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# Thermoresponsive Injectable Hydrogels Cross-Linked by Native Chemical Ligation

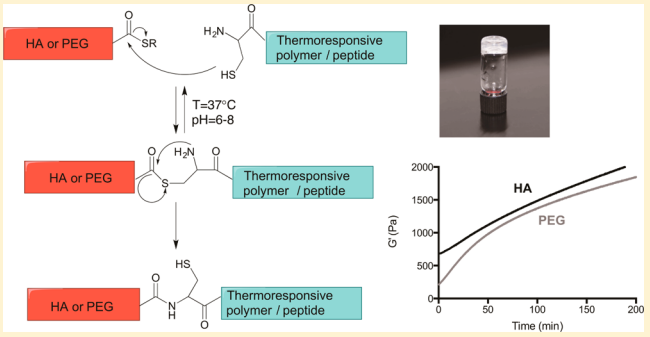
Kristel W. M. Boere,[†] Bram G. Soliman,[†] Dirk T. S. Rijkers,[‡] Wim E. Hennink,[†] and Tina Vermonden*[,†]

[†]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Faculty of Science, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands

[‡]Medicinal Chemistry & Chemical Biology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Faculty of Science, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands

Supporting Information

**ABSTRACT:** Temperature-induced physical gelation was combined with native chemical ligation (NCL) as a chemical cross-linking mechanism to yield rapid network formation and mechanically strong hydrogels. To this end, a novel monomer *N*-(2-hydroxypropyl)methacrylamide-cysteine (HPMA-Cys) was synthesized that copolymerizes with *N*-isopropylacrylamide (NIPAAm) to yield thermoresponsive polymers decorated with cysteine functionalities. Triblock copolymers consisting of a poly(ethylene glycol) (PEG) middle block flanked by random blocks of NIPAAm and HPMA-Cys were successfully synthesized and characterized. Additionally, thioester cross-linkers were synthesized based on PEG and hyaluronic acid, respectively. Upon mixing the thermoresponsive polymer with PEG or hyaluronic acid cross-linker, cysteine and thioester functionalities react under physiological conditions to generate a native peptide bond. An immediate physical network was formed after elevation of the temperature to 37 °C due to the self-assembly of the pNIPAAm chains. This network was stabilized in time by covalent cross-linking due to NCL reaction between thioester and cysteine functionalities, resulting in hydrogels with up to 10 times higher storage moduli than without chemical cross-links. Finally, a collagen mimicking peptide sequence was successfully ligated to this hydrogel using the same reaction mechanism, showing the potential of this hydrogel for tissue engineering applications.




## 1. INTRODUCTION

Hydrogels have been extensively studied for drug delivery and tissue engineering applications.[1−5] Their high water content allows the incorporation of cells and/or biomolecules and the exchange of nutrients and metabolites.[6] In particular, *in situ* cross-linkable systems that can be injected as liquid formulations into the body are attractive for minimal invasive treatments.[7−9] Moreover, *in situ* forming hydrogels are able to fill irregularly shaped defect sites, resulting in close contact with the surrounding tissue. Thermosensitive systems are an important class of injectable systems since they can form hydrogels upon temperature changes.[10,11] When thermosensitive polymers pass their lower critical solution temperature (LCST), their polymer chains collapse and aggregate. Although several thermosensitive systems have been investigated, poly(*N*-isopropylacrylamide) (pNIPAAm)-based materials are the most studied due to their sharp phase transition at 32 °C.[10] However, networks held together by physical interactions only form mechanically weak scaffolds, resulting in fast erosion or degradation before new tissue is formed.[9] Another drawback of solely physical networks is the often-encountered syneresis of the hydrogel, resulting in an incomplete filling of the defect.[12] For these reasons, chemically cross-linkable systems have been developed that allow the formation of covalent bonds *in situ*.[13] Such a dual hardening hydrogel has the advantage of forming an immediate physical network at 37 °C, followed by chemical cross-linking *in situ* to enhance the mechanical stability. The addition of thermogelation obviates the often-found problematic slow gelation kinetics of injectable systems due to slow chemical cross-linking. An attractive approach to combine physical and chemical gelation is a two-component system that can be mixed prior to injection. Such systems can be administered as liquid formulations that undergo gelation due to physical forces triggered by e.g. temperature and that are subsequently stabilized by chemical reactions between groups present of the hydrogel building blocks. It has been shown that the mechanical, degradation, and swelling characteristics of these two-component hydrogels can easily be tailored by changing the cross-linking density.[14] However, to develop a biocompatible *in situ* cross-linkable system without adverse side reactions remains a challenge. Frequently studied *in situ* cross-linking reactions, such as radical polymerization induced by UV

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irradiation,[15] Michael addition,[16,17] condensation reactions of aldehydes and amines,[18] or ring-opening of epoxides with amines,[19] all have the disadvantage of either forming free radicals or having nonselective reactivity that can cause undesired side reactions with nucleophiles present in biomolecules such as proteins, possibly resulting in toxicity and inflammation.[20] Besides, addition reactions are typically carried out in organic solvents, since water can also react with the cross-linking agents. On the other hand, chemoselective reactions[21] like click chemistry[22−24] generally require the use of toxic metal catalysts such as $Cu^{+}/Cu^{2+}$. Attempts have been made to eliminate the use of these toxic catalysts for biomedical applications.[25,26] Recently, Hu et al.[27] developed an injectable hydrogel using native chemical ligation, a fast and chemoselective cross-linking method. In native chemical ligation, a thioester and an N-terminal cysteine undergo a reversible transthioesterification, whereafter an N-to-S rearrangement occurs, resulting in the irreversible formation of a native peptide bond.[28] Native chemical ligation has been widely used for the synthesis of peptides and biologically active proteins,[29] and its reaction mechanism has been studied in detail.[30] However, only recently NCL has been applied for cross-linking hydrogels.[27] Apart from its chemoselective nature, another attractive feature of native chemical ligation is the possibility to covalently link other functionalities, e.g., bioactive peptides such as RGD to the polymer network. For tissue engineering, peptide-functionalized hydrogels have shown to promote their adhesion to body tissues as well as to enhance proliferation and differentiation of embedded cells.[31,32] Although native chemical ligation is normally applied using rather toxic catalytic thiols such as 4-mercaptophenylacetic acid (MPAA) or 2-mercaptoethanesulfonate (MESNa),[30] Hu et al. succeeded in preparing an injectable hydrogel without using such additives.[27] Furthermore, the group of Messersmith showed the compatibility of these hydrogels with extracellular matrix proteins and stem cells[33] and the use of oxo-ester-mediated native chemical ligation, resulting in faster gelation kinetics and thiol capture.[34] However, the application of NCL hydrogels as injectable materials is limited by its relatively slow cross-linking kinetics.

