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Thermogelling and Chemoselectively Cross-Linked Hydrogels with Controlled Mechanical Properties and Degradation Behavior

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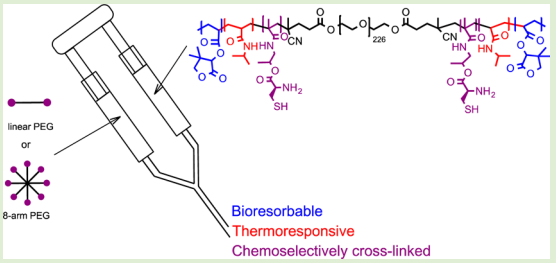
Supporting Information

ABSTRACT: Chemoselectively cross-linked hydrogels have recently gained increasing attention for the development of novel, injectable biomaterials given their limited side reactions. In this study, we compared the properties of hydrogels obtained by native chemical ligation (NCL) and its recently described variation termed oxo-ester-mediated native chemical ligation (OMNCL) in combination with temperature-induced physical gelation. Triblock copolymers consisting of cysteine functionalities, thermoresponsive *N*-isopropylacrylamide (NIPAAm) units and degradable moieties were mixed with functionalized poly(ethylene glycol) (PEG) cross-linkers. Thioester or *N*-hydroxysuccinimide (NHS) functionalities attached to PEG reacted with cysteine residues of the triblock copolymers via either an NCL or OMNCL pathway. The combined physical and chemical cross-linking resulted in rapid network formation and mechanically strong hydrogels. Stiffness of the hydrogels was highest for thermogels that were covalently linked via OMNCL. Specifically, the storage modulus after 4 h reached a value of 26 kPa, which was over a 100 times higher than hydrogels formed by solely thermal physical interactions. Endothelial cells showed high cell viability of 98 ± 2% in the presence of OMNCL cross-linked hydrogels after 16 h of incubation, in contrast to a low cell viability (13 ± 7%) for hydrogels obtained by NCL cross-linking. Lysozyme was loaded in the gels and after 2 days more than 90% was released, indicating that the cross-linking reaction was indeed chemoselective as the protein was not covalently grafted to the hydrogel network. Moreover, the degradation rates of these hydrogels under physiological conditions could be tailored from 12 days up to 6 months by incorporation of a monomer containing a hydrolyzable lactone ring in the thermosensitive triblock copolymer. These results demonstrate a high tunability of mechanical properties and degradation rates of these in situ forming hydrogels that could be used for a variety of biomedical applications.



INTRODUCTION

In situ forming hydrogels are an attractive class of biomaterials due to their capability of completely filling irregular tissue defects, using a minimally invasive treatment.[1−6] Particularly interesting are hydrogels that can be formed by both physical and chemical cross-linking, because physical cross-linking induces a quick stabilization of the network after injection, while chemical cross-linking ensures the formation of a mechanically stable network.[7−9] One of the most studied physically gelling hydrogels for biomedical applications is based on temperature sensitive poly(*N*-isopropylacrylamide) (pNIPAAm), because of its lower critical solution temperature (LCST) of 32 °C.[10] However, solely physically formed hydrogels are often mechanically weak, resulting in fast degradation or erosion. On the other hand, solely chemically formed hydrogels either cross-link too quickly, which leads to obstruction in the needle, or too slowly, resulting in leaching away of the polymer precursors from the application site. A dual gelation mechanism therefore combines the advantageous properties of both methods, providing an easy injection and strong network formation.

Although there is a wide variety of chemical cross-linking mechanisms available,[11] especially chemoselective reactions such as copper free click chemistry,[12] Diels−Alder[13,14] and native chemical ligation (NCL)[15] recently attracted great interest for application in drug delivery and tissue engineering. The chemoselective nature of these reactions results in efficient cross-linking between the hydrogel components without ligation to other biomolecules. NCL has been widely employed for peptide synthesis,[15] but is relatively unexplored in the field of biomaterials. It involves a reaction between an *N*-terminal cysteine and a thioester and proceeds via a reversible transthioesterification to an irreversible rearrangement that results in the formation of an amide bond. Groll et al. reported the synthesis of cysteine functionalized polymers for peptide

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conjugation by NCL.[16,17] Hydrogels formed by native chemical ligation without using a catalyst were first reported by the group of Messersmith.[18] Recently, we showed the formation of thermoresponsive hydrogels cross-linked by NCL.[19] The thermoresponsive component was composed of an ABA triblock copolymer having a poly(ethylene glycol) (PEG) B block and A blocks consisting of randomly distributed *N*-isopropylacrylamide (NIPAAm) and *N*-(2-hydroxypropyl)-methacrylamide-cysteine (HPMA-cysteine) monomers. Chemical cross-linking was accomplished after mixing this triblock copolymer with hyaluronic acid (HA) or PEG functionalized with thioester groups. The mild reaction conditions and possibility to further functionalize the hydrogel with peptides showed its attractiveness for biomedical applications.

Although these hydrogels were formed in a controlled manner, after cross-linking a thiol byproduct, ethyl thioglycolate, was released, which belongs to a group of compounds recently reported to be cytotoxic.[20] Strehin et al. proposed to use a variant of native chemical ligation, namely oxo-ester mediated native chemical ligation (OMNCL), thereby eliminating the release of thiol byproducts.[21] Moreover, they showed the chemoselectivity of the OMNCL mechanism by quantitative NMR analysis and the cytocompatibility of the obtained hydrogels both in vitro and in vivo. In contrast to NCL, OMNCL utilizes an ester instead of a thioester as reactive compound.[22] OMNCL has been first described by the group of Danishefsky as a simplification of native chemical ligation. They showed that the resulting ligation reactions had faster kinetics and higher yields, especially when the ligation took place at sterically hindered sites, yet still showing a high selectivity as proven in competitive experiments.[22] The chemoselectivity of OMNCL for cysteines over other amino acids was further proven in a microarray study.[23]

An ideal hydrogel for biomedical applications should have tunable mechanical properties and degradation rates for use in multiple applications. For tissue engineering purposes, the mechanical properties of the hydrogel should ideally match the stiffness of the target tissue.[24,25] Controlling and tuning the hydrogel stiffness has been reported in literature to have a significant effect on the performance of cells in terms of cell migration and differentiation.[26] In addition, the hydrogels should degrade in the same time frame as new tissue formation occurs. Although materials based on pNIPAAm homopolymers are widely used in tissue engineering applications, they are not resorbable. Several studies have shown the bioresorbability of NIPAAm based polymers after copolymerization of NIPAAm with hydrolyzable monomers, such as *N*-(2-hydroxypropyl)-methacrylamide lactate[27] and dimethyl-γ-butyrolactone acrylate (DBA).[28] After hydrolysis, the LCST of the polymers increases in time, resulting in dissolution of the polymers when an LCST of 37 °C is reached.

Copolymers of NIPAAm and DBA have been first reported by the group of Vernon.[28] An attractive feature of these copolymers is that degradation could be tuned by the incorporation of DBA and degradation under physiological conditions occurred without releasing low molecular weight degradation products. Further, a good biocompatibility of the obtained hydrogels was reported.[29] In addition, Mikos et al., described the formation of a physically and chemically gelling hydrogel that was bioresorbable after introduction of these DBA groups[30] and its application for bone regeneration.[31]

In this work, we further explored the opportunities of our previously developed thermoresponsive hydrogels cross-linked by native chemical ligation, involving the recent knowledge of oxo-ester mediated native chemical ligation. The aim of this study was to compare the properties of thermoresponsive, in situ forming hydrogels cross-linked by native chemical ligation and oxo-ester mediated native chemical ligation regarding their gelation kinetics, cell viability, and protein compatibility, thereby providing for the first time an in depth comparison of these chemoselectively cross-linked hydrogels. Finally, we present the ability to tune mechanical properties and degradation rates of our hydrogels by changing the polymer composition, toward controlling the eventual properties of these injectable hydrogels for the desired application.

