Published in final edited form as:
Biomacromolecules. 2009 August 10; 10(8): 2194–2200. doi:10.1021/bm900366e.

Hydrogels Cross-Linked by Native Chemical Ligation

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Abstract

We describe the use of native chemical ligation (NCL) reaction to covalently cross-link soluble polymers into hydrogels. Macromonomers consisting of a four-armed poly(ethylene glycol) (PEG) core end-functionalized with either thioester or *N*-terminal cysteine peptide were designed and synthesized. Upon mixing aqueous solutions of the thioester and *N*-terminal cysteine macromonomers, rigid hydrogels formed within minutes. The gelation time was affected by choice of buffer, pH, polymer concentration, reaction temperature, and chemical composition of the *N*-terminal cysteine conjugate. The kinetics of gel formation and the viscoelastic behavior of selected hydrogels were further studied by oscillatory rheology, which demonstrated a minimum gel formation time of approximately two minutes and the formation of an elastic cross-linked hydrogel via the NCL reaction. A useful feature of this hydrogel strategy is the regeneration of thiol functional groups as a result of the NCL reaction, thereby allowing functionalization of the polymer hydrogel with biomolecules. This was demonstrated by conjugation of a maleimide-GRGDSPG-NH_2 peptide to an NCL hydrogel, permitting the attachment of human mesenchymal stem cells (hMSCs) on the hydrogel. Due to the mild reaction conditions, chemoselectivity, and potential for biological functionalization, our approach may prove useful as a general method for hydrogel formation, including hydrogels intended for biomedical applications.

Introduction

Hydrogels are hydrophilic polymeric networks capable of absorbing and retaining large amounts of water.[1–4] Hydrogels are useful in controlled release systems for drug delivery,[5] tissue repair, and tissue engineering[6,7] and as surgical sealants and adhesives.[8,9] Although great progress in medical application of hydrogels has been made, it remains challenging to develop cross-linking methods that satisfy the demanding biological and handling requirements for medical treatment.[10] Hydrogel systems capable of deployment by minimally invasive methods and solidification under physiological conditions are desirable for many medical applications,[11–17] and identification of new and biocompatible cross-linking methods may lead to greater pharmaceutical and medical use of hydrogels.

In nature, thioesters participate in the synthesis of a number of cellular components and can be prepared as activated building blocks through chemical synthesis.[18,19] Although relatively unreactive to aminolysis, thioesters readily react with a thiol group through transesterification to form a new thioester.[20–22] The reaction between a thioester and an *N*-terminal-Cys yields

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an *S*-acyl covalent intermediate that spontaneously undergoes an *S*- to *N*-acyl migration to form a new amide bond through a five-member ring intermediate (Scheme 1).[22] This mild native chemical ligation (NCL) method has proven useful in chemical synthesis of large peptides and proteins[18,23–26] and dendrimers[27] and was combined with peptide self-assembly to generate polypeptides with repeated sequences.[28] A recent report on *β*-sheet forming peptides indicates that the NCL reaction increased the stiffness of preassembled peptide hydrogels through cross-linking of short self-assembled peptides.[29] However, the use of NCL to form covalently cross-linked hydrogels from soluble macromolecular precursors is unreported in the literature.

As a hydrogel cross-linking method, NCL is expected to have attractive features including chemoselectivity, that is, only an *N*-terminal-Cys is reactive, and high efficiency is achievable under mild aqueous conditions. Furthermore, compared to other reactions involving thiol containing functional groups previously reported for hydrogel formation,[11,15] the thiol side chain of the *N*-terminal Cys is preserved as a consequence of rearrangement during the NCL reaction, allowing functionalization of the polymer hydrogel network with bioactive compounds for modulation of the biological properties of in situ formed hydrogels.

A significant challenge for in situ forming NCL hydrogels lies in the relatively slow reaction rate of NCL. Although peptide ligation by NCL can proceed to high yield, in some cases the reaction has been reported to require hours or days for completion.[23,30] As a consequence, excess free low molecular weight thiols and reducing agents are commonly included in 10-fold or more molar excess to accelerate peptide ligations using NCL.[31] Given the possibility that high concentrations of organic thiols or strong reducing agents used for accelerating NCL may have adverse consequences on biological systems, catalyst-free approaches to NCL hydrogel formation are highly desirable.

Here we illustrate the use of NCL as a strategy to form covalently cross-linked polymer hydrogels under mild conditions and in the absence of catalysts. We report the synthesis of new thioester-polymer and *N*-terminal cysteine-polymer bioconjugates (Scheme 2), the reaction conditions leading to rapid hydrogel formation by NCL, the viscoelastic behavior of the hydrogels by oscillatory rheology, and stem cell culture on the formed hydrogel after biofunctionalization with a cell adhesion peptide.

Experimental Section

Materials

Four-armed poly(ethylene glycol) (PEG) with amine end groups (MW 10k; PEG4A) was purchased from SunBio PEG Shop (Orinda, CA). Diacrylated poly(ethylene glycol) (PEGDA) of molecular weight 3400 was purchased from Glycosan (Salt Lake City, UT). 3-Mercaptopropionic acid ethyl ester was purchased from TCI America (Portland, OR). 2-ClTrt chloride resin (1.55 mmol/g), benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), and 1-hydroxybenzotriazole (HOBt) were purchased from Peptide International (Louisville, KY). Protected amino acids were purchased from NovaBiochem (La Jolla, CA). Acetonitrile and MeOH were from Burdick and Jackson. Trifluoroacetic acid (TFA) and ether were from J. T. Baker. Succinic anhydride, *N*,*N*-dimethylformide (DMF), *N*,*N*-diisopropylethylamine (DIEA), piperidine, 4-dimethylaminopyridine (DMAP), ninhydrin, tris(2-carboxyethyl) phosphine hydrochloride (TCEP), ethyl 3-mercaptopropionate (EMP), and 2-hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Calcein AM was purchased from Invitrogen (Chicago, IL).

Synthesis of Ethyl 3-Mercaptopropionate-Succinic Acid (EMP-SA)

EMP (2.95 g, 22 mmol) was added under argon to a stirring solution of succinic anhydride (2.0 g, 20 mmol) and DMAP (122 mg, 1 mmol) in 25 mL of acetonitrile–pyridine (9:1). The reaction mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure and dried in vacuo. The residue was dissolved in 50 mL of EtOAc. The EtOAc solution was washed with 0.1 N HCl aqueous solution 30 mL (×3) and H_2O (×3) and dried over anhydrous $MgSO_4$. After filtration, the solution was concentrated to dryness under reduced pressure and in vacuo (Scheme 3). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 10.23 (s, br), 4.14 (2H, q, J = 7.0 Hz, -C$\underline{H}_2$-CH$_3$), 3.13 (2H, t, J = 7.0 Hz, −S-C$\underline{H}_2$-), 2.88 (2H, t, J = 7.0 Hz, -S-CO-C$\underline{H}_2$-), 2.69 (2H, t, J = 7.0 Hz, -S-CH$_2$-C$\underline{H}_2$-), 2.62 (2H, t, J = 7.0 Hz, -C$\underline{H}_2$-COOH), 1.25 (3H, t, J = 7.0 Hz, −CH$_2$-C$\underline{H}_3$).