The aim of this study is to develop a dual hardening *in situ* cross-linkable hydrogel formed by temperature-induced physical cross-linking and chemoselective cross-linking by native chemical ligation, without the addition of a catalyst. Combining thermogelation with native chemical ligation allows the formation of an immediate physical network that can be strengthened in time by chemical cross-links. For this aim, a triblock copolymer of pNIPAAm-*co*-HPMACys-PEG-pNIPAAm-*co*-HPMACys (PNC) was synthesized containing a newly designed monomer HPMA-cysteine to introduce cysteine functionalities in a thermosensitive polymer. The methacrylamide group of this monomer allows copolymerization with other (meth)acryl-containing monomers using radical polymerization. As the second component, two different thioester cross-linkers were synthesized: a PEG difunctionalized thioester and a partially functionalized hyaluronic acid (HA) thioester. HA is a natural polysaccharide, present in many tissues in the human body and widely used in hydrogels for biomedical applications due to its favorable biological properties.[35,36] Finally, the PEG thioester was partially functionalized with a peptide using native chemical ligation. As a model peptide, a short discoidin domain receptor-2 (DDR-2) binding peptide (CGPRGQOGVMGFO) was chosen since it was suggested that this sequence positively affects mesenchymal stem cell differentiation, proliferation, and cell adhesion.[37−39] In this paper, we show the use of NCL as a chemoselective cross-linking reaction in combination with thermosensitive polymers as well as for the chemoselective ligation of peptides to hydrogels.

## 2. MATERIALS AND METHODS

**2.1. Materials.** All commercial chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and used as received unless indicated otherwise. *N*-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized by reaction of methacryloyl chloride with 1-aminopropan-2-ol in dichloromethane, essentially according to Ulbrich.[40] Peptide grade dichloromethane (DCM) was obtained from Biosolve (Valkenswaard, The Netherlands). *N*,*N*′-Dimethylaminopyridine (DMAP) was purchased from Fluka (Zwijndrecht, The Netherlands). Boc-*S*-acetamidomethyl-L-cysteine (Boc-Cys(Acm)-OH was purchased from Bachem (Bubendorf, Switzerland). PEG10,000-ABCPA macroinitiator was synthesized as described by Neradovic et al.[41] Ethylthioglycolate succinic acid (ET-SA) was prepared according to a literature procedure.[27] Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained from Carl Roth (Karlsruhe, Germany). Hyaluronic acid (HA) with a molecular weight of 31 kDa was supplied by Lifecore (Chaska, MN). 4-(Dimethylamino)pyridinium-4-toluenesulfonate (DPTS) was synthesized according to the method described by Moore et al.[42] 2,4,6-Trinitrobenzenesulfonic acid (TNBSA) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) were obtained from Thermo Fischer Scientific (Rockford, IL). DDR-2 peptide CGPRGQOGVMGFO (O = hydroxyproline) was supplied by GenScript (Piscataway, NJ). PBS buffer pH 7.4 (8.2 g/L NaCl, 3.1 g/L $Na_2HPO_4{\cdot}12H_2O$, 0.3 g/L $NaH_2PO_4{\cdot}2H_2O$) was purchased from B. Braun (Melsungen, Germany).

**2.2. Synthesis of Boc-Cys(Acm)-HPMA.** In a typical procedure, Boc-Cys(Acm)-OH (4.0 g, 13.7 mmol), HPMA (2.0 g, 13.7 mmol), and DMAP (167 mg, 1.37 mmol) were dissolved in dry DCM (10 mL). To this solution DCC (2.8 g, 13.7 mmol) was added, and the reaction mixture was stirred for 16 h under a nitrogen atmosphere at room temperature. Subsequently, the suspension was cooled to 0 °C and filtered, and the filtrate was concentrated to 5 mL. The product was purified by silica gel chromatography, using DCM/MeOH (9:1 v/v) as eluent. The monomer Boc-Cys(Acm)-HPMA (HPMA-Cys) was obtained as a viscous oil (72% yield). $^1$H NMR ($CDCl_3$): $\delta$ = 6.78 (s, 1H, N*H*), 6.61 (s, 1H, N*H*), 5.74 (s, 1H, $H_2$C=CH), 5.51 (s, 1H, $CH_2CH(CH_3)O$), 5.34 (s, 1H, $H_2$C=CH), 5.09 (s, 1H, N*H*), 4.39 (m, 3H, $SCH_2NH$, $SCH_2CH$), 3.65 (m, 1H, $CH_2CH(CH_3)$), 3.38 (m, 1H, $CH_2CH(CH_3)$), 2.96 (m, 2H, $CHCH_2S$), 2.02 (s, 3H, $COCH_3$), 1.96 (s, 3H, $C{=}C(CH_3)$), 1.44 (s, 9H, $(CH_3)_3CO$), 1.28 (d, 3H, $OCHCH_3$) $^{13}$C NMR ($CDCl_3$): $\delta$ = 170.8 (O−*C*(O)CH, 168.9 $CH_3C{=}O$, 155.8 O−*C*(O)−N, 139.7 $CH_2{=}CH$, 120.1 $CH_2{=}CH$, 80.5 $(CH_3)_3C$, 72.0 $CH_2CH(CH_3)O$, 54.2 $SCH_2CH$, 43.9 $CH_2CH(CH_3)O$, 41.6 $SCH_2NH$, 33.5 $SCH_2CH$, 28.3 $(CH_3)_3C$, 23.2 $CH_3C(O)NH$, 18.7 $CH_3C{=}C$, 17.5 $CH_2CH(CH_3)O$. $R_t$ HPLC: 10.2 min.

**2.3. Synthesis of PEG2000 Thioester.** In a typical reaction,[43] ET-SA (880 mg, 4 mmol), DCC (824 mg, 4 mmol), and DPTS (59 mg, 0.4 mmol) were dissolved in dry DCM (5 mL). To this solution PEG 2000 (2 g, 2 mmol of OH functionalities) was added, and the obtained reaction mixture was stirred for 16 h at room temperature under a nitrogen atmosphere. Next, solid DCU was removed by filtration, and the desired polymer was precipitated in cold diethyl ether. The precipitate was harvested by filtration, dissolved in 10 mL of $H_2O$, and dialyzed for 2 days ($M_w$ cutoff: 2000 Da) against water at 4 °C, and the solution was subsequently lyophilized. A white powder was obtained in 92% yield, which was characterized by $^1$H NMR. The degree of substitution (DS), defined as the percentage of hydroxyl groups derivatized by thioester moieties, was determined by $^1$H NMR from the integral ratio of the methyl protons of the thioester functionalities at 1.3 ppm ($I_{TE}$) and the PEG protons between 3.2 and 4 ppm ($I_{PEG}$) using the equation


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$$\mathrm{DS} = \frac{I_{\mathrm{TE}}/6}{I_{\mathrm{PEG}}/182} \times 100\%$$

The degree of substitution (DS) was confirmed by adding two drops of trichloroacetyl isocyanate (TAIC) to the $^1$H NMR sample. TAIC causes a shift to 4.42 ppm for the $CH_2$ protons of PEG adjacent to the OH end groups.[44] To calculate the DS, the peak integral at 4.42 ppm was divided by the PEG proton signals between 3.2 and 4.0 ppm.