2. MATERIALS AND METHODS

2.1. Materials. All materials were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and used as received unless otherwise noted. *N*-(2-hydroxypropyl)methacrylamide-Boc-*S*-acetamidomethyl-L-cysteine (HPMA-Boc-Cys(Acm)) was synthesized according to a previously published procedure.[19] PEG 20 000 8-arm with a tripentaerythritol core was obtained from JenKem Technology USA (Plano, Tx, USA). PEG 10 000-(4,4′-azobis(4-cyanopentanoic acid) (ABCPA) macroinitiator and ethylthioglycolate succinic acid (ET-SA) were synthesized following established procedures.[18,32] Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained from Carl Roth (Karlsruhe, Germany). Peptide grade dichloromethane (DCM) was purchased from Biosolve (Valkenswaard, The Netherlands). *N*,*N*′-Dimethylaminopyridine (DMAP) was obtained from Fluka (Zwijndrecht, The Netherlands). 4-(Dimethylamino)pyridinium-4-toluene-sulfonate (DPTS) was prepared according to a reported procedure.[33] MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and Celltiter 96 kit #G3580 were purchased from Promega (Madison, WI, USA). DMEM cell culture medium (#D6429) containing 4.5 g/L glucose and 10% FBS was obtained from Sigma-Aldrich. Antibiotic−antimyotic solution containing penicillin−streptomycin and antimycotics was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). 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 PNCD Triblock Copolymers. ABA triblock copolymers (abbreviated as PNCD) were synthesized consisting of a poly(ethylene glycol) 10 kDa B block, following previously published procedures.[19] The A blocks consisted of *N*-isopropylacrylamide (NIPAAm), *N*-(2-hydroxypropyl)-methacrylamide-cysteine (HPMA-Cys) and dimethyl-γ-butyrolactone acrylate (DBA). Five different polymers were synthesized by varying the DBA feed (0, 2.5, 5, 7.5, and 10 mol % of total monomer content). The feed ratio of HPMA-Boc-Cys(Acm) was kept at 7 mol % of the total monomer feed. In short, PEG-ABCPA 10 000 Da, HPMA-Boc-Cys(Acm), NIPAAm and DBA (total approximately 3 g, monomer:PEG ratio 322:1) were dissolved in 40 mL dry acetonitrile under N_2 atmosphere and polymerized for 40 h at 70 °C under stirring. After cooling the mixture to room temperature, the formed polymers were precipitated in cold diethyl ether and collected as a white solid after filtration. Boc protecting groups of cysteine were removed by dissolving the polymer in DCM/trifluoroacetic acid (TFA) (1:1 v/v, 40 mL) and stirring for 2 h at room temperature. Subsequently, the solution was concentrated under reduced pressure to 10 mL, and the polymers were precipitated in diethyl ether. A white solid was

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obtained and further dried under vacuum at room temperature. Acm protecting groups were removed by dissolving the polymer in MeOH/H_2O (1:1 v/v, 100 mL) under a nitrogen atmosphere, and addition of 1 mL 1 M HCl and 16 mL 0.2 M iodine in MeOH/H_2O (1:1 v/v). The brownish mixture was stirred for 1 h at room temperature, followed by addition of a few drops of 1 M ascorbic acid to quench the excess of iodine. TCEP (1 g) was added to the obtained colorless solution to reduce disulfide bonds. The mixture was stirred for 16 h, dialyzed against water for 3 days at 4 °C (M_w cutoff: 12−14 kDa) and the polymers were obtained after lyophilization. The polymers were characterized by ^{1}H NMR and GPC. Additionally, primary amine and thiol groups of cysteine were quantified by 2,4,6-trinitrobenzene sulfonic acid (TNBSA) and Ellman's assay respectively, following reported procedures.[34,35] ^{1}H NMR ($CDCl_3$): δ = 5.45 and 5.32 (d, DBA), 3.97 (s, NIPAAm), 3.80 (t, terminal CH_2 PEG), 3.62 (m, CH_2 PEG backbone), 1.42 (s, Boc HPMA-Cys), 1.11 (s, NIPAAm).

2.3. Synthesis of Linear and 8-Arm PEG Thioester Cross-Linkers. Linear PEG 2000 Da and 8-arm PEG 20 000 Da with thioester functionalities (abbreviated as PEG linear thioester and PEG 8-arm thioester, respectively) were synthesized following literature procedures.[18,19] Briefly, 6 g linear PEG 2000 or 15 g 8-arm PEG 20 000 (corresponding to 6 mmol OH groups) was added to a solution of ET-SA (2.6 g, 12 mmol), DCC (2.5 g, 12 mmol) and DPTS (177 mg, 1.2 mmol) in dry DCM (100 mL), and the mixture was stirred for 16 h at room temperature. Dicyclohexylurea (DCU) was removed by filtration, and the formed polymer was precipitated in cold diethyl ether. PEG thioester was obtained after filtration, further dried under vacuum and characterized by ^{1}H NMR. Additionally, two drops of trichloroacetyl isocyanate (TAIC) were added to the ^{1}H NMR sample to confirm the calculated DS, since TAIC induces a shift of the CH_2 protons of PEG adjacent to the OH end groups to 4.42 ppm.[36,37] Yield: 92% for PEG linear, 90% for PEG 8-arm, ^{1}H NMR ($CDCl_3$): δ = 4.42 (2H, TAIC shift CH_2OH), 4.24 (2H, t, terminal PEG CH_2), 4.14 (q, 2H, CH_3CH_2O), (3.62 (PEG backbone), 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 Linear and 8-Arm PEG NHS Cross-Linkers. Linear and 8-arm PEG were functionalized with NHS groups in a two-step synthesis according to a literature procedure.[21] First, the terminal OH groups of PEG were converted into COOH groups by a reaction with glutaric anhydride. In detail, 10 g of 8-arm PEG 20 000 (4 mmol OH) or 10 g of linear PEG 2000 (10 mmol OH) was dissolved in 20 mL chloroform. A 5-fold molar excess of glutaric anhydride and pyridine relative to the OH end groups of PEG were added to the PEG solution. The mixture was brought under nitrogen atmosphere and refluxed for 24 h at 80 °C. The solution was cooled to room temperature, 100 mL of methanol was added, and the formed polymer was precipitated in cold diethyl ether. The product was collected after filtration and further dried under vacuum.

In a subsequent reaction, NHS functionalities were introduced on the glutaric acid groups. Glutaric acid-terminated PEG (10 g) was dissolved in 30 mL of DMSO together with a 10-fold molar excess of NHS and EDC relative to the COOH groups of PEG and stirred for 1 h at room temperature. Then, 200 mL of methanol was added, and the product was precipitated in cold diethyl ether. The precipitate was harvested by filtration, and the methanol washing, precipitation, and filtration procedure was repeated twice. A white powder was collected and further dried under vacuum. The polymer was characterized by ^{1}H NMR to calculate the degree of substitution (DS). DS was confirmed after addition of TAIC as described in section 2.3. Yield: 85% for PEG linear, 88% for PEG 8-arm, NMR ($CDCl_3$): δ = 4.42 (2H, TAIC shift CH_2OH) 4.24 (2H, t, terminal PEG CH_2), 3.62 (PEG backbone), 2.84 (4H, m, 2CH_2 NHS), 2.71 (2H, t, NOC(O)-CH_2), 2.49 (2H, t, NOC(O)$CH_2CH_2CH_2$), 2.06 (2H, p, NOC(O)CH_2CH_2).

2.5. Polymer Characterization. The obtained polymers were characterized by gel permeation chromatography (GPC) and NMR spectroscopy. The molecular weights were determined by GPC using a PLgel 5 μm MIXED-D column (Polymer Laboratories). The column temperature was set to 65 °C and DMF containing 10 mM LiCl was used as eluent. The elution rate was set to 1 mL/min and the sample concentration was 5 mg/mL. Calibration was performed using poly(ethylene glycol) standards of narrow and defined molecular weights (PSS Polymer Standards Service GmbH, Mainz, Germany).