Synthesis of Thioester Macromonomer 1

The synthetic approach is shown in Scheme 3. A solution of EMPSA (0.234 g, 1 mmol) in DCM (2 mL) was added into a vial containing PEG4A (1 g, 0.4 mmol of amine) and BOP (0.221 g, 1 mmol), followed by addition of DIEA (0.348 mL, 2 mmol). The mixture was vortexed for 5 min and rocked for 2 h. The reaction was monitored by silica gel TLC (solvent system DCM–MeOH–HOAc = 100:3:1). The spots on the TLC plate were visualized by spray of 1% ninhydrin solution in ethanol containing 3% HOAc followed by heating at 105 °C. The purification of the product was performed by dilution with MeOH to a final volume of 50 mL. The solution was shaken thoroughly and frozen at −20 °C. The precipitate was collected by centrifugation (−9 °C, 6000 rpm, 20 min) and decanting the solvent. The purification cycle of dissolution in MeOH at room temperature, freezing at −20 °C, centrifugation at −9 °C, and decanting the MeOH was repeated four times, followed by precipitation with diethyl ether, and drying in vacuo. The ninhydrin test of the purified product gave a yellowish solution. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.15 (2H, q, J = 7.0 Hz, -C$\underline{H}_2$-CH$_3$), 3.79−3.41 (m, -O-CH$_2$-CH$_2$-O-), 3.12 (2H, t, J = 7.0 Hz, -S-C$\underline{H}_2$-), 2.92 (2H, t, J = 7.0 Hz, -S-CO-C$\underline{H}_2$-), 2.61 (2H, t, J = 7.0 Hz, -S-CH$_2$-C$\underline{H}_2$-), 2.52 (2H, t, J = 7.0 Hz, -C$\underline{H}_2$-COOH), 1.26 (3H, t, J = 7.0 Hz, -CH$_2$-C$\underline{H}_3$).

Protected Dipeptide Fragment Synthesis

Fully protected dipeptides Boc-Cys(Trt)-Gly-OH, Boc-Cys(Trt)-Glu(OtBu)-OH, Boc-Cys (Trt)-Asp(OtBu)-OH, Boc-Cys(Trt)-Trp(Boc)-OH, and Boc-Cys(Trt)-Arg(Pbf)-OH were synthesized manually as protected peptide fragments by Fmoc strategy on a 2-chlorotrityl chloride resin (Scheme S1). A typical procedure of solid-phase synthesis is described below, performed with 2-chlorotrityl chloride resin (1.0 g, 1.55 mmol/g). The solution of Fmoc-amino acid-OH (1 mmol) dissolved in DCM (10 mL) and DIEA (1 mL) was added to a reaction vessel containing resin and rocked for 30 min, followed by washing with DMF three times. The resin was treated with DCM–MeOH–DIEA (8:1:1) for 20 min and washed with DMF three times. Fmoc was removed by treatment with 20% piperidine in DMF for 20 min and washed with DMF four times. The ninhydrin test of the resin gave a positive result. Boc-Cys(Trt)-OH (0.92 g, 2 mmol) and BOP (0.88 g, 2 mmol) were dissolved in DCM (8 mL), followed by addition of DIEA (522 μL, 3 mmol). After 10 min, the solution was added to the resin and rocked for 2 h, followed by washing with DMF and MeOH, three times each. The resin was dried in vacuo and the ninhydrin test of the resin gave a yellowish color. Then, protected peptide fragments were obtained by treatment of the resin with 1% TFA in dichloromethane (DCM), and the cleaved peptide sequences were confirmed by MALDI TOF-MS analysis. The analysis of the products by silica gel TLC (solvent system DCM–MeOH–HOAc = 100:3:1) gave a single spot on the plate.

Synthesis of *N*-Terminal Cysteine Macromonomers 2, 3a–e

The synthetic approach used for macromonomers **3a**–**e** is shown in Scheme 4. The method used for macromonomer **2** was similar except for the use of Boc-Cys(Trt)-OH instead of dipeptide. The solution of Boc-Cys(Trt)-OH or protected dipeptide Boc-Cys(Trt)-AA-OH (0.25 mmol) in DCM (2 mL) was added into a vial containing PEG4A (0.5 g, 0.2 mmol of amino group) and BOP (0.11 g, 0.25 mmol), followed by addition of DIEA (44 μL, 0.25 mmol). The mixture was vortexed for 5 min, subsequently rocked for 2 h, and concentrated under a N_2 flow. The residue was dissolved in 50 mL of MeOH and frozen at −20 °C. The precipitate was collected by centrifugation (−9 °C, 6000 rpm, 20 min) and decanting the solvent. The purification cycle of dissolution in MeOH at room temperature, freezing at −20 °C, centrifugation at −9 °C, and decanting the MeOH was repeated four times, followed by precipitation with diethyl ether, and drying in vacuo. The analysis of the products by silica gel TLC (solvent system DCM–MeOH–HOAc = 100:3:1) gave a single spot on the origin and no spot from Boc-Cys(Trt)-OH or protected dipeptides. The ninhydrin test of the purified product gave a yellowish solution. Proton NMR ($CDCl_3$, 500 MHz) spectra of the protected cysteine-PEG4A conjugates confirmed their structures.

Protected cysteine-PEG4A conjugates were then treated with 30 mL of TFA containing TIS (1 mL) and EDT (1 mL) at room temperature for 2 h and concentrated under reduced pressure, respectively. The residue was dissolved in 50 mL of MeOH and frozen at −20 °C. The precipitate was collected by centrifugation (−9 °C, 6000 rpm, 20 min) and decanting the solvent. The purification cycle of dissolution in MeOH at room temperature, freezing at −20 °C, centrifugation at −9 °C, and decanting the MeOH was repeated four times, followed by precipitation with diethyl ether, and drying in vacuo to generate the conjugates **2** and **3a**–**e** TFA salt. Ninhydrin test gave a dark blue color, indicating that the Boc protection group was removed. Finally, the conjugates **2** and **3a**–**e** TFA salt were dissolved in 0.1 M NH_4HCO_3 aqueous solution (25 mL), bubbled with argon for 20 min, frozen at −20 °C, and lyophilized to produce the salt-free conjugates **2** and **3a**–**e**.

Hydrogel Formation

Gel formation was accomplished by mixing equimolar amounts of thioester macromonomer **1** in pure water (solution A) with one of the *N*-terminal cysteine macromonomers **2** and **3a**–**e** in buffer solution (buffer concentration, ×2; solution B) at 23 or 37 °C. *N*-Terminal cysteine macromonomers were used in either TFA salt form (Table S1) or salt-free form (Table S2). The molar ratio of thioester macromonomer (**1**) to *N*-terminal cysteine macromonomer (**2**, **3a**) was generally 1:1 unless specifically noted, and the final polymer concentration ranged from 2–20% (w/v). No exogenous free thiol or reducing agent was added during the NCL reaction.

