi}\right) \exp\left(-Y \left(\frac{v_2}{1-v_2}\right)\right) \quad \text{Eq. 3}$$

To experimentally verify the effect of the gel on protein diffusion out of the network, we prepared a set of hydrogels that did not contain the activated disulfide, and incubated these gels in a solution of FITC-labeled bovine serum albumin (BSA, $M_n \sim 66,500$) overnight. We monitored the diffusion of BSA out of the gels, and found that the BSA is completely released within three hours (Figure 2a). Therefore, proteins and peptides of the same or smaller size should be able to diffuse into and out of these hydrogels completely within a few hours.

In order to test the utility of this system for sequestering proteins, hydrogels containing the activated disulfide were incubated with a solution of BSA (which contains a free thiol²⁹), but no disulfide exchange occurred, even under extended incubation (>48 hours). Because BSA diffuses into and out of the gel within a few hours, we presume the photodegradable tether is sterically inaccessible to larger proteins. To confirm, we synthesized a new linker, PEG-10K-methacrylate-4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-yl)disulfanyl)ethoxy)butanamido)ethyl)phenoxy)butanoate (abbreviated PEG-10K-MA-*o*-NB-SSpyr). The PEG chain in this macromer is significantly longer ($M_n=10,000$ vs. $M_n=536$ Da), which allows greater distance between the network crosslink site and the activated disulfide (227 ethylene oxide repeat units vs. eleven). We copolymerized PEG-10K-MA-*o*-NB-SSpyr with PEG 10K dimethacrylate and infused the hydrogels with a solution of BSA. Pyridine-2-thione was released, confirming that sterics were likely limiting the interaction of protein with the photodegradable linker. Despite the significantly longer tether, only approximately 10% of the disulfide groups underwent exchange, reinforcing our hypothesis that sterics play an important role in conjugating proteins to these hydrogels post-fabrication.³⁰

If a protein is stable to the polymerization conditions, it can undergo disulfide exchange with PEG-10K-MA-*o*-NB-SSpyr prior to incorporation into the hydrogel (Scheme 5a). We incubated BSA in a buffered solution of PEG-10K-MA-*o*-NB-SSpyr at 4 °C overnight; pyridine-2-thione release indicates complete exchange occurred. The PEG-10K-MA-*o*-NB-S-BSA conjugate was copolymerized with PEG10K dimethacrylate into a hydrogel. After washing to remove any unreacted materials, hydrogels were exposed to 365 nm light ($I_0=10 \text{ mW/cm}^2$), allowed to equilibrate in buffered solution overnight at 4 °C, and protein release was quantified via UV-Vis spectroscopy ($\lambda=280 \text{ nm}$). The release profile of BSA was exponential (Figure 2b). The actual concentration of BSA released after complete degradation ($126 \pm 8 \text{ }\mu\text{g/mL}$) was slightly lower than expected ($155 \text{ }\mu\text{g/mL}$); this difference may be due to hydrolysis of the tether prior to fabrication, incomplete reactive incorporation of the tethered protein during polymerization, or slight sequestration of the released BSA into the hydrogel. The enzymatic activity of the released BSA was quantified using *p*-nitrophenyl acetate as the substrate. The released BSA exhibits identical esterase activity compared to the native BSA that did not experience sequestration and release ($\lambda=405 \text{ nm}$ Native: $A = 0.185 \pm 0.006$; Released: $A = 0.196 \pm 0.006$). These results demonstrate that moderate molecular weight proteins can be sequestered and released from hydrogels using light while maintaining their enzymatic activity.