The polymers were also characterized with ^{1}H NMR spectroscopy on an Agilent 400 MHz spectrometer. Chemical shifts were referred to the residual solvent peak (δ = 7.26 ppm for $CDCl_3$ and 4.79 ppm for D_2O). Number-average molecular weight (M_n) determination by ^{1}H NMR was based on the integral ratio of the PEG mid block (904 protons) to monomer proton content.

2.6. Differential Scanning Calorimetry (DSC). The LCST of the obtained deprotected PEG-NIP-HPMACys-DBA (PNC(D)) triblock polymers was measured using a Discovery DSC (TA Instruments, New Castle, DE, USA). PNC(D) was allowed to dissolve for 4 h at 4 °C in a concentration of 10 wt % in PBS before measuring. Ten microliters was transferred into an aluminum sample pan and the pan was hermetically capped. Thermograms were recorded in triplicate from 0 to 70 °C, using a heating rate of 5 °C/min. The LCST was determined as the onset temperature in the thermogram.

2.7. Rheological Characterization. Rheological analysis of the hydrogels was performed on a Discovery HR-2 rheometer (TA Instruments, New Castle, DE, USA), using a 20 mm steel cone (1°) geometry equipped with a solvent trap. Temperature sweeps were performed from 4 to 50 °C at a heating rate of 1 °C/min. Time sweeps were performed for 4 h at 37 °C. For all measurements a frequency of 1 Hz and a strain of 1% was applied. This strain and frequency were previously determined to be within the linear viscoelastic region of these polymer solutions and hydrogels.[19]

2.8. Hydrogel Preparation. Cylindrically shaped hydrogels of 100 μL were prepared in plastic molds with a diameter of 4 mm. Separate solutions of PNC(D) and PEG cross-linker were prepared in volumes of 75 and 25 μL PBS, respectively. PNC(D) polymers were dissolved at 4 °C for 3 h, while PEG cross-linkers were dissolved for 30 min at 4 °C to limit premature hydrolysis. Upon complete dissolution, the PEG cross-linker solution was added to the PNCD solution, shortly mixed, and transferred into the mold using a positive displacement pipet. The molds were capped and placed at 37 °C for 3 h to allow physical and chemical cross-linking.

2.9. Dynamic Mechanical Analysis. Hydrogels were prepared as described in section 2.8. The Young's modulus of the obtained hydrogels was determined on a Q800 DMA (TA Instruments, New Castle, DE, USA) in triplicate after 3 or 24 h

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cross-linking at 37 °C. Additionally, 24 h cross-linked hydrogels were incubated for 3 h in PBS or PBS containing 20 mM TCEP to reduce possibly formed disulfide bonds and were subsequently measured by DMA. A compression of 0.01 N/min until 0.1 N was applied to the hydrogels at room temperature. The Young's modulus was determined from the linear region of the stress−strain curve between 10 and 15% strain.

2.10. GPC Analysis of Lysozyme Ligation to the Hydrogel Components. To study the selectivity of the chemical cross-linking reactions, the possible ligation of lysozyme to the hydrogel components was analyzed by GPC. PEG 8-arm thioester, PEG 8-arm NHS, and PNC were dissolved in a concentration of 10 mg/mL in PBS, either with or without 10 mg/mL lysozyme. After 5 h of incubation at room temperature, the molecular weights of the polymers and proteins present in the mixtures were measured by GPC using a Superdex 75 column and PBS as solvent. Analysis was performed by RI detection and UV detection at 210 and 280 nm. A flow of 0.5 mL/min and a run time of 60 min were applied.

2.11. Lysozyme Ligation to Dual Cross-Linked Hydrogels. Possible protein ligation to PNC−PEG 8-arm thioester and PNC−PEG 8-arm NHS hydrogels was studied using lysozyme as a model protein by measuring the amount of nongrafted protein by release experiments. In detail, stock solutions of 22 wt % PNC, 33 wt % PEG cross-linker and 50 mg/mL lysozyme were prepared. PEG cross-linker (80 μL) and lysozyme (40 μL) were added to PNC (280 μL), mixed, and three samples of 100 μL were transferred into a cylindrical glass vial (diameter of 5 mm). The final solutions consisted of 15 wt % PNC, 7.5 wt % PEG cross-linker and 0.004 wt % lysozyme. The hydrogels were allowed to form for 16 h at 37 °C, after which 0.9 mL of PBS buffer pH 7.4 was added on top of the gels. In time, samples of 0.15 mL were taken and replaced by 0.15 mL fresh PBS buffer. The concentration of lysozyme in the different release samples was determined with Acquity UPLC using a BEH C18 1.7 μm, 2.1 × 50 mm column. As eluent, a gradient from 0 to 100% of eluent A was used, where eluent A was H_2O/acetonitrile/TFA 95/5/0.1% and eluent B was 100/0.1% acetonitrile/TFA. The injection volumes were 5 μL, the flow rate was set at 0.25 mL/min and detection was done at 210 nm.

2.12. Cell Viability Assays. An MTS assay was performed to assess cytocompatibility of the hydrogels described in this study. C166 mouse endothelial cell line was chosen as a representative of healthy tissue. The cells were seeded on a 96 well plate with 6000 cells per well and 100 μL of DMEM medium including antibiotics was added. Two different experiments were performed. First, hydrogels of 50 mg wet weight consisting of 7.5 wt % PNC and 3.8 wt % PEG cross-linker, were allowed to form for 3 h as described in section 2.8 and were subsequently introduced in the medium on the cell layer. After 16 h of incubation at 37 °C, the hydrogels were removed, and the medium was washed four times to remove reaction products that could interfere with the MTS assay. In the second experiment, 50 mg prefabricated hydrogels were added to 100 μL of medium without cells and incubated for 2 h. Then, 50 μL of this medium was added to the cell layer and further incubated for 16 h. In this leachables assay, the influence of soluble products that are released from the hydrogels on the cell viability was tested. Again, the medium was washed four times with PBS and subsequently replaced with 100 μL medium. Then, 20 μL MTS reagent was added and further incubated for approximately 2 h to develop the color. After mixing, absorption was measured at 490 nm on a Biochrom EZ microplate reader. Metabolic activity of the cells was normalized to a negative control (medium, 100% value) and compared to a positive control (medium +100 μM SDS, 0% value). Brightfield microscopy pictures were taken using a Keyence BZ-9000E microscope before adding MTS to analyze the cell shape with a 20x Nikon objective.

Since a potential application of the hydrogels of this study is, e.g., for cartilage tissue engineering,[25] a Live/Dead viability assay (calcein AM/ethidium homodimer, Life Sciences, USA) was performed on chondrocytes following previously reported procedures.[38,39] In short, equine chondrocytes were harvested from full thickness cartilage. Chondrocytes were encapsulated in the hydrogels at a concentration of 5×10^6 cells/mL. Hydrogels containing cells were allowed to form for 3 h before addition of DMEM medium. Viability was visualized after 7 days of culture using a light microscope (Olympus, BX51, USA) with excitation/emission filters set at 488/530 nm and 530/580 nm to detect living (green) cells and dead (red) cells, respectively.

2.13. Swelling and Degradation Study. Hydrogels of 100 μL were prepared as described in section 2.8. Three different groups were analyzed in this study, containing 15 wt % PNC(D) and 7.5 wt % PEG 8-arm NHS, 7.5 wt % PNC(D) and 3.8 wt % PEG 8-arm NHS or 15 wt % PNC(D) and 3 wt % PEG linear NHS, all corresponding to a 1:1 molar ratio of functional groups. After incubation of the polymer solutions at 37 °C for 3 h, the formed hydrogels (4.3 mm diameter, 5.0 mm height) were pushed out from the mold and transferred into a 2 mL glass vial. The hydrogel weight was recorded, and 1 mL of PBS containing 0.02% NaN_3 was added. At regular time intervals, excess of buffer was removed, the weight of the hydrogels was measured, and 1 mL of fresh PBS was added. Measurements were performed twice a week during the first 5 weeks and then once a week until complete degradation or until 6 months. The swelling ratio is defined as the weight at a certain time point (W_t) divided by the initial hydrogel weight (W_0) (Swelling ratio = W_t/W_0). After measuring, 1 mL of fresh buffer was added, and the hydrogels were further incubated at 37 °C. Finally, solutions after full degradation were lyophilized, redissolved in D_2O, and analyzed by ^{1}H NMR.