Screening of Gel Formation Time and Rheological Characterization

Rapid screening of gel formation time was performed by visual inspection as follows: to a stirring solution A (250 μL) in a test tube (100 × 13 mm) with a stirring bar (10 × 3 mm; 200 rpm) was added solution B (250 μL) and a stopwatch was started. Gel formation time was recorded when the stirring bar stopped rotating as a result of gel formation (Tables S1 and S2). More detailed hydrogel characterization was performed using dynamic rheology in which gel formation occurred in situ. All oscillatory rheological experiments were performed with a Paar Physica MCR300 Rheometer with a Peltier temperature control device maintained at 20 °C using either a stainless steel cone/plate fixture (50 mm diameter, 1° cone) or a stainless steel parallel plate fixture (25 mm diameter, 1 mm gap). Two methods were used.

Method 1. A total of 500 μL each of solutions A and B were added to a vial. After vortexing, 590 μL of the mixture was immediately loaded onto the thermostatted rheometer plate, and a

50 mm/1° cone was positioned to confine the solution in a 0.05 mm gap at the center between the cone and the plate. After moisturized Kimwipes paper was applied to surround the cone/plate fixture for evaporation control, data were collected every 20 s over 140 min. The measurements of the storage modulus and loss modulus were taken in the oscillatory mode at 1 Hz frequency and 1% strain during cross-linking. At the conclusion of the gelation experiment, a frequency sweep experiment was performed from 0.01 to 10 Hz with 19 data points at 1% strain. Finally, a strain sweep experiment was performed with strain from 1 to 100% at 1 Hz frequency. Data obtained using this method are shown in Figures 3 and S1–S4.

Method 2. Solutions A and B were mixed as described above, 500 μL of the mixture was immediately loaded onto the thermostatted rheometer plate (20 °C), and a parallel plate (25 mm in diameter) was positioned to confine the solution within a 1 mm gap. After moisturized Kimwipes paper was applied to surround the parallel plate fixture for evaporation control, data were collected every 20–30 s for up to 300 min. The measurements of the storage modulus and loss modulus were taken at 20 °C in the oscillatory mode at 1 Hz frequency and 1% strain during measurement. In selected experiments, frequency and strain sweeps were performed after the storage modulus reached a stable plateau value. Frequency sweeps were performed from 0.01 to 10 Hz at 1% strain. Strain sweeps were performed from 1 to 100% strain at 1 Hz frequency. Data obtained using this method are shown in Figures 1, 2, and S5–S8.

Synthesis of Cell Adhesive Peptides

Peptides maleimide-GRGDSPG-NH_2 and Ac-CGRGDSPG-NH_2 were synthesized using standard solid phase peptide synthesis protocols on Rink amide resin (Anaspec, San Diego, CA) at 0.1 mmol scale. Each coupling step was carried out by mixing 3 equiv of Fmoc-protected amino acids, PyBop, and *N*-methyl morphiline with the resin beads for 4 h on a rocker. Upon completion of coupling indicated by Kaiser's test, the resin beads were washed thoroughly with DMF and then 20% piperidine in DMF was used to deprotect Fmoc group to expose the amine groups on the beads for the next coupling. After the last amino acid was conjugated, either maleimide-OSU ester (2 equiv) in DMF was used to attach the maleimide moieties, or acetyl anhydride (20 equiv) and triethylamine (5 equiv) in DMF was used to cap the *N*-terminal of resin-bound peptides. Cleavage of the peptides from the resin and deprotection of the amino acid side chains were accomplished by treating the resin with 95% (v/v) TFA, 2.5% H_2O, and 2.5% TIS for 2 h at room temperature, after which the cleaved peptide solution was collected by filtration. Solvent was removed using a rotary evaporator; the product residues were dissolved in a minimal amount of TFA and precipitated with cold ether and by centrifugation at 4 °C. The product pellets were dissolved in deionized water, frozen, and lyophilized. Crude products were purified by preparative RP-HPLC and peptides were confirmed by MALDI-TOF MS.

Preparation of Peptide Functionalized NCL Hydrogel

Macromonomers **1** and **2** were dissolved at 10% (w/v) in H_2O and 0.2 M $NaHCO_3$ respectively. A total of 15 μL of each solution was pipetted into a 1 mL disposable syringe with the tip cut off. Gel was allowed to form for 15 min at 37 °C. Hydrogel disks (diameter = 5 mm, thickness = 1 mm) were removed from the syringe and placed into a well of a 96-well cell culture plate, washed with PBS, and then immersed in 100 μL of a solution of maleimide-GRGDSPG-NH_2 in PBS (10 mM, pH 7.2) for 15 min. The gel disks were then washed thoroughly with PBS prior to seeding of hMSCs.

Human Mesenchymal Stem Cell Culture

Human mesenchymal stem cells (hMSC) were obtained from Lonza (Walkersville, MD) and used as received. In 75 cm^2 tissue-culture treated flasks, 4×10^5 cells were seeded and cultured in hMSC basal media with mesenchymal cell growth supplements, L-glutamine, amphotericin-

B, and gentamicin purchased from Lonza. Cells were split 1:5 approximately once a week and fed every 3 days until utilized, and cells that had been passaged twice were used for culture on hydrogel surfaces. Hydrogel disks formed by NCL were seeded with hMSCs at a density of 5000 cells/cm^2 and cultured in hMSC growth media for 24 h. Cells were stained with Calcein AM (Invitrogen, Chicago, IL) and imaged by fluorescence microscopy to determine cell adhesion. Images are shown in grayscale and 80× original magnification. Cell adhesion on hydrogel surfaces was quantified using Metamorph Image Analysis software for eight fields per condition. Projected cell surface areas were measured in Metamorph to determine the percentage of hydrogel surface covered by cells.

Results and Discussion

Synthesis of Thioester and *N*-Terminal Cysteine Macromonomers

To simplify the chemical synthesis of the thioester macromonomer, a thioester was first prepared and subsequently coupled with an amine terminated poly(ethylene glycol) (PEG4A, 10 kDa) to afford the product. Based on a previous report,[32] ethyl 3-mercaptopropionate (EMP) was reacted with succinic anhydride in pyridine to produce ethyl 3-mercaptopropionate-succinic acid monothioester (EMPSA). EMPSA was coupled with PEG4A in the presence of BOP and DIEA in dichloromethane (DCM) to generate the thioester macromonomer **1** (Scheme 3).