A second approach to determine the DS was performed by hydrolyzing the PEG thioester in a 0.2 M NaOH solution for 2 h under stirring. Quantification of free SH groups was performed using the Ellman's assay.[45]

$^1$H NMR ($CDCl_3$): $\delta$ = 4.42 (t, 2H, shift TAIC), 4.14 (q, 2H, $CH_3CH_2O$), 3.62 (182H, PEG protons), 3.13 (t, 2H, $SC(O)CH_2CH_2$), 2.89 (t, 2H, $SC(O)CH_2CH_2$), 1.21 (t, 3H, $CH_3CH_2O$).

**2.4. Synthesis of HA Thioester.** Hyaluronic acid ($M_w$ 31 kDa) was partially functionalized with ET-SA to introduce thioester functionalities at the primary hydroxyl groups, using EDCI as the coupling agent. In a typical procedure, HA (500 mg, 1.27 mmol of primary OH groups), ET-SA (800 mg, 1.82 mmol), and EDCI (500 mg, 2.61 mmol) were dissolved in $H_2O$ (10 mL). The solution was stirred at room temperature for 16 h, while maintaining the pH at 5 with 1 N HCl. The HA thioester derivative was purified by dialysis ($M_w$ cutoff: 12−14 kDa) against water for 4 days at 4 °C and subsequently lyophilized. The degree of substitution (DS) was determined by hydrolyzing the HA thioester in a 0.2 M NaOH solution for 2 h under stirring and subsequent quantification of formed SH bonds using Ellman's reagent.

$^1$H NMR ($D_2O$): $\delta$ = 4.6−3.3 (protons of hyaluronic acid), 2.86 (s, $CHCH_2O$) 1.98 (s, $CH_3CONH$) 1.28 (t, $CH_3CH_2O$).

**2.5. Synthesis of PEG−NIP−HPMA-Cys Triblock Copolymers.** In a typical procedure, PEG-ABCPA 10 000 (0.23 g), Boc-Cys(Acm)-HPMA (0.31 g, 0.74 mmol), and NIPAAm (1.0 g, 8.85 mmol, feed ratio Boc-Cys(Acm)-HPMA:NIPAAm 7:93) were dissolved in dry acetonitrile (10 mL) in an airtight screw-cap glass vial. The reaction mixture was flushed with nitrogen for 15 min and subsequently stirred for 40 h at 70 °C. Next, the polymer was precipitated in cold diethyl ether (100 mL), and the precipitate was filtrated, washed with diethyl ether, and dried. The synthesized polymer was obtained in a yield of 77%.

To remove the Boc groups of cysteine, the obtained triblock copolymer was dissolved in TFA/DCM (1:1 v/v, 10 mL) under a nitrogen atmosphere and stirred for 2 h. The solvents were evaporated under reduced pressure, and the residual solid was dissolved in water (10 mL). The triblock copolymer was purified by dialysis for 2 days at 4 °C against water ($M_w$ cutoff: 12−14 kDa) and lyophilized. To remove the acetamidomethyl (Acm) functionality, 1.0 g of polymer was dissolved in MeOH/$H_2O$ (1:1 v/v, 40 mL) under a $N_2$ atmosphere and 1 N HCl (540 $\mu$L) was added, followed by the immediate addition of 0.2 M iodine in MeOH/$H_2O$ (1:1 v/v/, 8 mL). The obtained brownish mixture was stirred for 1 h at room temperature. The excess iodine was quenched by addition of 4 drops of 1 M ascorbic acid. The colorless solution was treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 500 mg) to reduce possibly formed disulfide bonds between cysteine moieties for 16 h and subsequently dialyzed against water for 2 days ($M_w$ cutoff: 12−14 kDa). The final deprotected polymer was obtained after lyophilization as a white powder and characterized by GPC and $^1$H NMR. The cysteine content was quantified by detection of free thiols by Ellman's method,[45] and free primary amines were detected by TNBSA assay.[46]

**2.6. Ligation of Peptide to PEG Thioester.** The PEG thioester construct (20 mg, 0.014 mmol of thioester functionalities) was dissolved in PBS (400 $\mu$L). A 10-fold molar excess of sodium 2-sulfonylethanesulfonate (MESNa, 16 mg, 0.1 mmol) was added as catalyst. In a separate vial, the peptide CGPRGQOGVMGFO (14 or 7 mg for a cysteine: thioester ratio of 1:1 or 0.5:1 mol/mol, respectively) was dissolved in PBS (100 $\mu$L), in the presence of TCEP (4 mg) to keep SH groups in their reduced form. These molar ratios were chosen to obtain a high degree of substitution (DS) and thereby enable characterization of the reaction products. Both solutions were mixed and allowed to react for 20 h at room temperature. After reaction, the mixture was dialyzed ($M_w$ cutoff: 2000 Da) for 2 days, and the obtained solution was subsequently lyophilized. The ligation product was analyzed by $^1$H NMR and GPC. The degree of substitution was calculated by NMR from the integrals of the characteristic peptide peaks at $\delta$ = 0.8 and 7.2 ppm of valine and phenylalanine, respectively. DS was confirmed by making use of the reduced intensity of the signal at $\delta$ = 1.2 ppm of the cleaved thioester after NCL reaction.

**2.7. NMR Spectroscopy.** The obtained monomers and polymers were characterized on a Varian Mercury Plus 300 spectrometer. For $^1$H NMR spectra, chemical shifts were referred to the residual solvent peak ($\delta$ = 7.26 ppm for $CDCl_3$, 4.79 ppm for $D_2O$, and 2.50 ppm for DMSO-$d_6$). For $^{13}$C NMR spectra, the central line in the chloroform triplet at $\delta$ = 77.16 ppm was used as the reference line.

**2.8. Gel Permeation Chromatography (GPC).** The molecular weights of the polymers were determined by GPC using a Plgel 5 $\mu$m MIXED-D column (Polymer Laboratories) with a column temperature of 65 °C. DMF containing 10 mM LiCl was used as eluent with an elution rate of 1 mL/min, and the sample concentration was 5 mg/mL. Poly(ethylene glycols) of narrow and defined molecular weights were used as calibration standards. For the samples containing peptide, 10 mM TCEP was added to the GPC samples to keep SH groups in their reduced form.

**2.9. Determination of Cloud Point.** The cloud point (CP) of the polymers was measured with a Shimadzu UV/vis-2450 spectrophotometer. The polymers were dissolved at low and high concentrations of 0.3 and 16 wt % in PBS (pH 7.4), respectively (Supporting Information Figure 6). The heating rate was approximately 1 °C/min, and the absorbance was measured from 4 to 45 °C at 650 nm. The CP was defined as the onset of increasing scattering intensity.[41]

**2.10. HPLC.** Purity of the synthesized Boc-Cys(Acm)-HPMA monomer was analyzed by HPLC using a C18 sunfire column. The monomer was dissolved in $H_2O$/MeCN (9:1 v/v) at a concentration of 1 mg/mL. A mobile phase gradient, from 100% of eluent A ($H_2O$/MeCN 9:1 v/v) to 100% of B (MeCN), with a 20 min run time was used. The injection volume of the samples was 5 $\mu$L, the flow rate was 1 mL/min, and absorbance was measured at $\lambda$ = 210 nm.