3. RESULTS AND DISCUSSION

3.1. Polymer Synthesis and Characterization. ABA triblock copolymers consisting of a hydrophilic PEG B block were synthesized by free radical polymerization. The A block consisted of three different monomers: (1) NIPAAm to render thermoresponsive polymers, (2) HPMA-Cys to allow chemoselective cross-linking, and (3) DBA to obtain gels with tunable degradation rates. The polymer structure is shown in Figure 1. After polymerization, the protecting groups present on the amine and thiol groups of cysteine were removed by TFA and iodine treatment, respectively, as described previously.[19] The successful deprotection was confirmed by ^{1}H NMR, TNBSA and Ellman's assays following established procedures.[19] The average molar incorporation of cysteine was 6%, corresponding to an average of 21 cysteine moieties per polymer chain, which was only slightly lower than the feed percentage of 7% for this monomer. As reported in Table 1, the molar ratios of the three monomers in the obtained polymer as calculated by ^{1}H NMR were in close agreement with the feed ratios. The synthesized

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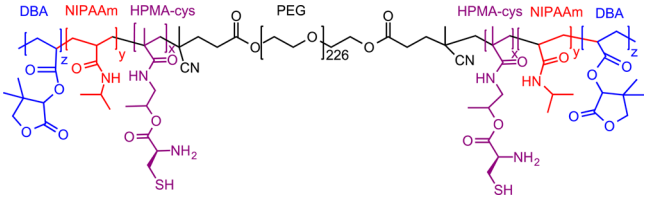

Figure 1. Chemical structure of PNCD polymers, consisting of a 10 kDa PEG mid block, flanked by random monomers of HPMA-cys, NIPAAm, and DBA.

polymers are abbreviated as PNC, PNCD2.5, PNCD5, PNCD7.5 and PNCD10 for a molar content of DBA of 0, 2.5, 5, 7.5 and 10%, respectively. The number-average molecular weights measured by GPC were in line with the values calculated from ^{1}H NMR analysis, whereas GPC measurements showed polydispersities ranging from 2.1 to 2.4, which is often found for free radical polymerization of multiblock copolymers.[40] Based on ^{1}H NMR and GPC, PEG 10 kDa B blocks were flanked by A blocks of an average molecular weight of 21 kDa. The polymers were synthesized in a high yield of 88% and obtained in a yield of 53% after full deprotection.

PEG cross-linkers either based on a linear PEG having a molecular weight of 2000 Da or an 8-arm PEG of 20 000 Da with thioester or NHS terminal reactive groups were synthesized. The polymer structures and corresponding ^{1}H NMR spectra are shown in Figure 2. Calculations of the degree of substitution (DS) for PEG thioester cross-linkers were based on the integral of the terminal CH_3 group at 1.21 ppm, while for NHS functionalized PEG calculations were performed using the peak at 2.84 ppm, which corresponds to the 2 CH_2 groups of the NHS ring. DS was confirmed by quantifying the unreacted PEG OH groups after addition of trichloroacetyl isocyanate (TAIC) to the NMR sample. PEG linear cross-linkers were synthesized with a high DS of 88 and 90% for thioester and NHS functionalities, respectively. Also, 8-arm PEG cross-linkers were obtained with a high DS of 92% for PEG-thioester and 94% for PEG-NHS, respectively.

3.2. Gel Formation and Mechanical Properties. The PNCD polymers were dissolved in PBS at 4 °C, and the thermoresponsive properties of the obtained polymer solutions were measured both by rheology and DSC (Figure 3). Rheological analysis showed that with increasing temperature, a sudden increase in storage modulus (G') as well as a crossover of G' and G'' (SI-Figure 1A) was observed, which indicates the phase transition from a liquid solution to a physically cross-linked network. By increasing the DBA content in the polymers, this phase transition occurred at lower temperatures as a result of an increase in hydrophobicity of the polymers. DSC results confirmed that the LCST values of the obtained polymers decreased with increasing DBA content in the polymer. Noteworthy, a linear relationship was obtained between the molar percentage of DBA in the polymers and their LCST (Figure 3B), which is in line with the literature.[28]

Physically and chemically cross-linked hydrogels were prepared by mixing an aqueous solution of thermoresponsive cysteine functionalized PNCD polymers with a solution of (thio)ester functionalized PEG cross-linkers at 37 °C. The mechanism of chemical cross-linking is shown in Figure 4. An immediate network formation was visualized at 37 °C by a lack of flow when tilting the vial upside down, as expected from the thermosensitive nature of the polymers. The gelation kinetics and mechanical properties were further analyzed by rheology at 37 °C. Again, an immediately formed physically cross-linked network was obtained characterized by a higher storage modulus than loss modulus (SI-Figure 1B). In time, the storage modulus increased further, indicating the formation of a more densely cross-linked network due to additional chemical cross-linking. This resulted in 8−120-fold higher G' values after 4 h compared to solely thermal physically cross-linked PNC hydrogels. As depicted in Figure 5A and B, when changing from a PEG linear to a PEG 8-arm cross-linker, the storage moduli after 3 h of network formation increased from 1.8 to 8.8 kPa for thioester functionalized PEG and from 4.7 to 24.3 kPa for NHS functionalized PEG. As expected, the increase in number of functional groups per cross-linker was associated with a 5-fold increase in mechanical strength. Similarly, replacing thioester groups by NHS groups resulted in approximately a 2.5 fold increase in storage moduli. The higher mechanical strength of the OMNCL cross-linked hydrogels is most likely a result of the higher reaction efficiency, in line with previous studies.[21] Furthermore, increasing the total polymer concentration resulted in an increase in mechanical strength (Figure 5C), again in line with the literature.[41] Additionally, the mechanical properties of the obtained hydrogels after 3 or 24 h of hydrogel formation were measured by compression measurements. As shown in Figure 5D, stiffer hydrogels were obtained using NHS functionalized PEG compared to thioester functionalized PEG, similar to the data obtained with oscillatory shear rheological measurements. The reported Young's modulus showed a further increase in mechanical strength in time. This could be ascribed to the formation of additional cross-links by disulfide bonds that form with slower kinetics than (oxo-ester mediated) native chemical ligation.[21] After NCL or OMNCL, an amide bond is formed and a free thiol remains available for additional cross-linking, which could further stabilize the hydrogel structure. The formation of these additional disulfide bonds was demonstrated by incubating 24 h cross-linked PNC−PEG 8-arm hydrogels in either PBS or PBS containing 20 mM TCEP

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

	% Cys		% NIPAAm		% DBA				
	feed	NMR	feed	NMR	feed	NMR	M_n (kDa)[a]	M_n (kDa)[b]	M_w/M_n[b]
PNC	7.0	6.2	93.0	93.8	0	0	20−10−20	43.0	2.36
PNCD 2.5	7.0	6.9	90.5	90.8	2.5	2.3	23−10−23	53.9	2.16
PNCD 5	7.0	5.7	88.0	89.0	5.0	5.3	20−10−20	52.4	2.17
PNCD 7.5	7.0	5.9	85.5	86.1	7.5	8.0	19−10−19	53.2	2.19
PNCD 10	7.0	5.9	83.0	83.8	10.0	10.3	21−10−21	53.0	2.15

[a]Determined by ^{1}H NMR. [b]Determined by GPC. [*]PNCD: PEG-NIP-HPMACys-DBA triblock; structure shown in Figure 1.