In designing the *N*-terminal cysteine macromonomers (Scheme 2), a family of six *N*-terminal cysteine–polymer bioconjugates were designed and synthesized, including an *N*-terminal cysteine macromonomer (**2**) as well as five *N*-terminal cysteine dipeptide macromonomers (**3a**–**e**); the latter was used to examine the effect of the neighboring amino acid residue on the reactivity of the *N*-terminal cysteine. To synthesize the *N*-terminal cysteine macromonomers, a commercially available protected cysteine Boc-Cys(Trt)-OH and five fully protected cysteine dipeptides prepared by standard Fmoc solid-phase peptide synthesis on a 2-chlorotrityl resin (Scheme S1) were coupled with PEG4A by activation of BOP and DIEA in DCM to produce the protected *N*-terminal cysteine macromonomers. The coupling reactions were monitored by silica gel TLC and visualization under UV 254 nm and by ninhydrin reaction. After purification, the protected *N*-terminal cysteine macromonomers were treated by TFA with scavengers to remove the protecting groups to afford the TFA salts of *N*-terminal cysteine macromonomers **2** and **3a**–**e** (Scheme 4), which were further converted to the salt-free conjugates **2** and **3a**–**e** by neutralization with aqueous ammonium bicarbonate and lyophilization.

Formation of NCL Hydrogels

Combining aqueous solutions of **1**, **2**, and **3a**–**e** resulted in hydrogel formation. The effects of buffer system, macromonomer concentration, and reaction temperature were first explored in screening experiments using mixtures of **1** and the TFA salt of **2**. The results showed that the hydrogel formation time was significantly affected by buffer system, bioconjugate concentration and reaction temperature (Table S1). In general, an inverse relationship between gelation time and pH, temperature and macromonomer concentration was found, with gel formation times determined by visual inspection ranging from 6 min to 3 days. Among the conditions tested during this screening, the hydrogel formed fastest (~6 min) at a total polymer concentration of 20% (w/v) in 0.1 M sodium bicarbonate buffer pH 8.3 at 37 °C. Subsequent experiments comparing the TFA and salt-free forms of *N*-terminal cysteine forms of macromonomers **2** and **3a**–**e** showed that the salt-free forms produced gels more rapidly than the corresponding TFA salts (Table S2).

In addition to amide bond formation by NCL (Scheme 1), other potential chemical reactions giving rise to gel formation in mixtures of thioester and *N*-terminal cysteine macromonomers include disulfide bond formation and new thioester bond formation by thioester exchange rather than a spontaneous *S*- to *N*-acyl migration. Several control experiments were performed that led us to conclude that hydrogel formation was a result of the NCL reaction between the thioester of **1** and *N*-terminal cysteine groups of **2** and **3a**–**e**. In one control experiment, rheological assessment of a 10% solution of *N*-terminal cysteine macromonomer **2** only in sodium bicarbonate buffer revealed no evidence of elastic gel formation within 5 h as indicated by values of G' several orders of magnitude below G'' (Figure 1). At higher concentration (20%), a solution of **2** alone under the same conditions remained fluid for at least three hours and became a gel overnight at room temperature (Table S1), suggesting the possibility of very slow gelation via spontaneous intermolecular disulfide bond formation between macromonomers of **2**. Furthermore, the gel of **2** was readily soluble upon addition of equal volume of aqueous 20 mM tricarboxyeth-ylphosphine (TCEP) or 100 mM 2-mercaptoethanol in water, providing strong evidence for formation of hydrogel through very slow formation of disulfide bonds. Dissolution of the hydrogel presumably occurred by reduction of the intermolecular disulfide bonds by TCEP or by thiol exchange with 2-mercaptoethanol. In contrast, the hydrogel produced by mixing **1** and **2** formed within 10 min, and was stable upon addition of an equal volume of 20 mM TCEP in water. The hydrogel was also stable upon addition of 100 mM 2-mercaptoethanol in water, which should dissolve gels formed by thioester exchange reactions between macromonomers **1** and **2**. From these results we concluded that mixtures of macromonomers **1** and **2** formed hydrogel mainly by cross-linking through NCL rather than disulfide or thioester bond formation. We cannot exclude the possibility that disulfide bond formation may contribute partly to network formation, however this is likely to be the case only when the NCL reaction kinetics are very slow NCL (e.g., several hours).

There is evidence in the literature that the rate of gelation in Michael addition-type cross-linking is influenced by the charge of the neighboring amino acid residue near the thiol.[33] Therefore, we explored the effect of neighboring amino acid on the reactivity of the *N*-terminal cysteine toward NCL reaction. To probe a possible effect, we synthesized a limited number of *N*-terminal cysteine dipeptide macromonomers (**3a**–**e**) and determined the gel formation time in reaction mixtures with macromonomer **1**. The results are summarized in Table S2 and reveal mixed effects of flanking amino acid on the reactivity of the *N*-terminal cysteine. Experiments involving mixtures of **1** with *N*-terminal cys macromonomers indicated that insertion of a neighboring Gly (**3a**), Glu (**3b**), or Arg (**3e**) residue between the *N*-terminal Cys and the polymer had little influence on the reactivity of the *N*-terminal Cys as judged by gel formation time. All of these formulations had gel times of 3–4 min as observed by simple visual inspection. In the case of Asp (**3c**), a mild retardation of gel formation was observed (gel time >6 min) compared to macromonomers **2**, **3a**, **3b**, and **3e**. An interesting effect was observed for insertion of the aromatic residue Trp between *N*-terminal cysteine and polymer (**3d**). Macromonomer **3d** formed a hydrogel immediately upon vortexing with water or buffer. This effect did not appear to result from simple swelling of the polymer by water but rather involved dissolution followed by immediate gelation. Dissolution of **3d** in TCEP containing buffer followed by mixing with macromonomer **1** resulted in gelation in just under 10 min; likewise, dissolution of **3d** in 2-mercaptoethanol containing buffer followed by mixing with macromonomer **1** resulted in gelation in just under 10 min. From these results we can conclude that the Trp residue has a retarding effect on the *N*-terminal Cys residue toward the NCL reaction but an accelerating effect of the *N*-terminal Cys macromonomer toward intermolecular cross-linking via disulfide bond formation by *N*-terminal Cys residues. More detailed studies will be necessary to fully explain the rapid solidification of macromonomer **3d**.

Mechanical Characterization of NCL Hydrogels

To characterize the viscoelastic behavior of the hydrogels formed by NCL, selected hydrogel systems were analyzed further by oscillatory rheology. The time-dependent changes in storage modulus (G') and loss modulus (G'') for NCL hydrogel compositions tested were characteristic of elastic hydrogel formation (Figure 2), as indicated by a low initial G', a G'/G'' crossover point representing a theoretical gel condition,[34] followed by rapid increase in G' to a plateau value within 2 h as the NCL reaction becomes complete. For the 10% mixture of **1** and **2**, the characteristic crossover point was reached after 3 min; however, for the 20% mixture of **1** and **3b** gel formation was so rapid that the crossover point was missed when the rheological measurements started ($G' > G''$; Figure S2), as was the case even when the polymer concentration was decreased to 10% (Figure S4). Given the delay necessary for mixing and transferring solutions and initiating data collection, we estimate that in these cases the mixtures formed gels in approximately two minutes or less. The stiffness of the fully cured NCL hydrogels was high, as illustrated by a plateau modulus value of nearly 40 kPa for the 20% mixture of **1** and **3b** shown in Figure 3. Frequency and strain sweep experiments conducted after G' reached a plateau indicated the storage modulus was frequency and strain independent as expected for a covalently cross-linked hydrogel. Additional rheological characterization of hydrogels derived from mixtures of macromonomers **1**, **2**, and **3a**–**e** are provided in the Supporting Information, Figures S1–S8.