**2.11. Hydrogel Formation.** Thermosensitive triblock copolymer PEG−NIP−HPMA-Cys (PNC), either protected or deprotected, was dissolved at a concentration of 20 wt % in PBS at 4 °C for at least 5 h prior to hydrogel formation. PEG thioesters and HA thioesters were dissolved for 1 h at room temperature at a concentration of 15 wt % in the same solvent. For hydrogel formation, the appropriate thioester solution was added to the PNC solution, shortly mixed with a pipet tip, and incubated at 37 °C. NCL hydrogels were prepared at a final concentration of 16 wt % PNC and 11 wt % HA thioester or 4 wt % PEG thioester (corresponding to a 1:1 ratio of thioester:cysteine). For vial tilting experiments, hydrogel formation was reported after immobility for at least 15 min with vials upside down.

**2.12. Rheological Characterization.** Rheological analysis of hydrogels was performed on an AR-G2 rheometer (TA Instruments), using a 20 mm steel cone (1°) geometry equipped with a solvent trap. For each measurement 70 $\mu$L of sample was used. Temperature sweeps of triblock copolymer solutions/gels were performed from 4 to 50 °C at a heating rate of 1 °C/min. Cross-linking of the hydrogels in time was measured *in situ* for 3.5 h at 37 °C. A 1% strain (within the linear viscosity region as is shown in Supporting Information Figure 5) and a 1 Hz frequency were applied.

**2.13. Dynamic Mechanical Analysis (DMA).** Elastic modulus measurements were performed on a DMA 2980 dynamic mechanical analyzer (TA Instruments). Hydrogels were formed from 16 wt % PNC and 4 wt % PEG thioester with or without peptide functionalization. The polymers were incubated at 37 °C for 16 h before measuring. Hydrogels of approximately 3 × 4 mm (height × diameter) were placed between the parallel plates, and a force ramp was applied at a rate of 0.1 N/min from 0.01 to 0.1 N at 37 °C. A Mann−Whitney U test was used for comparing Young's moduli between two groups. Raw data were processed in Prism GraphPad

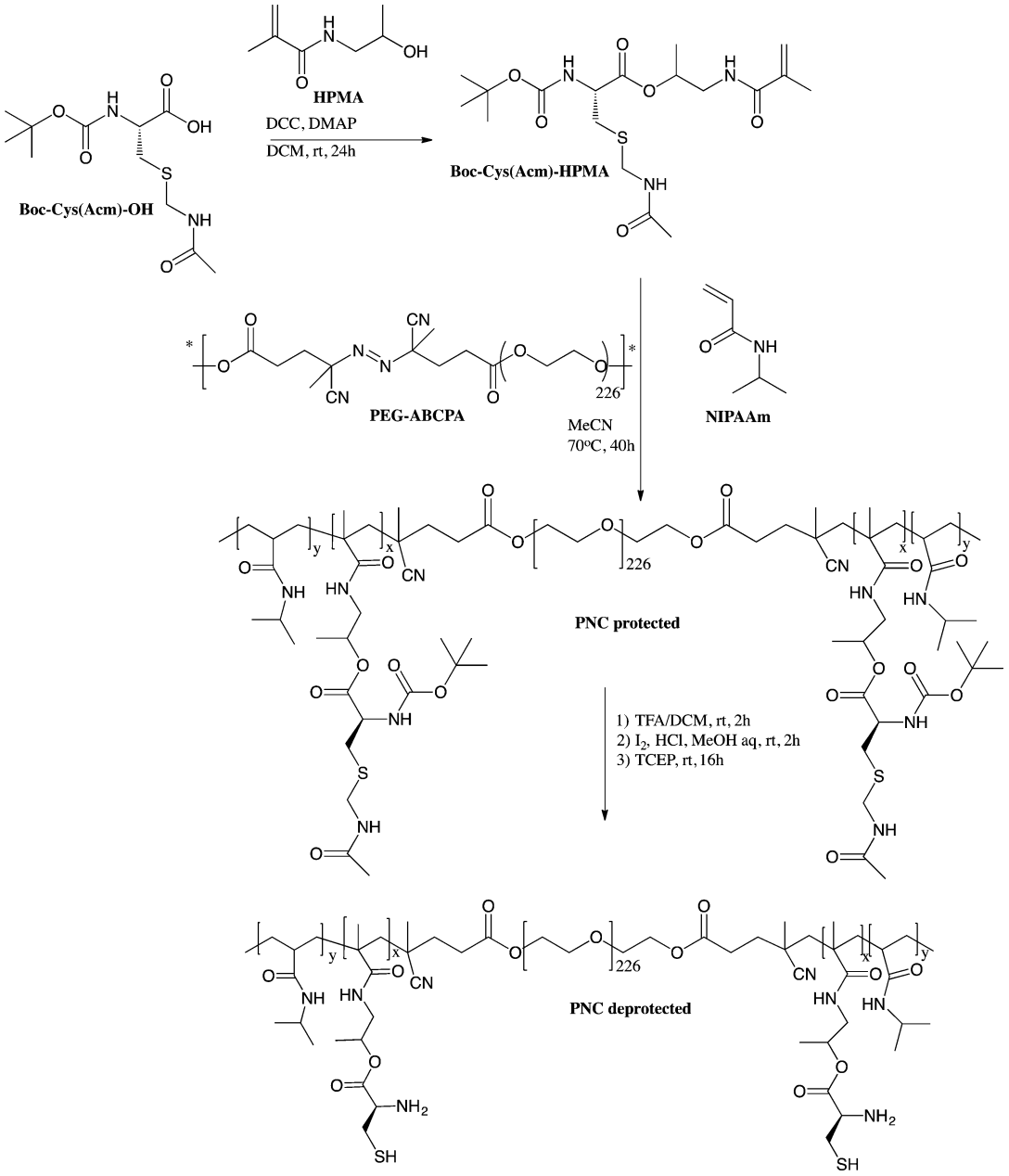


**Figure 1.** Synthesis route of ABA triblock copolymers consisting of a $PEG_{10000}$ B-block and random A-blocks of NIPAAm and HPMA-Cys.

Version 6.0 and $p < 0.05$ was considered significant. Data are represented as means ± standard deviations.

**2.14. TNBSA Assay.** Free primary amine groups of the synthesized triblock copolymer were quantified by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBSA).[46] Glycine standards were prepared at concentrations ranging from 0 to 0.15 mM in a 0.1 M sodium bicarbonate buffer (pH 8.5). The triblock copolymer was dissolved at a concentration of 0.5 mg/mL in the same buffer. Then, 0.25 mL of 0.01% (w/v) solution of TNBSA in buffer was added to 0.5 mL of polymer solution, and the samples were incubated at 37 °C for 2 h. Subsequently, 0.25 mL of 10% aqueous SDS and 0.125 mL of 1 N HCl were added to the solution, and the absorbance was measured in triplicate at $\lambda$ = 335 nm using a BMG Spectrostar nano well plate reader.

**2.15. Ellman's Assay.** Ellman's reaction was performed to detect free SH groups.[45] Cysteine hydrochloride monohydrate standards were prepared at concentrations ranging from 0 to 1.5 mM in a 0.1 M sodium phosphate buffer (pH 8.0) supplemented with 1 mM EDTA. The synthesized polymer was dissolved in a concentration of 2.5 mg/mL in the same buffer. The absorbance of the samples containing 50 $\mu$L Ellman's reagent (4 mg/mL), 2.5 mL buffer and 250 $\mu$L of each sample was measured in triplicate at $\lambda$= 412 nm using a BMG Spectrostar nano well plate reader.