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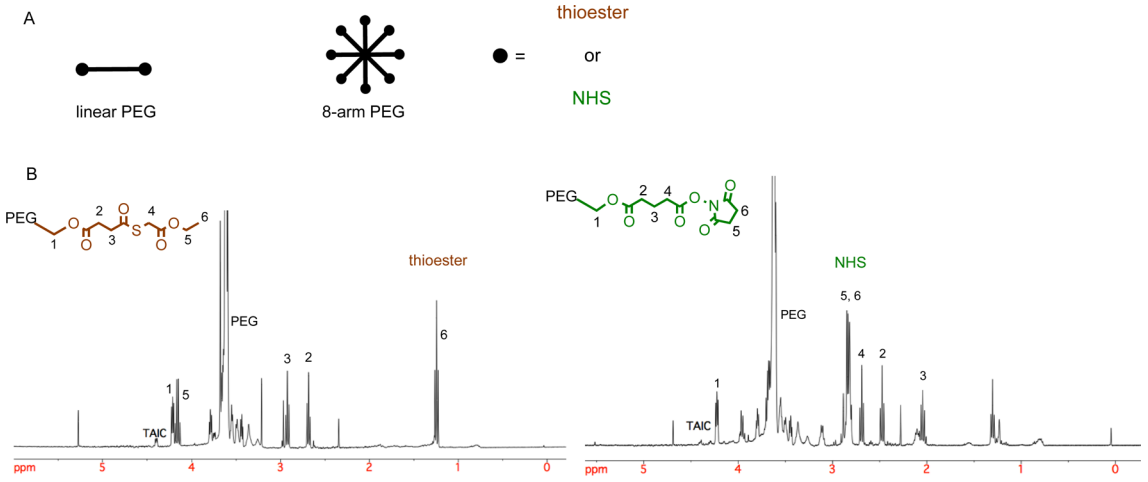

Figure 2. (A) Schematic display of the cross-linkers used in this study, synthesized from a linear PEG 2000 or 8-arm PEG 20 000 with either thioester or NHS functionalities. (B) ^{1}H NMR of 8-arm PEG thioester and NHS cross-linkers in $CDCl_3$. Peak 6 of PEG thioester at 1.21 ppm and Peak 5,6 of PEG NHS at 2.84 ppm indicate peaks that were used to calculate the degree of substitution for thioester and NHS groups, respectively.

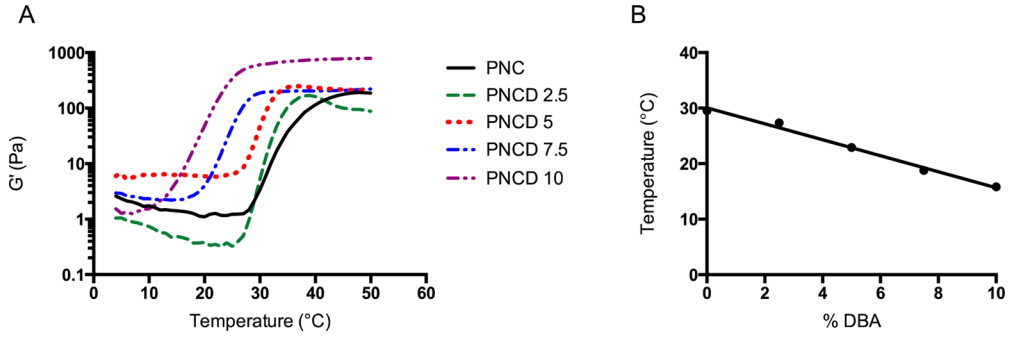

Figure 3. Effect of DBA content in thermosensitive PNCD polymers on (A) gelation temperature as measured by rheology in 20 wt % concentration and (B) LCST as measured by DSC in 10 wt % concentration.

to reduce disulfide bonds, following previously reported procedures.[18] After 3 h of incubation, the stiffness of the hydrogels was measured with dynamic mechanical analysis (DMA). As expected, the Young's modulus of hydrogels that were incubated in 20 mM TCEP decreased from 98 ± 4 kPa to 61 ± 5 kPa for PNC−PEG NHS hydrogels and from 11 ± 1 kPa to 9 ± 1 kPa for PNC−PEG thioester hydrogels as a result of the reduction of disulfide bonds (Figure 5D).

Additionally, we investigated the influence of a non-stoichiometric ratio of cysteine:thioester functional groups on the gel formation. Interestingly, changing this ratio did not have a significant effect on the mechanical properties in case of the formulations using an 8-arm PEG cross-linker (Figure 6). In contrast, changing the cysteine:thioester ratio from 1:1 to 1:0.5 for the linear PEG cross-linkers led to a 25% lower storage modulus after 4 h, while changing the ratio to 1:2 resulted in a 51% lower G'. An excess of thioester functionalities blocks the cysteine groups that are available, resulting in dangling chains that form a dead end. On the contrary, a shortage of thioester groups results in a less tightly cross-linked network. As the PEG 8-arm thioester has 8 functionalities per chain, not all thioester groups need to react before a cross-link is formed. Similar results were obtained for fully cross-linked hydrogels that were measured after 24 h by DMA(Figure 6C), showing that a nonstoichiometric ratio in case of linear PEG cross-linkers not only influenced the gelation kinetics, but also the final mechanical properties. While a shortage of thioester groups had a considerable effect on the gel formation kinetics, an excess of thioester groups also led to lower final moduli of the hydrogel as a result of the dangling chain ends. In the case of the 8-arm cross-linker, only small differences in the final moduli were found. Therefore, besides increasing the overall mechanical properties, hydrogels with 8-arm cross-linkers have the advantage to be less susceptible to a nonstoichiometric ratio of functional groups.

Taken altogether, with limited changes in the overall polymer concentration, the mechanical strength of the hydrogels significantly increased by using an 8-arm PEG cross-linker and using NHS functionalities instead of thioester groups.

3.3. Protein compatibility. Injectable hydrogels for biomedical applications require in many cases the incorporation of proteins, e.g., in drug delivery or growth factors for tissue engineering purposes.[42,43] Therefore, it is of utmost importance to use cross-linkers with functional groups that do not interfere with the protein structure and still allow release of the incorporated active proteins.[44] Lysozyme was used as a model protein since its size (molecular weight = 14 kDa, hydrodynamic diameter = 4.1 nm[45]) and positive charge at physiological pH (isoelectric point = 11.35[46]), are similar to many growth factors.[47] We first studied the potential ligation of lysozyme to the individual hydrogel components under physiological conditions by GPC. The molar ratio of NH_2 of lysozyme to NHS or thioester functionalities of PEG in the mixtures was 1:1, while the molar ratio of NH_2 of lysozyme to

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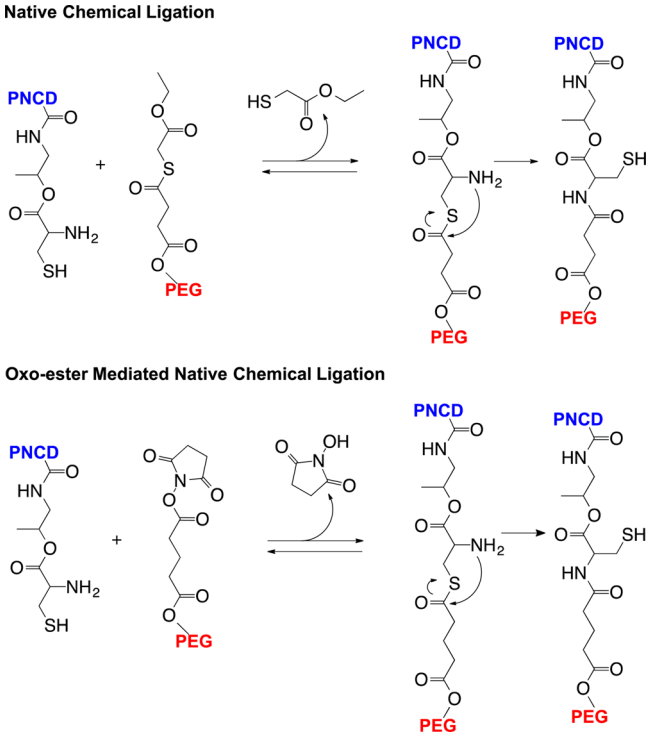

Figure 4. Native chemical ligation (NCL) and oxo-ester mediated native chemical ligation (OMNCL) cross-linking mechanisms between cysteine functionalities of PNCD and thioester or oxo-ester functionalities of PEG cross-linkers.