These results are encouraging, but in order to use this system to deliver chemical cues to cells, we need the ability to incorporate more sensitive biomolecules such as growth factors. TGF- β 1 is a growth factor important in wound healing and implicated in many diseases such as fibrosis and cancer. It has a moderate molecular weight ($\sim 25 \text{ kDa}$) and contains nine cysteine residues; eight form disulfide bonds, while one is free, allowing its facile exchange with the activated disulfide^{31,32}. TGF- β 1 was incubated with PEG-10K-MA-*o*-NB-SS-Pyr for 12 h at 4 °C and pyridine-2-thione release was monitored. The TGF- β 1 photodegradable macromer conjugate was copolymerized with PEG10K dimethacrylate into hydrogels. After washing to remove any unreacted materials, the gels were exposed to 365 nm light ($I_0=10 \text{ mW/cm}^2$, $t=10 \text{ min}$) and allowed to equilibrate in buffer for two hours, to release a final concentration of 5.2 ng/mL TGF- β 1 (quantified by ELISA). The solutions were applied without dilution to plated hMSCs, which undergo chondrogenesis in the presence of TGF- β 1^{33,34}. Glycosaminoglycan (GAG) production was visualized via toluidine blue staining (Figure 3a–c). After three days hMSCs treated with the released TGF- β 1 produce GAGs (Figure 3c, observed as dark granules in the cytoplasm) and appear similar to the positive control (Figure 3b, hMSCs treated with 10 ng/mL TGF- β 1 for three days), while the untreated hMSCs do not stain with toluidine blue (Figure 3a, except for the cell nucleus). GAG production was also measured via dimethylmethylene blue (DMMB) assay and normalized to the number of cells (measured via PicoGreen assay) (Figure 3d). Despite relatively large error in the measurements, it is clear that GAG production is greater in both the positive control and the cells treated with photoreleased TGF- β 1. The combination of the differences in toluidine blue staining and the qualitative differences in GAG production demonstrate that the sequestered and released TGF- β 1 retains its biological activity and is able to induce differentiation of hMSCs.

Through examples above, we have demonstrated that this platform can be used to incorporate and release biomolecules and therapeutics of various sizes predictably and controllably. This library of *o*-NB-containing macromers should allow direct conjugation of many different functional groups to the macromer, either before or after hydrogel fabrication. The acrylate and pyridyldisulfide moieties should react directly with free thiols either before or after incorporation (respectively) of the macromer into a hydrogel depot. The NHS-ester allows conjugation of any protein through lysine residues or N-terminal amines. While conjugation prior to hydrogel fabrication is more efficient, NHS-esters can survive radical polymerizations and thus should allow post-fabrication incorporation (as demonstrated using phenylalanine as a model compound). The carboxylic acid functionality will allow conjugation to alcohols and amines via ester and amide formation. The alcohol functionality provides conjugation to carboxylic acids via ester formation, or conjugation to molecules with good leaving groups via nucleophilic substitution (Chart 1). Only the acrylate and the benzyl bromide should be sensitive to standard free radical polymerization conditions, requiring their conjugation to biomolecules prior to hydrogel fabrication. All other groups allow post-fabrication incorporation of biomolecules into the hydrogel.

Conclusions

Here we report the synthesis of a library of *o*-NB macromers containing different functionalities at the benzylic position. As proof-of-concept, the N-hydroxysuccinyl ester macromer was incorporated into hydrogels, and then reacted with phenylalanine. Upon exposure to light ($\lambda=365$ nm, 10 mW/cm², 10 min) 81.3% of theoretical load of phenylalanine was released from the gel, demonstrating the utility of these linkers for incorporating and releasing therapeutics such as peptides and proteins. We successfully demonstrated the quantifiable conjugation of a bioactive peptide (GCGYGRGDSPG), an enzymatically active protein (BSA) and a bioactive growth factor (TGF- β 1) into hydrogels via disulfide exchange, and demonstrated that these biomolecules can be released controllably from the hydrogels using light. Neither the incorporation process nor photorelease has any apparent effect on their bioactivity. This platform provides researchers with an array of chemistries that should allow for direct conjugation of nearly any type of therapeutic agent to the linker, and its subsequent controlled release using light. Because light is an externally controlled trigger, this approach allows precise spatial and temporal patterning of biological signal within a hydrogel matrix. Precise control over the delivery of therapeutics is critical to recapture the complex signaling cascades found in nature. External control of the temporal and spatial distribution of different signals may introduce a pathway to engineering complex tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for AMK for this work was provided by UCLA HSSEAS Start-up funds, UCLA/CNSI IRG Seed funding, Millipore Corporation and the National Institutes of Health through the NIH Director's New Innovator Award Program, 1-DP2-OD008533. HDM thanks the NIH (NIBIB R01 EB 136774-01A1) for funding.