Cell Adhesion and Growth on NCL Hydrogels

Incorporation of bioactive compounds into PEG hydrogels through thioreactive functional groups was shown to be an effective approach for tissue engineering.[35] A useful feature of the NCL hydrogel is the presence of thiol functional groups in the final hydrogel as a result of the NCL reaction. Endogenous thiol groups found in NCL hydrogels can be easily derivatized to confer specific functional properties on the hydrogels. Thiol groups can be utilized for attaching bioactive molecules through various methods, for example, by Michael-type addition reaction with maleimide-containing molecules or through disulfide formation with other thiol-containing molecules. This property distinguishes the NCL gelation chemistry from previously reported methods of hydrogel formation wherein endogenous sites for functionalization are often not available after hydrogel formation.[15]

As an illustration of NCL hydrogel biofunctionalization, we derivatized an NCL hydrogel with a cell adhesive peptide and studied the attachment of human mesenchymal stem cells (hMSCs) on the derivatized hydrogel. First, a NCL hydrogel was formed as described above through the reaction of a 10% solution of macromonomers **1** and **2** in 100 mM NH_4HCO_3. The resulting NCL hydrogel was functionalized by reacting polymer-bound thiols with maleimide-GRGDSPG-NH_2 to facilitate attachment of hMSCs via interaction between polymer bound peptide and integrin receptors. The RGD peptide functionalized NCL hydrogel promoted hMSC adhesion on the gel surface during a 24 h cell culture experiment, whereas the underivatized NCL hydrogel did not support adhesion of hMSCs under the same conditions (Figure 4).

Conclusion

In summary, an approach to formation of polymer hydrogels by NCL reaction was reported. In this approach, a thioester–polymer bioconjugate reacts with an *N*-terminal cysteine-polymer to rapidly form hydrogels with high storage modulus. The methods developed for the synthesis and purification of the bioconjugates are simple and amenable to scale-up, and the NCL reaction occurs rapidly in the absence of exogenous thiols, catalysts, and other accelerating agents. Control experiments confirmed that the hydrogels formed through NCL cross-linking rather than disulfide bond formation or thioester exchange reactions. The presence of another

amino acid adjacent to the *N*-terminal cysteine resulted in mild retarding or accelerating effects on the kinetics of hydrogel formation by NCL, which depended on the composition of the amino acid flanking the *N*-terminal cysteine. As for the thioester macromonomer composition, further improvements in the NCL hydrogel system could be achieved in the future through optimization of the thioester–polymer leaving group.[31,36] Leaving groups other than EMP may be used, however, the nature of the leaving group may have an impact on gelation kinetics and other properties of the NCL hydrogels. Finally, biofunctionalization of NCL hydrogels is straightforward through biomolecule conjugation to endogenous cysteine thiol functional groups on the polymer network, as demonstrated by adhesion of hMSCs on an RGD peptide functionalized NCL hydrogel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to acknowledge funding from NIH Grants DE 013030 and EB 003806.

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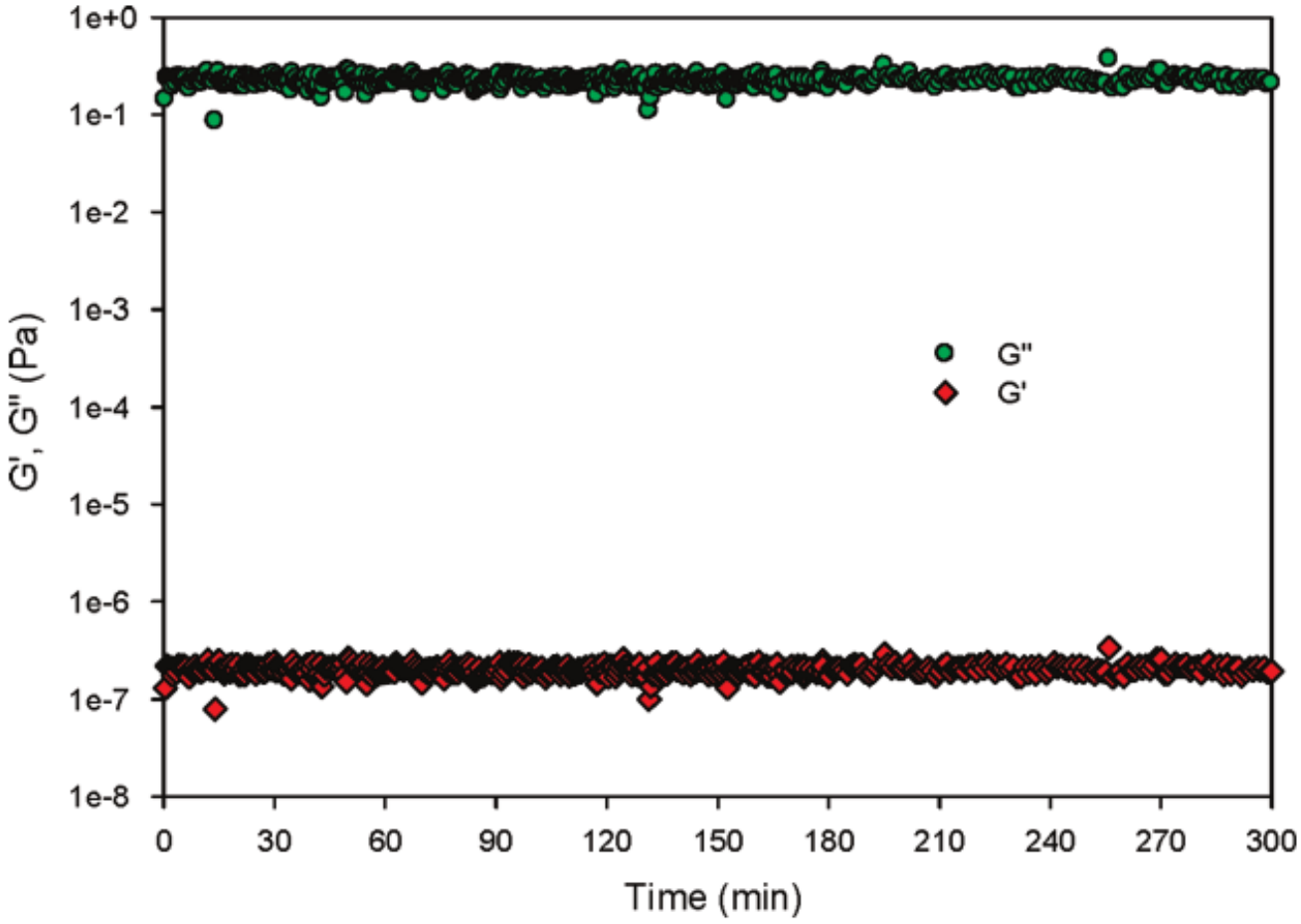


Figure 1.
Oscillatory rheology of 10% macromonomer **2** in 100 mM NH_4HCO_3, pH 8.3. Storage (*G′*) and loss (*G″*) modulus vs time are shown (rheology method 2).