SH of PNC was 2:1. Figure 7 shows the GPC chromatograms with UV detection after at least 4 h incubation of the hydrogel building blocks with lysozyme. Native lysozyme displays a peak at 32 min, and no shift was found for lysozyme mixed with either PNC or PEG thioester. Therefore, it can be concluded that mixing lysozyme with PNC or PEG thioester did not cause any significant ligation between the functional groups of the protein and the polymers. This was expected since all eight cysteines present in lysozyme are paired in disulfide bridges.[48] By contrast, lysozyme mixed with PEG NHS showed much shorter retention times of only 16 min, indicating that approximately 90% of lysozyme was conjugated to the PEG NHS functionalized cross-linkers, resulting in the formation of a higher molecular weight product. This result is not surprising, as it is well-known that NHS groups can react with amino groups of, e.g., lysine moieties that are present in proteins.[49,50] In the experiments reported above, we only studied the potential reaction of lysozyme with the individual hydrogel components. However, during hydrogel preparation thiol side groups of thermosensitive polymers are present that compete with the amines of lysozyme for reaction with the NHS esters. In the hydrogels, the molar ratio of NH_2 of lysozyme was 1:20 to NHS and SH groups. As has been previously reported in the literature, in a competitive reaction between amines and cysteines, NHS-activated carboxylic acids preferentially react with the thiol functionality of cysteine.[21,22] Because of the high molar access of SH over NH_2 groups and their higher reactivity, the probability of reaction of lysozyme with PEG NHS is likely low. To investigate this, a protein release study was performed to study whether lysozyme reacted with the hydrogel components during hydrogel formation. These network-grafted

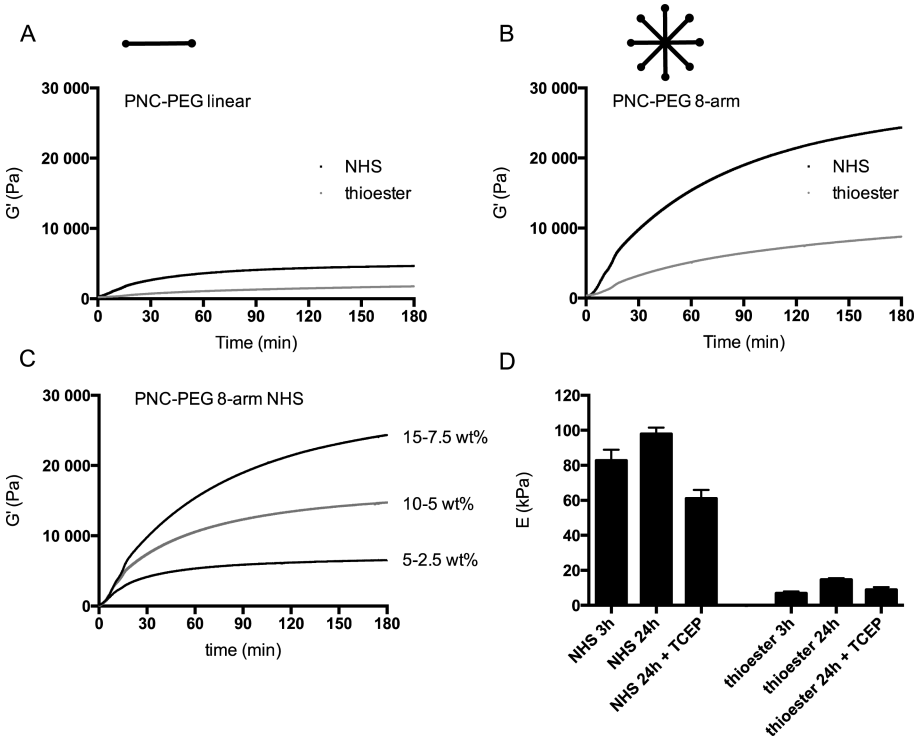

Figure 5. (A) Storage modulus (G') as a function of time for hydrogels containing linear PEG cross-linker: 15 wt % PNC + 3 wt % PEG linear. (B) G' as a function of time for hydrogels containing 8-arm PEG cross-linker: 15 wt % PNC + 7.5 wt % PEG 8-arm. (C) G' for three different polymer concentrations of PNC−PEG 8-arm NHS: 15−7.5 wt %, 10−5 wt % and 5−2.5 wt %. (D) Young's modulus as measured by DMA: 15 wt % PNC + 7.5 wt % PEG 8-arm after different times of gelation and after incubation in either PBS or PBS containing 20 mM TCEP. Data are shown as mean ± standard deviation (n = 3).

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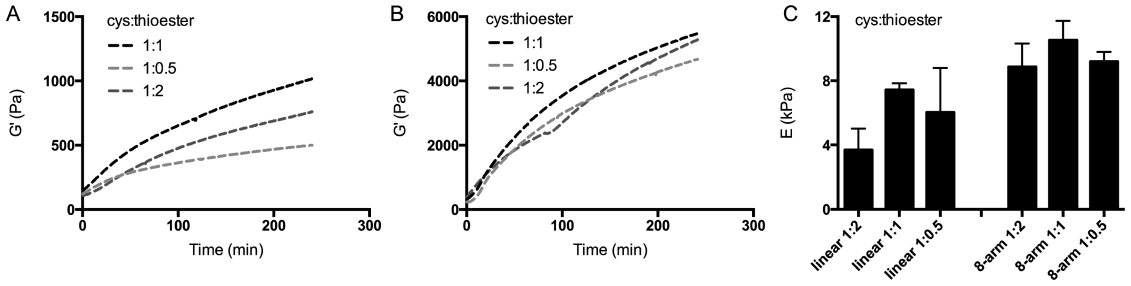

Figure 6. Effect of changing cysteine:thioester ratio on mechanical properties as measured by rheology for (A) linear PEG cross-linkers and (B) 8-arm PEG cross-linkers. (C) Young's modulus as measured by DMA for different cysteine:thioester ratios after 24 h of gel formation. Data are shown as mean ± standard deviation (n = 3).

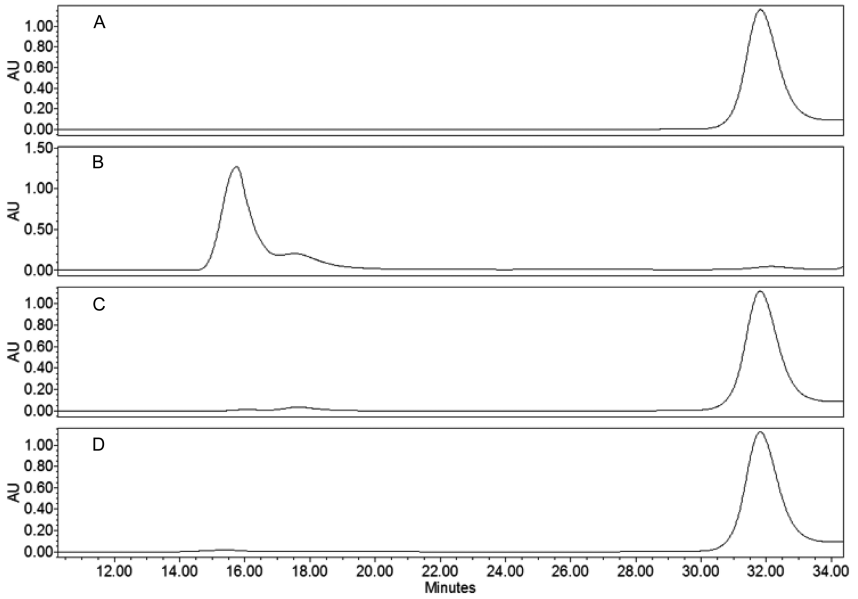

Figure 7. GPC chromatograms of lysozyme incubated with different hydrogel components: PNC, PEG NHS, or PEG thioester in PBS buffer as measured at 210 nm. (A) Lysozyme; (B) Lysozyme + PEG NHS; (C) Lysozyme + PEG thioester; (D) Lysozyme + PNC.