References

1. Vermonden T, Censi R, Hennink WE. Chem Rev. 2012; 112:2853. [PubMed: 22360637]
2. Kim H, Kim HW, Suh H. Biomaterials. 2003; 24:4671. [PubMed: 12951010]
3. Benoit DSW, Nuttelman CR, Collins SD, Anseth KS. Biomaterials. 2006; 27:6102. [PubMed: 16860387]
4. Zisch AH, Lutolf MP, Ehrbar M, Raeber GP, Rizzi SC, Davies N, Schmokel H, Bezuidenhout D, Djonov V, Zilla P, Hubbell JA. Faseb J. 2003; 17:2260. [PubMed: 14563693]
5. Wilcox M, Viola RW, Johnson KW, Billington aP, Carpenter BK, Mccray Ja, Guzikowski aP, Hess GP. J Org Chem. 1990; 55:1585.
6. Kloxin AM, Kasko AM, Salinas CN, Anseth KS. Science. 2009; 324:59. [PubMed: 19342581]
7. Kloxin AM, Tibbitt MW, Kasko AM, Fairbairn JA, Anseth KS. Adv Mater. 2010; 22:61. [PubMed: 20217698]
8. Sarig-Nadir O, Livnat N, Zajdman R, Shoham S, Seliktar D. Biophys J. 2009; 96:4743. [PubMed: 19486697]
9. Tibbitt MW, Kloxin AM, Dyamenahalli KU, Anseth KS. Soft Matter. 2010; 6:5100. [PubMed: 21984881]
10. Hahn MS, Miller JS, West JL. Adv Mater. 2006; 18:2679.
11. Hahn MS, Taite LJ, Moon JJ, Rowland MC, Ruffino KA, West JL. Biomaterials. 2006; 27:2519. [PubMed: 16375965]
12. Luo Y, Shoichet MS. Nat Mater. 2004; 3:249. [PubMed: 15034559]
13. Luo Y, Shoichet MS. Biomacromolecules. 2004; 5:2315. [PubMed: 15530047]
14. Griffin DR, Patterson J, Kasko AM. Biotechnology and Bioengineering. 2010; 107:1012. [PubMed: 20661910]
15. Griffin DR, Kasko AM. J Am Chem Soc. 2012; 134:13103. [PubMed: 22765384]
16. Griffin DR, Kasko AM. ACS Macro Letters. 2012; 1:1330. [PubMed: 25285242]
17. Bontempo D, Heredia KL, Fish BA, Maynard HD. Journal of the American Chemical Society. 2004; 126:15372. [PubMed: 15563151]
18. Mohsen-Nia M, Modarress H, Rasa H. Journal of Chemical and Engineering Data. 2005; 50:1662.
19. Bryant, SJ.; Anseth, KS. Scaffolding in Tissue Engineering. CRC Press; Boca Raton: 2006.
20. Grover GN, Alconcel SNS, Matsumoto NM, Maynard HD. Macromolecules. 2009; 42:7657. [PubMed: 21552430]
21. Zhu H, Guo ZK, Jiang XX, Li H, Wang XY, Yao HY, Zhang Y, Mao N. Nat Protoc. 2010; 5:550. [PubMed: 20203670]
22. Hwang NS, Varghese S, Elisseeff J. Methods in Molecular Biology. 2007; 407:351. [PubMed: 18453267]
23. Holmes CP, Jones DG. J Org Chem. 1995; 60:2318.
24. Burakowska E, Zimmerman SC, Haag R. Small. 2009; 5:2199. [PubMed: 19572327]
25. Hermanson, GT. Bioconjugate Techniques. Academic Press; San Diego, CA: 2008.
26. Navath RS, Pabbisetty KB, Hu LQ. Tetrahedron Lett. 2006; 47:389.
27. Mather BD, Viswanathan K, Miller KM, Long TE. Progress in Polymer Science. 2006; 31:487.
28. Schlosser JL, Kerr J, Wong D, Kasko AM. manuscript in preparation.
29. Suttiaprasit P, Krisdhasima V, McGuire J. Journal of Colloid and Interface Science. 1992; 154:316.
30. Matsumoto NM, Gonzalez-Toro DC, Chacko RT, Maynard HD, Thayumanavan S. Polymer Chemistry. 2013
31. Roberts, AB.; Sporn, MB. Peptide Growth Factors and Their Receptors. Springer; Berlin: 1991.
32. Daopin S, Piez KA, Ogawa Y, Davies DR. Science. 1992; 257:369. [PubMed: 1631557]
33. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. Exp Cell Res. 1998; 238:265. [PubMed: 9457080]
34. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Tissue Eng. 1998; 4:415. [PubMed: 9916173]