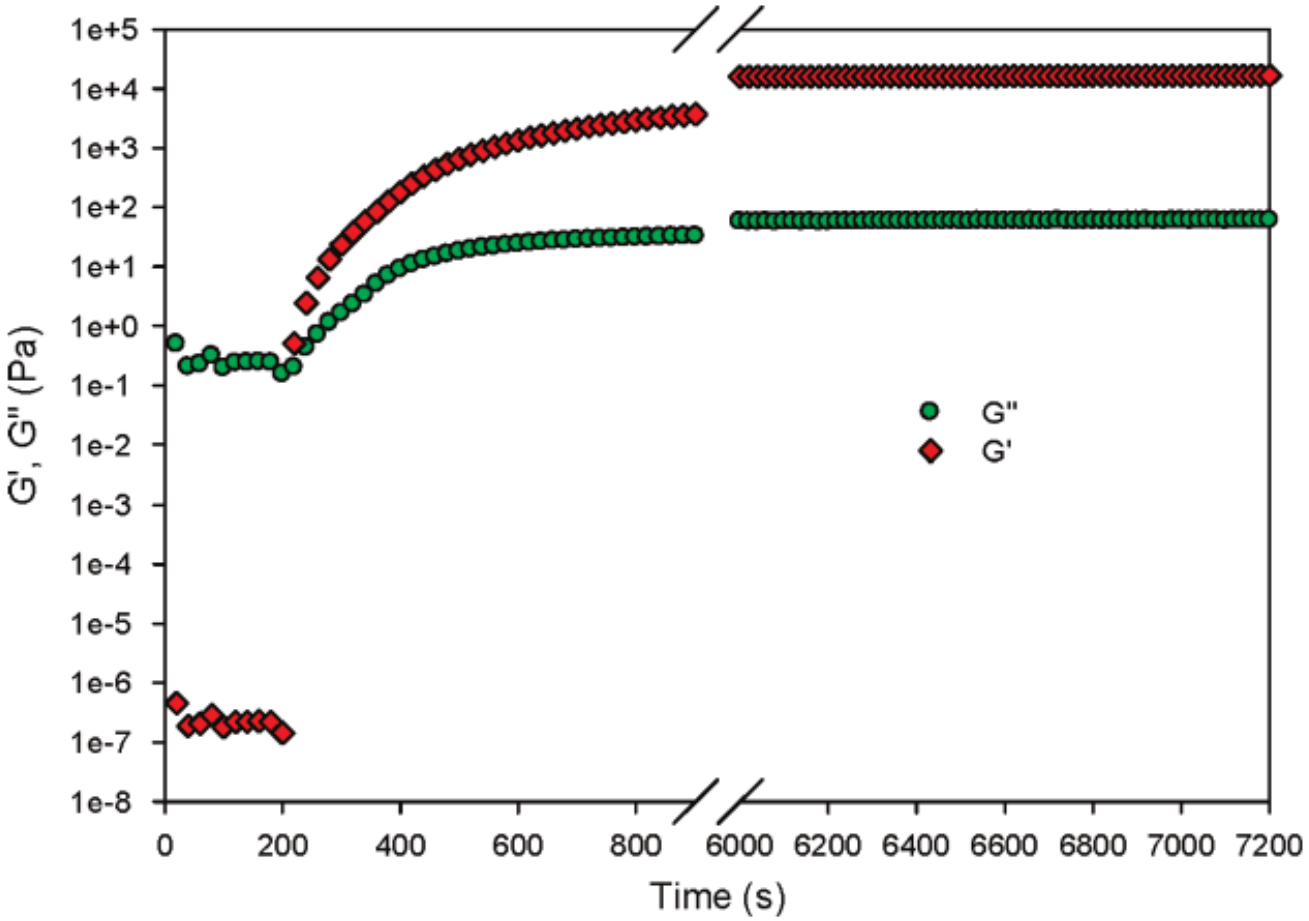


Figure 2.
Oscillatory rheology of a mixture of 10% macromonomer **1** and 10% macromonomer **2** in 100 mM NH_4HCO_3, pH 8.3. Storage (G') and loss (G'') modulus vs time are shown (rheology method 2).