## 3. RESULTS AND DISCUSSION

**3.1. Boc-Cys(Acm)-HPMA Synthesis.** The monomer Boc-Cys(Acm)-HPMA (Figure 1) was synthesized via a DCC-mediated esterification between the hydroxyl functional group of HPMA and the carboxyl functional group of Boc-Cys(Acm)-OH, using DCC as coupling agent and DMAP as catalyst. Boc and Acm protecting groups were carefully chosen to design a monomer that could be copolymerized and subsequently deprotected without affecting the final polymer structure. The monomer was purified by column chromatography over silica gel using DCM/MeOH (9:1 v/v) as eluent and obtained in a yield of 72%. HPLC analysis based on UV detection confirmed


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a high purity of >96% ($R_t$ = 10.2 min, Supporting Information Figure 1). The structure of Boc-Cys(Acm)-HPMA was confirmed by $^1$H NMR and $^{13}$C NMR (Supporting Information Figure 2).

**3.2. Synthesis of Triblock Copolymer PEG−NIP−HPMA-Cys (PNC).** A thermosensitive ABA triblock copolymer containing HPMA-Cys was synthesized via free radical polymerization using a PEG10,000-ABCPA macroinitiator and NIPAAm and Boc-Cys(Acm)-HPMA as monomers in a feed molar ratio of 93:7 (Figure 1). A triblock copolymer with NIPAAm and Boc-Cys(Acm)-HPMA as the outer blocks and PEG as the midblock (abbreviated as PNC) was obtained in a yield of 77% with a $M_n$ based on NMR of 64 kDa and a PDI of 2.5.

This triblock copolymer was first treated with TFA/DCM (1:1 v/v) to remove the Boc protecting groups from the $NH_2$ groups of cysteine. Second, the acetamidomethyl (Acm) groups were removed from the SH groups of cysteine by iodine, followed by exposure to TCEP to reduce possibly formed disulfide bonds. Removal of Boc and Acm groups of cysteines resulted in an increase in cloud point of the triblock copolymer from 18 to 33 °C, most likely due to its increased hydrophilicity. The removal of the Boc protecting group was confirmed by $^1$H NMR by the disappearance of the signal at $\delta$ = 1.4 ppm. Furthermore, the free $NH_2$ and SH groups of the cysteine units of the synthesized block copolymer were quantified by the TNBSA and Ellman's assay, respectively, and found to be lower than the feed (6.2% $NH_2$ and 5.4% SH compared to a feed of 7.0%). The difference between $NH_2$ and SH content was within the experimental error of these measurements.[47] As determined by GPC, deprotection resulted in a slight decrease in molecular weight, indicating that disulfide bond formation was negligible. Nonetheless, the molecular weights measured by GPC were higher than those determined by $^1$H NMR, which can be ascribed to the previously described phenomenon that pNIPAAm-based polymers have the tendency to aggregate in DMF.[48] Therefore, $M_n$ of these triblock copolymers was based on NMR, in line with the literature.[49] The polymer characteristics are summarized in Table 1.

**Table 1. Polymer Characteristics of ABA Triblock Copolymers Synthesized by Radical Polymerization Having a PEG10000 B Block and Random A Blocks of NIPAAm and HPMA-Cys (Triblock Abbreviated as PNC)**

| | $M_n$[a] (kDa) | $M_n$[b] (kDa) | $M_w/M_n$[b] | % $NH_2$[c] | % SH[d] | cloud point[e] (°C) |
|---|---|---|---|---|---|---|
| PNC[f] (protected) | 64 | 135 | 2.5 | | | 18 |
| PNC[f] (deprotected) | 58 | 113 | 2.6 | 6.2 | 5.4 | 33 |

[a]Determined by $^1$H NMR. [b]Determined by GPC. [c]Determined by TNBSA assay, expressed as mol % of A block. [d]Determined by Ellman's assay, expressed as mol % of A block. [e]Determined by light scattering at 650 nm. [f]PNC: PEG-NIP-HPMA-Cys triblock, structures shown in Figure 1.

**3.3. Synthesis of PEG and HA Thioester.** Two cross-linkers were synthesized by introduction of thioester functionalities at the hydroxyl groups of poly(ethylene glycol) (PEG) and hyaluronic acid (HA) using ethylthioglycolate succinic acid (ET-SA, Supporting Information Figure 3) and DCC or EDCI as coupling agent, respectively (Figure 2). A high degree of substitution (DS) is necessary to use PEG as a cross-linker, since only difunctionalized PEG can contribute to network formation. When *N,N′*-dimethylaminopyridine (DMAP) was used as the catalyst, a maximum DS of 50% was obtained. Therefore, 4-(dimethylamino)pyridinium-4-toluenesulfonate (DPTS) was chosen as catalyst, which has shown to enhance esterification reactions by suppression of the commonly observed side reaction leading to nonreactive *N*-acylurea derivatives.[42] Indeed, using this catalyst, the DS of PEG as determined by $^1$H NMR (shown in Supporting Information Figure 4A) was 85%, which was further confirmed by hydrolyzing the thioester and subsequent analysis of free thiol content by Ellman's assay (DS = 88%).

Hyaluronic acid was partially functionalized with thioester groups as alternative for the PEG cross-linker. A similar functionalization procedure was followed as for the synthesis of PEG thioester; however, the reaction was performed in aqueous solution because of the limited solubility of hyaluronic acid in organic solvents, and consequently a water-soluble cardodiimide derivative (EDCI) was selected as the coupling agent. Because of overlap of relevant peaks, the DS of thioester-functionalized HA could not be determined by NMR analysis (spectrum shown in Supporting Information Figure 4B). Therefore, DS, defined as percentage of disaccharide units of HA derivatized with thioester functionalities, was measured by thioester hydrolysis and subsequent thiol quantification and was found to be 11%.

**3.4. Hydrogel Characterization.** The gelation characteristics of the triblock copolymer PEG−NIP−HPMA-Cys (PNC) were studied by rheology. A temperature sweep experiment showed an increase in storage modulus ($G'$) and loss modulus ($G''$) with increasing temperature reflecting the thermosensitive behavior of these polymers in aqueous solution (Figure 3). The deprotected triblock formed a hydrogel at higher temperatures than the protected triblock, which was expected from the higher hydrophilicity of the unprotected cysteine moieties and was in line with the cloud point measurements. A slight decrease of $G'$ was shown for the protected triblock above 30 °C, most likely caused by an increased mobility of the polymers at high temperatures.[50] Hydrogels of PEG−NIP−HPMA-Cys with either PEG thioester or HA thioester were formed by dissolving the individual compounds in PBS and mixing them at room temperature in a concentration of 16 wt % PNC and 4 wt % PEG or 11 wt % HA thioester, achieving a 1:1 functional group molar ratio. Possible gel formation was visually assessed immediately after mixing and after 30 min and 3 h (Figure 4). When the solutions were mixed at room temperature, a homogeneous liquid solution was obtained. Incubation of this polymer solution at 37 °C for 30 min resulted in the formation of a gel. However, cooling the mixture to room temperature resulted again in a liquid solution, which means that at that time point the system was a reversible (physical) gel. Importantly, after 3 h of incubation at 37 °C followed by cooling down to room temperature, the hydrogel remained intact, indicating that cross-linking by native chemical ligation indeed had occurred. In principle, hydrogel formation could also be the result of disulfide formation of cysteines in the PNC macromers. However, a 16 wt % PNC solution without thioester cross-linker was still liquid at room temperature after 3 h incubation at 37 °C, indicating that the contribution of disulfide bridges to the network formation was negligible. These results demonstrate that an irreversible chemically cross-linked hydrogel after 3 h incubation was only obtained after