Figure 1.

a) Fractional release of GCGYGRGDSPG as a function of exposure time ($\lambda=365$ nm, $I_0=10.0 \pm 0.2$ mW/cm²) b) changes in morphology of fibroblasts seeded onto an unexposed gel (left) and an exposed gel (right) ($\lambda=365$ nm, $I_0=10$ mW/cm², $t=20$ min; images acquired 60 minutes after seeding).

Figure 2.

a) Diffusion of FITC-labeled BSA out of hydrogels as a function of time b) BSA release as a function of exposure time at 365 nm ($\lambda=365$ nm, $I_0=10.0 \pm 0.2$ mW/cm²).

Figure 3.

Expression of GAGs visualized by toluidine blue staining after three days of culture. (a) Untreated hMSCs only show nuclear staining (negative control), while (b) hMSCs treated with 10 ng/mL TGF-β1 (positive control) or (c) 5.2 ng/mL photoreleased TGF-β1 exhibit dark granules in the cell cytoplasm, indicating GAG production. (d) GAG production per cell, normalized to untreated hMSCs (- control).

Scheme 1.

Synthesis of PEG526-methacrylate-4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate.

Scheme 2.

Synthesis of polymerizable *o*-NB modified with an activated ester and an activated disulfide.

Scheme 3.

Synthesis of PEG526-methacrylate-4-(4-(1-aminoethyl)-2-methoxy-5-nitrophenoxy)butanoate.

Scheme 4.

Synthesis of PEG526-methacrylate-4-(4-(1-acryloyloxyethyl)-2-methoxy-5-nitrophenoxy)butanoate.

Scheme 5.

Incorporation of thiol-containing biomolecules into hydrogels a) Disulfide exchange of PEG-10K-methacrylate-4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-yl)disulfanyl)ethoxy)butanamido)ethyl)phenoxy) butanoate, followed by copolymerization into a hydrogel network and b) copolymerization of PEG526-methacrylate-4-(2-methoxy-5-nitro-4-(1-((4-oxo-4-(2-(pyridin-2-yl)disulfanyl) ethoxy)butanoyl)oxy)butanoate or PEG-10K-methacrylate-4-(2-methoxy-5-nitro-4-(1-(4-oxo-4-(2-(pyridin-2-yl)disulfanyl) ethoxy)butanamido)ethyl)phenoxy)butanoate into a hydrogel, followed by disulfide exchange.

Macromer Structure	Reactive Group	Functional group reactivity
Alcohol	Carboxylic acid, alkyl halides
Alkyl halide	Alcohols, nucleophiles
Amine	Carboxylic acids
Carboxylic acid	Amines, alcohols, alkyl halides
NHS-ester	Amines
Pyridyl disulfide	Thiols
Acrylate	Thiols, amines (pseudo-Michael addition)
Biotin	Streptavidin

Chart 1.

Reactivity of *o*-NB conjugates towards different functional groups found on biomolecules.