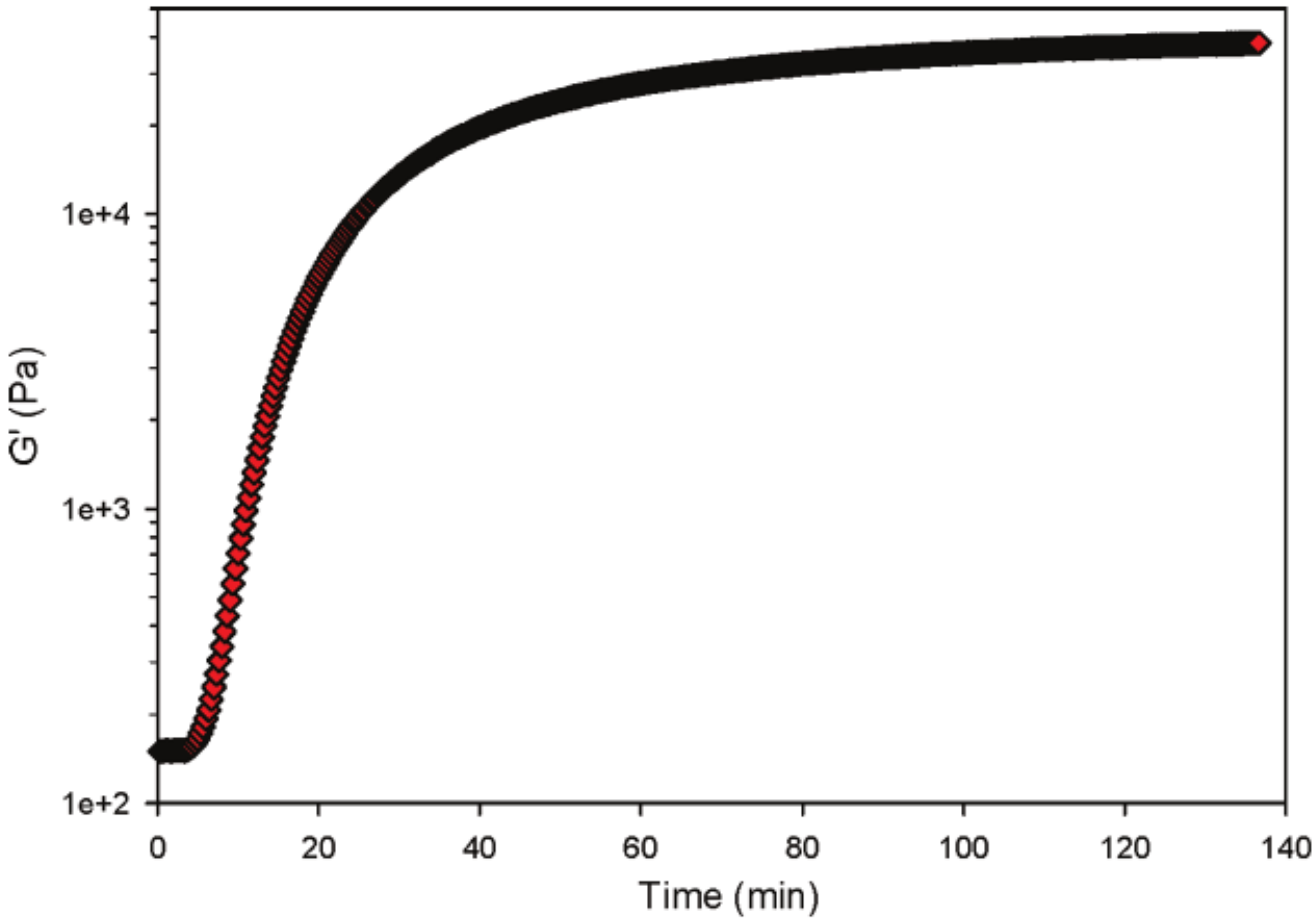


Figure 3.
Oscillatory rheology of a mixture of 20% macromonomer **1** and 20% macromonomer **3b** in 100 mM NH_4HCO_3, pH 8.3. Storage (*G′*) modulus vs time is shown (rheology method 1).

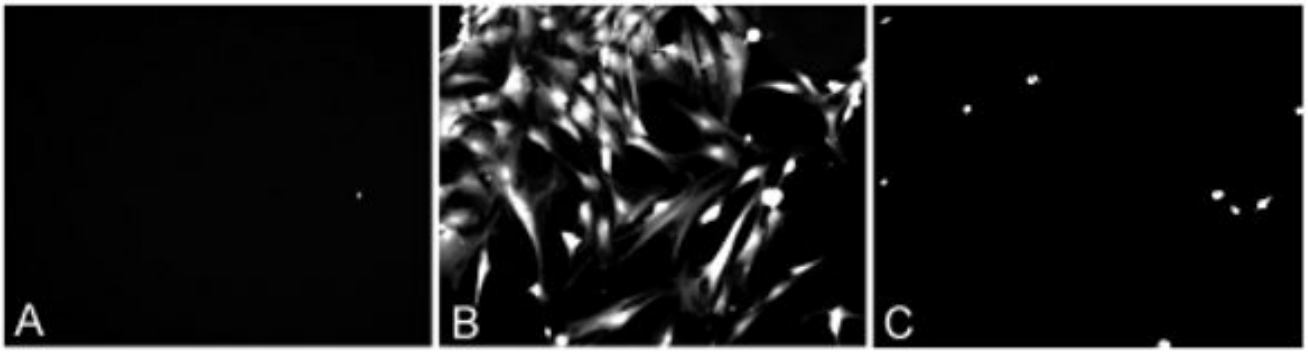
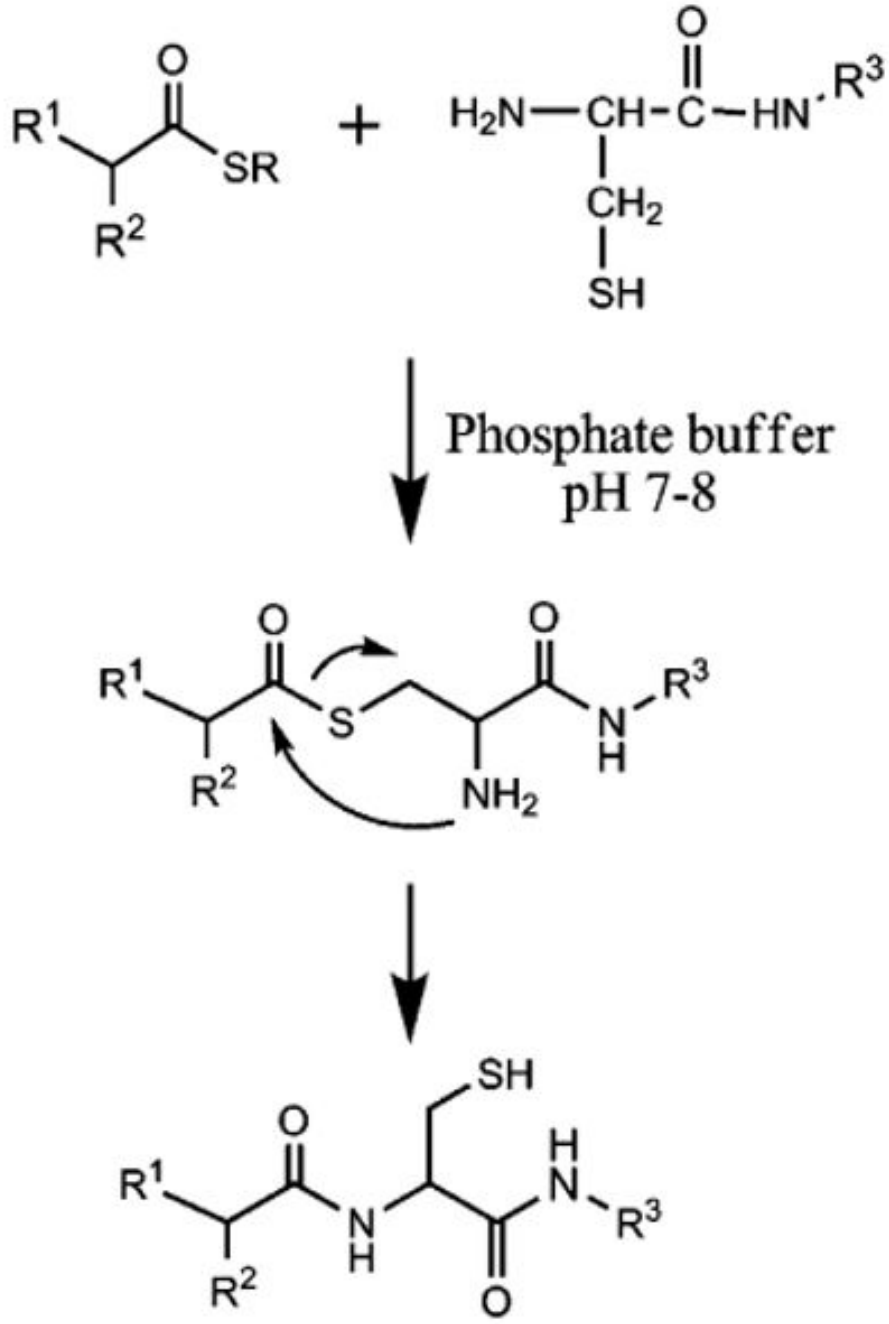
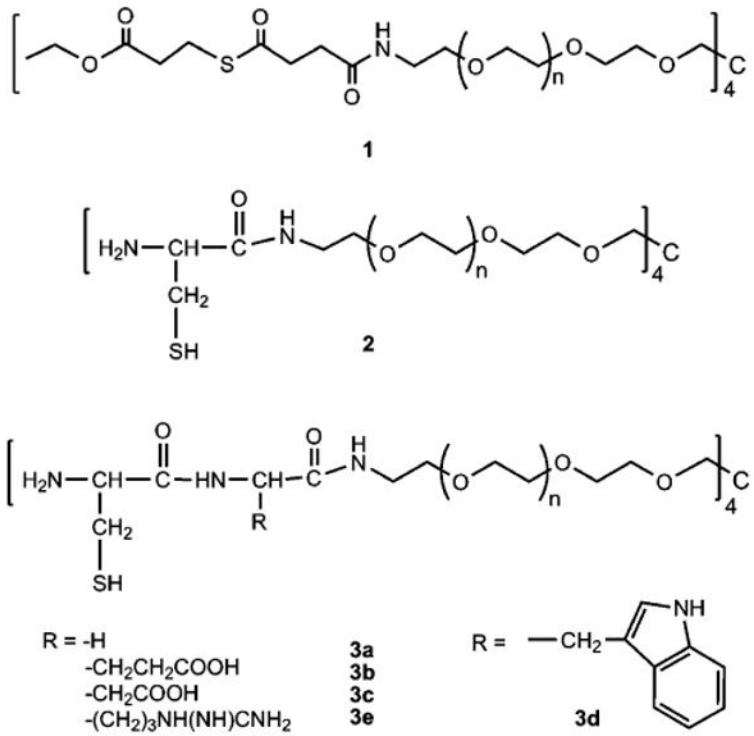