protein molecules are immobile and therefore unable to release in the surrounding medium. The purpose of this release study was to compare the extent of lysozyme release from the PNC−PEG thioester and PNC−PEG NHS hydrogels and to correlate this with the potential grafting of lysozyme to the hydrogel building blocks. Figure 8 shows the release of lysozyme from two hydrogel formulations, using either thioester or NHS functionalized PEG 8-arm cross-linkers. As anticipated, the extent of grafting was low and more than 90% of the loaded lysozyme was released from both gels after 2 days, at which time a plateau in release was obtained. This indeed confirms that the reaction between NHS and cysteine is preferred over a reaction between NHS and amino groups of lysine residues of lysozyme. The amount of lysozyme that was incorporated in the hydrogels was based on literature procedures.[51] It must be noted that when more lysozyme will be loaded in the hydrogel, this will likely increase the potential of a side reaction of lysozyme with the NHS groups, which could have an effect on swelling, volume transition temperature and degradation. In addition, the presence of native proteins may have some effect on the hydrogel properties after injection of the hydrogel precursors in vivo. On the contrary, in some applications such as growth factor incorporation, covalent attachment of a protein to the hydrogel can induce beneficial cell differentiation, migration or proliferation, so grafting as such is not necessarily unfavorable.[43,52,53] In line with the mechanical characteristics, hydrogels containing NHS groups released lysozyme over a slightly longer time frame than hydrogels containing thioester groups. Fast release of lysozyme from the hydrogels was expected, as lysozyme is a relatively small protein, and this finding is in line with previous studies.[51] When longer release times are needed, this could potentially be

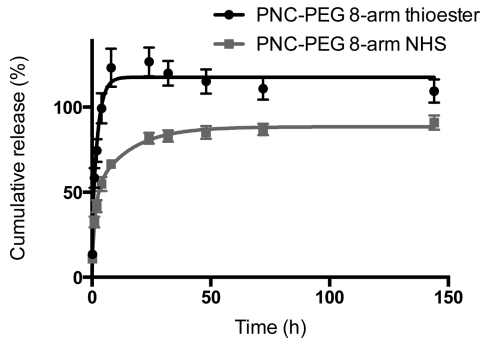

Figure 8. Cumulative release of lysozyme from PNC−PEG 8-arm thioester or PNC−PEG 8-arm NHS hydrogels. Hydrogel concentrations were 15 wt % PNC + 7.5 wt % 8-arm PEG. Data are shown as mean ± standard deviation (n = 3).

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accomplished by increasing polymer concentrations, increasing the number of functional groups per polymer chain or by incorporating lysozyme in microparticles that are embedded in the hydrogel.[41,54]

3.4. Cell Viability. Cell survival in the presence of chemoselectively cross-linked PNC−PEG 8-arm NHS or PNC−PEG 8-arm thioester hydrogels was assessed using two different cell viability assays. Equine chondrocytes were encapsulated in the hydrogels, and their viability was studied after 7 days of culture using a live/dead assay (SI-Figure 2). Most cells simultaneously showed green cytoplasmic fluorescence and red nuclear fluorescence in the live/dead assay after 7 days. Therefore, it was difficult to draw conclusions about their viability, although overlay pictures might indicate a better cell viability for PNC−PEG 8-arm NHS hydrogels. For this reason, the effect of the hydrogel leachables on the metabolic activity of a mouse endothelial cell line was analyzed with an MTS assay. Also taking into consideration that an MTS assay gives quantitative results, evaluation of the cell viability using this assay was preferred over a live/dead assay. In an MTS assay, a tetrazolium salt is converted to an aqueous soluble formazan product by mitochondrial activity of viable cells, which can be measured colorimetrically.[55] Two different experimental set-ups were used to evaluate the cell viability. In the first setup, prefabricated hydrogels, without extraction of possible leachable products, were added to medium containing cells and incubated for 16 h. After removal of the gels and several washing steps, the metabolic activity was measured. As shown in Figure 9, cell viability was not affected by the PNC−PEGNHS hydrogels, while addition of the PNC−PEGthioester hydrogels caused a decrease in viability to 13 ± 7%. Based on literature findings, this decrease in cell viability is most likely caused by the release of ethyl thioglycolate after cross-linking (as schematically shown in Figure 4).[20] In the second setup, we further tested this hypothesis using an assay developed to test the cytotoxicity of leachables that are extracted from gels.[56] After incubating prefabricated hydrogels for 2 h in medium, the medium without the gel was added to the cells and further incubated for 16 h. The MTS results showed a much lower cell viability upon incubation of the extract of PNC−PEGthioester hydrogel as compared to PNC−PEGNHS hydrogel extract. Finally, additional confirmation was obtained after visual analysis of the cell shape (SI-Figure 3). While the cell shape was hardly affected after contact with the leachables of the PNC−PEGNHS hydrogels, the cells clearly lost their healthy, stretched shape upon incubation with the extract of the PNC−PEGthioester hydrogel. Taken together, PNC−PEGNHS hydrogels have a substantially better cytocompatibility than the PNC−PEGthioester hydrogels, which likely can be ascribed to the reaction product formed (namely ethyl thioglycolate) during the formation of the PNC−PEGthioester hydrogels.

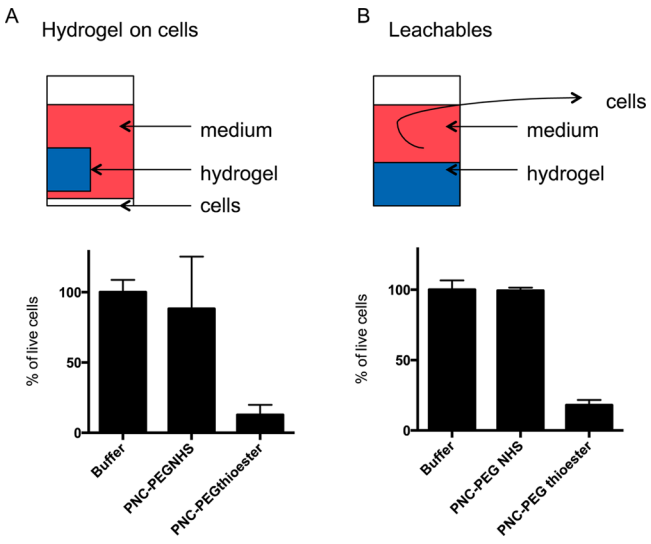

Figure 9. Cell viability as tested by MTS assay of endothelial cells with PNC−PEG 8-arm hydrogels (7.5−3.8 wt %) using (A) contact assay (hydrogel on cells) and (B) leachables assay. Data are shown as mean ± standard deviation (n = 4).