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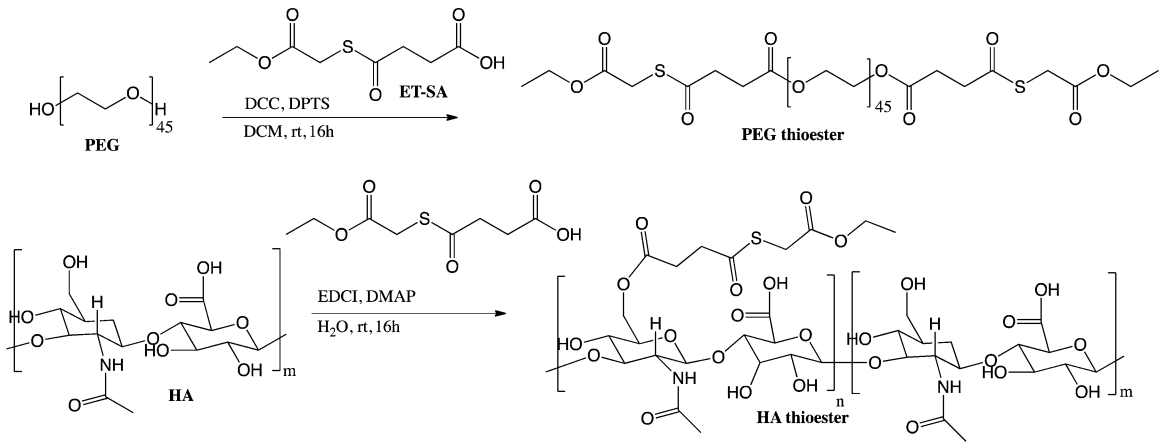


**Figure 2.** Synthesis route of PEG thioester and HA thioester.

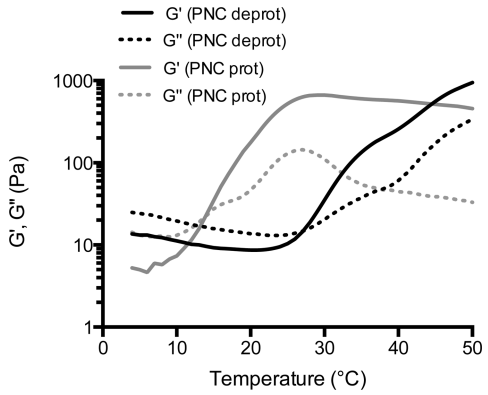


**Figure 3.** Storage ($G'$) and loss ($G''$) moduli as a function of temperature for PNC protected (PNC prot) and PNC deprotected (PNC deprot) at a concentration of 16 wt % in PBS.

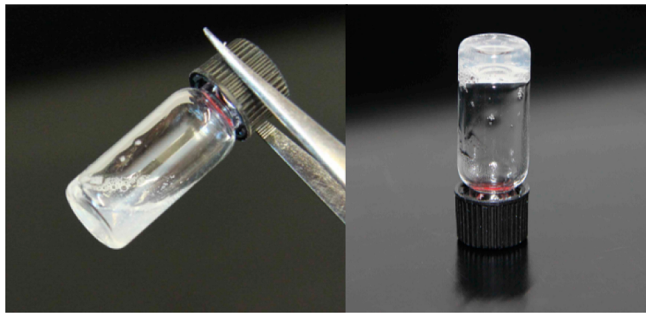

**Figure 4.** Hydrogel formation of 16 wt % PNC and 4 wt % PEG thioester in PBS at 37 °C. Left: photo taken at $t$ = 0; right: photo taken at $t$ = 3 h. Pictures were taken at room temperature.

addition of a thioester cross-linker. The gelation kinetics were followed by rheology measurements for 3.5 h (Figure 5A). After mixing the two components, for both the PEG and HA cross-linkers an immediate gelation at 37 °C was shown by $G'$ values between 0.2 and 0.7 kPa and tan delta of 0.4. This rapid gel formation is the result of physical interactions between the pNIPAAm chains. In time, cross-linking proceeded through native chemical ligation, resulting in up to 10 times higher $G'$ values than for networks formed by only physical interactions. However, cross-linking after 3.5 h was not completed, but the kinetics could not be further measured accurately due to evaporation of water. Systems containing either HA or PEG thioester cross-linkers showed similar rheological behavior in time (Figure 5A) in terms of gel formation kinetics as well as $G'$ values. It was shown that immediately after mixing HA−PNC networks showed higher $G'$ and $G''$ values. This is most likely caused by the higher thioester functionalities per cross-linker of the HA formulation. On average, the HA cross-linker contained 8 thioester moieties per chain compared to 2 thioester moieties for the PEG cross-linker. Therefore, using the HA cross-linker a strong chemically cross-linked network was obtained rapidly after mixing with PNC. Moreover, the higher viscosity of the HA formulation also contributed to higher initial moduli as compared to the PEG system. To demonstrate again that the cross-linking indeed proceeded through native chemical ligation rather than disulfide formation of the cysteine groups, a sample of 16 wt % of deprotected triblock copolymer in PBS was analyzed by rheology without adding a thioester cross-linker (Figure 5B). The sample was first heated from 4 to 50 °C at a heating rate of 1 °C/min and further followed at 37 °C for 3 h. During the heating step, an increase in storage modulus ($G'$) showed the thermosensitive behavior of the polymer, while at 37 °C no further increase in $G'$ was detected for 3 h for this sample without cross-linker. This observation proved that in this time frame negligible disulfide formation occurred, and the contribution to gel formation in PNC−PEG and PNC−HA samples was the result of cross-linking by NCL.