Figure 4.

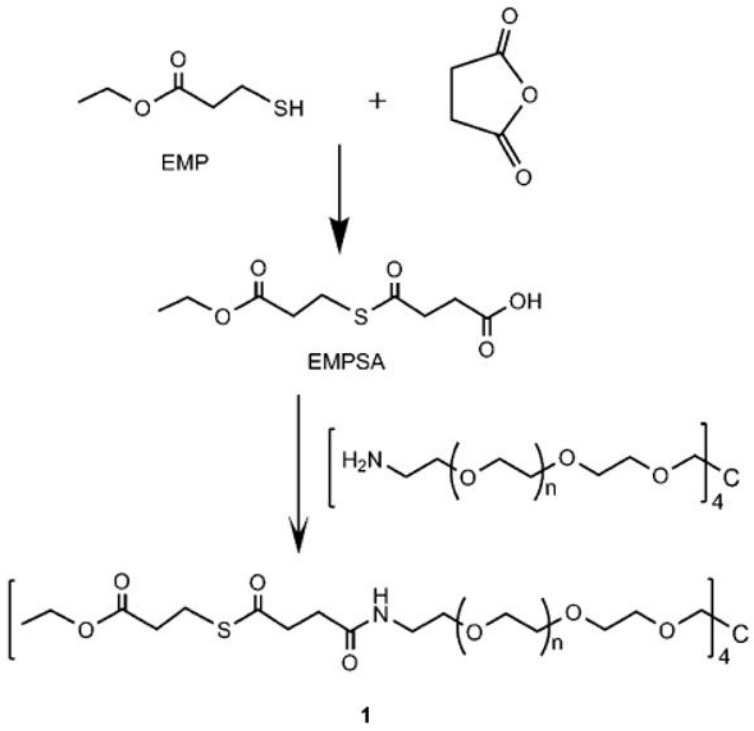
Human mesenchymal stem cell culture on NCL hydrogel of **1** and **2** (10%) before (A) and after functionalization with maleimide-GRGDSPG-NH_2 peptide (B). Image analysis revealed cell adhesion on nonmodified NCL hydrogel to be <0.1% of available surface area (A), whereas cell adhesion increased to 68 ± 7.2% of available surface area on hydrogel functionalized with maleimide-GRGDSPG-NH_2 peptide (B). Only minimal cell adhesion was observed on hydrogel surface that was treated with nonthiol reactive Ac-GRGDSPG-NH_2 peptide (C).



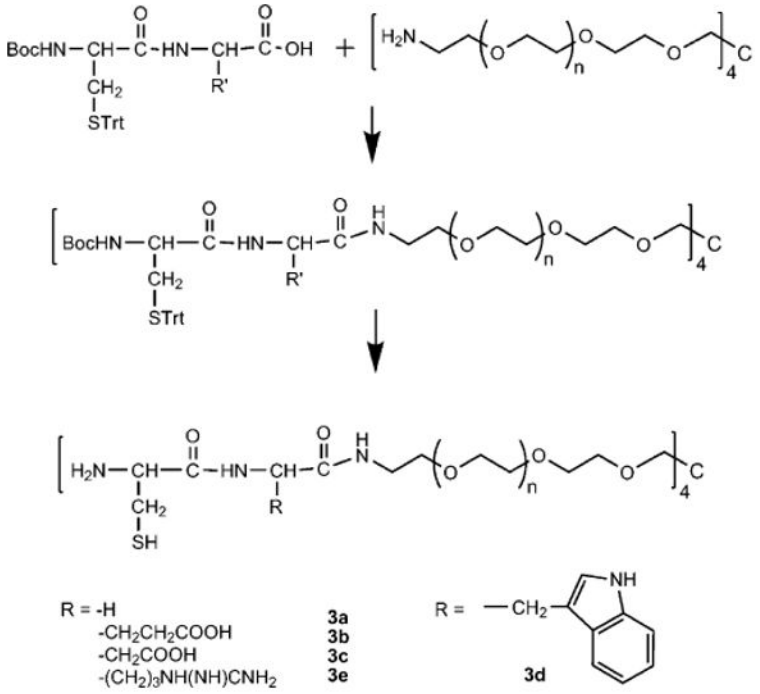
Scheme 1.
Chemistry of Native Chemical Ligation



Scheme 2.
Structures of Thioester-Polymer **1** and *N*-Terminal Cysteine-Polymer Bioconjugates **2** and **3a**–**e**



Scheme 3.
Synthesis of Macromonomer **1**



Scheme 4.
Synthesis of *N*-Terminal Cysteine Macromonomers **3a**–**e**