3.5. Hydrogel Degradation. The degradation of the dual cross-linked hydrogels was assessed under physiological conditions at 37 °C and pH 7.4. Only the NHS-functionalized PEG cross-linkers were included in this study, due to their favorable gelation kinetics, mechanical properties, and cytocompatibility. Hydrolysis of the ester bonds present in the hydrogel network can occur at three different positions: (1) hydrolysis of the ester bonds between the PEG mid block and thermosensitive outer blocks; (2) hydrolysis of ester bonds within the chemical cross-links (Figure 4); (3) hydrolysis of the ester bonds in the lactone ring of DBA. Hydrolysis of ester bonds at positions 1 and 2 leads to dissolution of the hydrogel, whereas hydrolysis at position 3 increases hydrophilicity and thus the swelling of the hydrogel network. Previous studies have shown that at physiological pH, only the ester bonds in the DBA ring are hydrolyzed and not the ester bond between the ring and the polymer backbone, thereby preventing formation of additional soluble byproducts.[28] Three different hydrogel formulations were tested, exhibiting storage moduli between 5 and 24 kPa after 3 h of hydrogel formation (SI-Figure 4). These formulations were chosen to assess the influence of concentration and the difference between a linear and 8-arm PEG cross-linker on the degradation rates. PNC was mixed with a linear PEG cross-linker in a total polymer concentration of 18 wt % and is further abbreviated as "linear". PNC−PEG 8-arm hydrogels were studied at a total polymer concentration of 11 or 22 wt % and abbreviated as "8-arm low" and "8-arm high", respectively. In all cases, a 1:1 molar ratio between NHS and cysteine functionalities was used to obtain efficient cross-linking. A low concentration, linear PEG hydrogel formulation was not included in the degradation study, since the mechanical properties were too weak to allow the formation of a stable hydrogel. As expected from our rheology experiments (Figure 5), hydrogels containing 8-arm PEG cross-linkers and higher polymer concentration formed stronger hydrogels. Degradation studies of the PNC−PEGNHS hydrogels lacking DBA functionalities, showed slow degradation and even after 6 months the hydrogels were still not fully degraded into water-soluble products (Figure 10). Likely, the ester bonds are located in the hydrophobic domains of the hydrogel network, therefore limiting the accessibility of water molecules. Interestingly, "8-arm low" and "linear" did not show any increase in swelling during the degradation study, while "8-arm high" hydrogels swelled in the first couple of days to two times of their original weight (SI-Figure 5). The denser network of this hydrogel formulation was most likely not fully cross-linked when it was placed in buffer. Therefore, an increase in swelling was possible until thermodynamic equilibrium was reached. After 6 months, "linear", "8-arm low" and "8-arm high" had remaining gel weights of 29%, 12% and 13% respectively.

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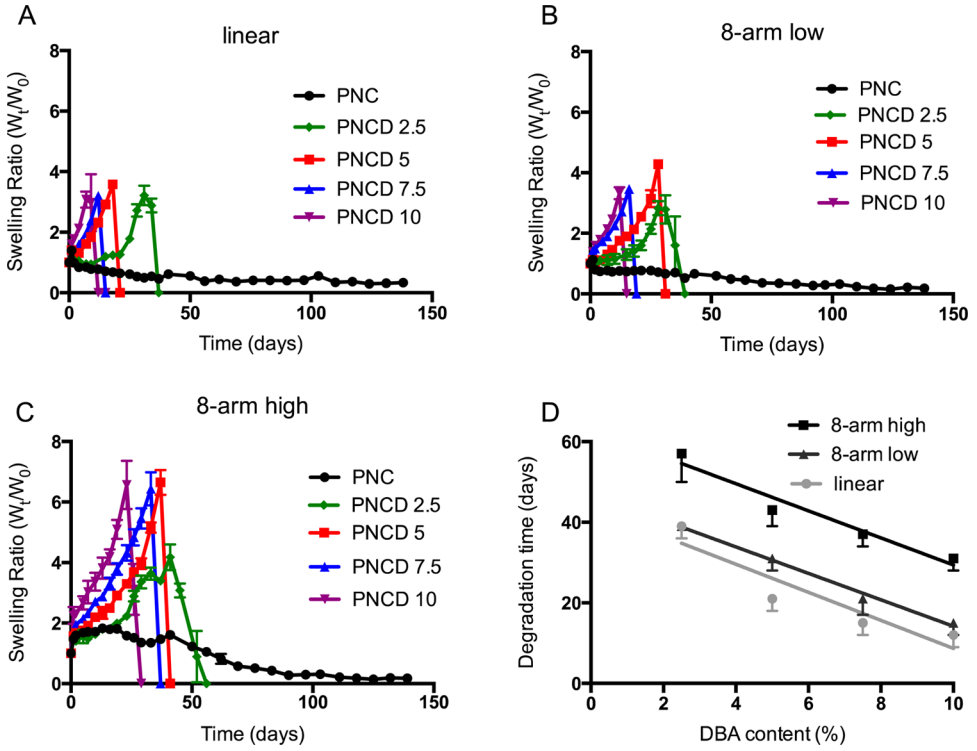

Figure 10. Swelling ratio (W_t/W_0) in time of three different hydrogel formulations with 0, 2.5, 5, 7.5 and 10% DBA content. (A) 15 wt % PNC(D) + 3 wt % PEG linear. (B) 7.5 wt % PNC(D) + 3.8 wt % PEG 8-arm. (C) 15 wt % PNC(D) + 7.5 wt % PEG 8-arm. Data are shown as mean ± standard deviation (n = 3). (D) Effect of DBA content on time until full degradation.

The highest remaining gel weight of "linear" was probably caused by the shorter PEG chains that are incorporated in the network, thereby reducing the overall hydrophilicity of the polymer network. A similar influence of the molecular weight of the PEG cross-links on the degradation rate has been previously reported by Zustiak et al.[57]

To increase the degradability of the hydrogels, PNCD polymers containing DBA moieties were mixed with PEG-NHS cross-linkers, using the concentrations as described above. As depicted in Figure 10, the degradation rate accelerated and decreased from more than 6 months for the formulations without DBA moieties, to only 12 to 57 days for formulations containing different amounts of DBA, which is in line with previous studies.[28,30] During hydrolysis, the lactone ring of DBA opens and yields at pH 7.4 networks with negatively charged carboxylate ions. Hence, the hydrophilicity of the polymer chain increases and the polymers exhibit a charge repulsion between the polymer chains in the hydrogel, which causes an increase in water uptake. As a consequence, the ester groups in the cross-links are more exposed to water and therefore more susceptible for hydrolysis. Indeed, during incubation the hydrogels swell up to 7 times of their original weight, after which they fully dissolve. As was expected from the rheology measurements, the hydrogels of PNCD−PEG 8-arm high were the most stable, as a result of their high cross-link density. We expect that an even larger range of degradation times could be obtained by further varying the DBA content. Figure 10D summarizes the degradation times of the different hydrogel formulations and shows a linear relationship between DBA content and time until full dissolution, thereby underlining the ability to control the degradation rates using these polymeric hydrogels.

Solutions after full degradation were lyophilized, redissolved in D_2O and measured by ^1H NMR to analyze the chemical nature of the soluble degradation products. The results showed the formation of a copolymer with completely hydrolyzed DBA lactone ring (SI-Figure 6), as has also been demonstrated in previous work.[28]

CONCLUSION

This study evaluated the mechanical properties, cytocompatibility, protein compatibility, and degradation kinetics of chemoselective and thermosensitive dual cross-linked hydrogels. After mixing two liquid precursor solutions, consisting of a thermoresponsive polymer and PEG cross-linker, physically cross-linked hydrogels were immediately formed at 37 °C. Mechanically stable hydrogels were obtained after chemoselective cross-linking of the two hydrogel components by native chemical ligation (NCL) or oxo-ester mediated native chemical ligation (OMNCL). The mechanical properties could be enhanced using OMNCL or 8-arm PEG cross-linkers. Additionally, degradation rates were tuned and controlled after incorporation of hydrolyzable DBA groups in the hydrogel network. Although the NCL cross-linked hydrogels showed cytotoxicity to endothelial cells, cell viability was not affected after incubation with OMNCL cross-linked hydrogels. The favorable and controllable properties of the studied OMNCL cross-linked hydrogels are attractive for further evaluation in biomedical applications.

ASSOCIATED CONTENT

Supporting Information

Storage and loss modulus of hydrogels (SI-Figure 1); live−dead overlay pictures (SI-Figure 2); microscopy pictures endothelial cells (SI-Figure 3); rheology time sweeps of hydrogels in

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degradation study (SI-Figure 4); swelling ratio of PNC−PEG hydrogels during first 10 days (SI-Figure 5); ^{1}H NMR before and after degradation (SI-Figure 6). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.5b00802.

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Notes

The authors declare no competing financial interest.

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