**3.5. Peptide Ligation.** The DDR-2 binding peptide CGPRGQOGVMGFO, known for its beneficial properties in cell differentiation, proliferation, and adhesion,[37−39] was ligated to part of the available PEG thioester functionalities. An *N*-terminal cysteine was introduced in the peptide sequence to allow ligation via NCL to the thioester functionality of PEG. PEG−peptide conjugates were formed with two different degrees of substitution (DS), using molar feed ratios of 1:1 and 0.5:1 thioester:cysteine peptide using MESNa as catalyst. These ratios were chosen to enable characterization of covalent ligation of this peptide to PEG thioester. After ligation, the NMR spectrum of the obtained conjugate clearly showed characteristic signals of peptide protons at $\delta$ = 0.8 and 7.2 ppm from valine and phenylalanine, respectively (Figure 6). Furthermore, reduced intensity of the $CH_3$ signal at $\delta$ = 1.2 ppm of the distal $CH_3$ group of the thioester indicated cleavage of the thioester due to the native chemical ligation reaction. Based on NMR integrals, DS was found to be 75 and 27% for molar feed ratios of 1:1 and 0.5:1 thioester:cysteine peptide, respectively. The same DS was found based on the decrease in


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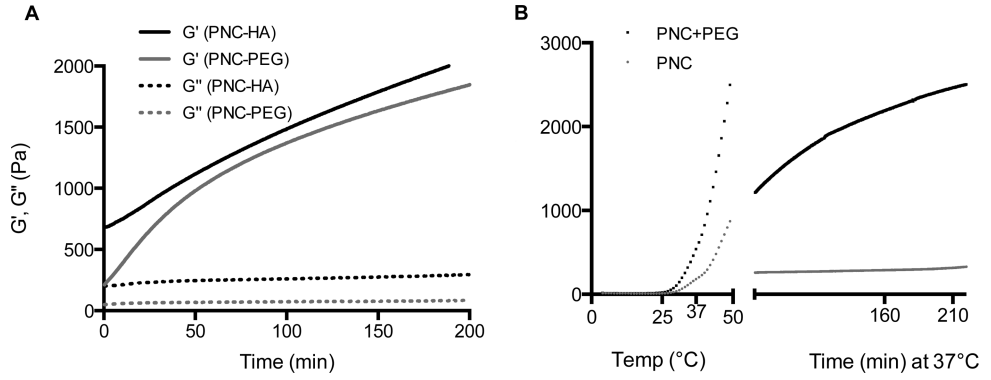


**Figure 5.** (A) Storage ($G'$) and loss ($G''$) moduli as a function of time of a mixture of 4 wt % PEG or 11 wt % HA thioester cross-linker with 16 wt % unprotected PNC at 37 °C in PBS. (B) Storage ($G'$) modulus of 16 wt % PNC deprotected with and without 4 wt % PEG thioester cross-linker. Samples were heated from 4 to 50 °C at 1 °C/min, subsequently cooled to 37 °C within 1 min, and further measured for 3 h at 37 °C.

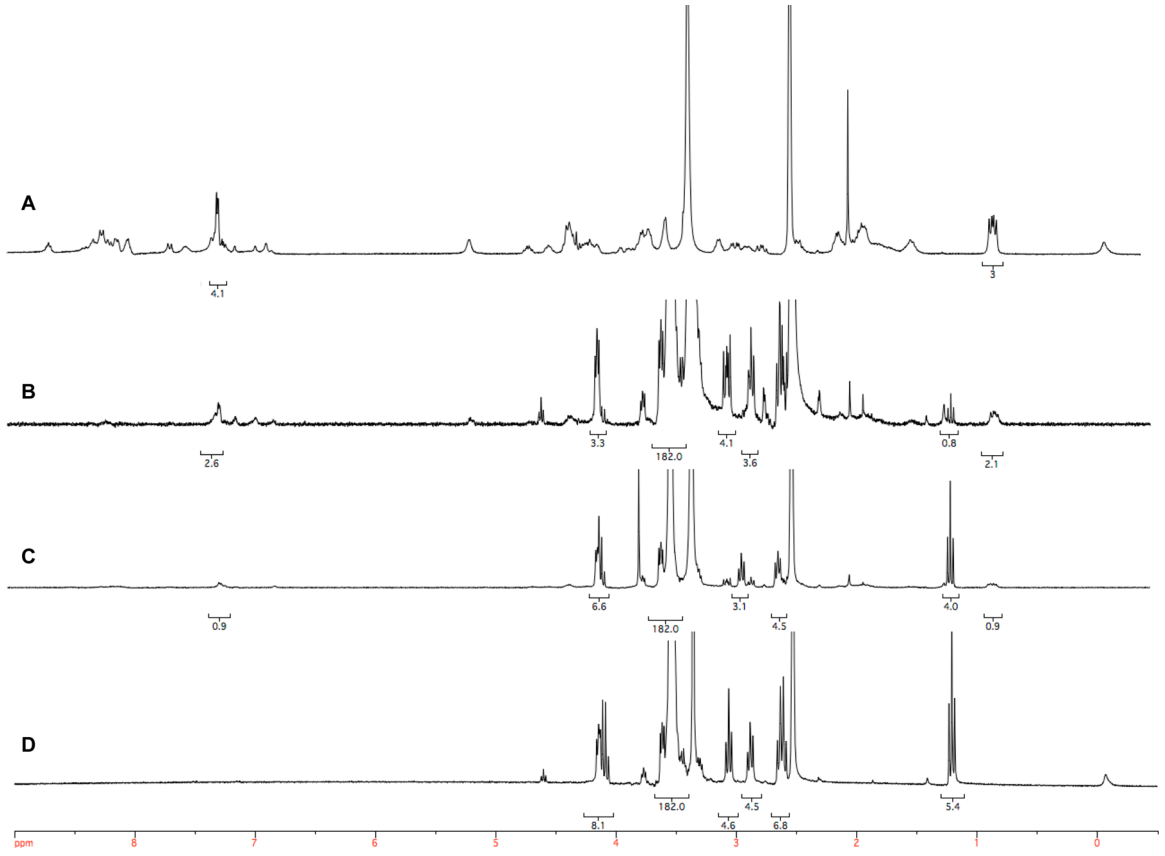


**Figure 6.** $^1$H NMR spectra of (A) peptide, (B) reaction product DS = 75%, (C) reaction product DS = 27%, and (D) PEG thioester. The peaks at 0.8 and 7.2 ppm show the peptide signals used to calculate the degree of substitution. The reduced intensity of the peak at 1.2 ppm (triplet of $CH_3$) demonstrates clearly the cleavage of the thioester ethyl functionality, an indication that the peptide is linked via NCL to the polymer.

thioester signal at 1.2 ppm as well as integrals of peptide signals. This indicates that the peptide was indeed successfully ligated, and nonfunctionalized peptide was completely removed by dialysis from the reaction mixture. Between 4 and 4.3 ppm, a quartet corresponding to the $O-CH_2$ protons adjacent to the terminal $CH_3$ (ethyl ester), which partially overlaps with a triplet corresponding to the terminal PEG $CH_2$ protons is visible in Figure 6D. A reduction of the peak integral of the $CH_2$ quartet at 4.2 ppm indicates that the terminal fragment of PEG thioester is removed after ligation, as is further confirmed by the displayed integral values. In contrast, the NMR spectrum of the reaction product with DS = 75% clearly shows that the signals of the $CH_2$ protons between PEG and the thioester groups are still present after ligation at 2.7 and 2.9 ppm. A slight difference in chemical shift is most likely caused by the peptide coupling. These results confirm the structure of the PEG–peptide conjugate as expected after NCL reaction.

Additional proof of peptide ligation was obtained by GPC analysis (Figure 7). After ligation, apart from the non-functionalized PEG–thioester a new peak appeared, showing that the molecular weight of the PEG thioester increased from 2.2–2.4 kDa to 3.8–3.9 kDa. This molecular weight was in good agreement with the theoretical molecular weight of a PEG thioester and one conjugated peptide (2.4 and 1.3 kDa,


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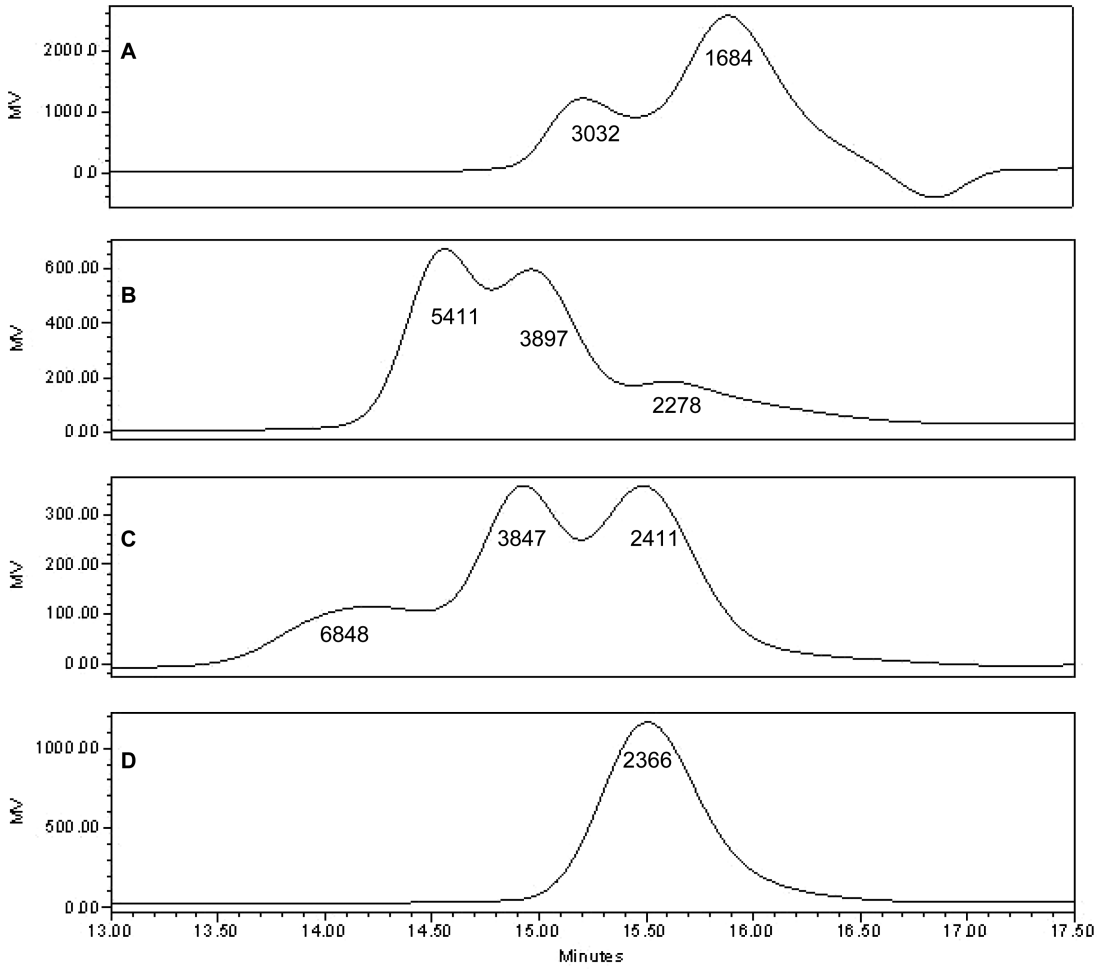


**Figure 7.** GPC chromatograms of (A) peptide, (B) reaction product DS = 75%, (C) reaction product DS = 27%, and (D) PEG thioester. Displayed values are the peak molecular weights based on RI detection and PEG standards. Ligation resulted in an increase in molecular weight. Disulfide formation was shown for both peptide and the ligated product.

respectively). Furthermore, the peaks at 5.4 and 6.8 kDa showed the presence of difunctionalized PEG-peptide conjugate (2.4 plus 2 × 1.3 kDa). After NCL reaction thiol functionalities remain that could potentially form disulfide bonds. Although TCEP was added as reducing agent to the GPC samples, a small fraction of disulfide bridged peptide and reaction product was visible at higher molecular weights. Based on GPC integrals for the product with DS = 27%, the reaction product contained approximately 40% nonfunctionalized PEG thioester and 43% PEG−thioester peptide conjugate. The remaining 17% contained PEG thioester with two peptides ligated and some disulfide product. The same agreement between GPC and NMR was found for the product with DS = 75%. These results showed that the PEG thioester was successfully functionalized with peptide with different degrees of substitution. However, it is important to realize that in hydrogel formulations for cell encapsulation experiments a lower functionalization of peptides to the cross-linker is desired (1−5%) to balance the peptide's biological activity[51] with the cross-linker capacity of the PEG thioester. An alternative approach would be to mix the DS 27% PEG thioester−peptide conjugate with nonfunctionalized PEG thioester to obtain a degree of functionalization of approximately 5 mol % peptide in the hydrogel. Using this approach, hydrogels were formed from PNC and PEG thioester functionalized with and without peptide conjugate (DS = 27%). The Young's moduli of the obtained hydrogels were measured with a dynamic mechanical analyzer (DMA). Incorporation of 25% PEG−peptide conjugate in the hydrogel formulation resulted in a slight reduction in the gel stiffness (from 5.3 ± 0.7 to 4.4 ± 0.8 kPa) that can be explained by the reduced number of available thioester groups for network formation by NCL. A Mann−Whitney U test showed a nonsignificant difference between these two groups ($P$ = 0.4), and therefore it can be concluded that peptide incorporation had a limited effect on hydrogel formation.

## 4. CONCLUSIONS

This study shows that thermoresponsive polymers that can undergo NCL reaction allow the immediate formation of a physical network that is strengthened in time due to the formation of covalent bonds between the hydrogel constituents. Both thioester cross-linkers based on hyaluronic acid and PEG contributed to the formation of a mechanically enhanced hydrogel. Control experiments proved that network formation proceeded through NCL rather than disulfide formation. This hydrogel is particularly attractive for biomedical and pharmaceutical applications because of the rapid network formation, further chemoselective chemical cross-linking of this network by NCL under mild reaction conditions, and the


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ability to covalently link a variety of peptides and other bioactive moieties.

## ■ ASSOCIATED CONTENT

### Supporting Information

HPLC chromatogram of Boc-Cys(Acm)-HPMA (SI-Figure 1); $^{1}$H NMR spectrum of ET-SA (SI-Figure 2); $^{1}$H and $^{13}$C NMR of Boc-Cys(Acm)-HPMA (SI-Figure 3); $^{1}$H NMR of PEG and HA thioester (SI-Figure 4); rheology amplitude sweeps (SI-Figure 5); cloud point measurement (SI-Figure 6). This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION


### Corresponding Author

*Ph (+31)620291631; e-mail T.Vermonden@uu.nl (T.V.).


### Notes

The authors declare no competing financial interest.

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