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In Situ Forming Hydrogels by Tandem Thermal Gelling and Michael Addition Reaction between Thermosensitive Triblock Copolymers and Thiolated Hyaluronan

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ABSTRACT: This study reports on the synthesis, characterization and peptide release behavior of an *in situ* physically and chemically cross-linking hydrogel. (Meth)acrylate bearing ABA-triblock copolymers consisting of a poly(ethylene glycol) (PEG) middle block, flanked by thermosensitive blocks of random *N*-isopropylacrylamide (pNIPAm)/*N*-(2-hydroxypropyl) methacrylamide dilactate (pHPMAm$_{lac2}$) and exhibiting lower critical solution temperature behavior in aqueous solution were synthesized. Upon body temperature induced physical gelation, these polymers were cured by Michael type addition reaction with thiolated hyaluronic acid (HA-SH) to yield injectable *in situ* gelling, biodegradable but structurally stable and biocompatible hydrogels. These stable and elastic networks were prepared by mixing (meth)acrylated ABA-triblock copolymers and thiolated hyaluronic acid at a ratio thiol/(meth)acrylate groups of 1/1. The simultaneous physical and chemical gelation kinetics, investigated by rheological measurements, demonstrated that the physical networks were progressively stabilized as the Michael addition reaction between (meth)acrylate and thiol groups proceeded and that acrylated thermosensitive polymers had a higher reactivity with thiol groups, as compared to methacrylate analogues, resulting in a faster gel formation. The networks, characterized by a remarkable initial structural stability, degraded in time at physiological conditions. The degradability is ensured by the presence of hydrolytically sensitive ester bonds in the cross-links, as well as in the lactate side chains and between PEG and thermosensitive blocks. Methacrylated polymer gels loaded with a model peptide (bradykinin), showed a diffusion controlled release of this peptide, tailorable by the polymer concentration. This tandem system, displaying *in situ* physical and chemical gelation has a high potential for biomedical applications, such as delivery of peptide and protein biopharmaceuticals.

Introduction

Most systemically administered pharmaceutical peptides and proteins undergo rapid elimination and cause high plasma peak concentrations, which often results in undesired side effects. In this study, a noninvasive and patient-friendly delivery system, capable to protect and release polypeptides in a sustained manner, is investigated. We designed an injectable hydrogel that exhibits a body temperature induced gelation at the site of injection, for which the structure is stabilized through a Michael addition reaction.

Injectable, self-assembling hydrogels are receiving increasing attention because of their desirable properties for drug delivery and tissue engineering applications. Their advantages over preformed chemically cross-linked polymer hydrogels are the use of minimally invasive methods for administration, *in vivo* shape adaptation, and ease of drug encapsulation.[1,2] Different physical interactions can be exploited for the design of *in situ* gelling systems, among which the use of thermosensitive polymers is of particular importance. Polymers exhibiting LCST (lower critical solution temperature) behavior are soluble in aqueous solutions at low temperature and self-assemble above their cloud point (CP), being suitable as injectable materials if their CP is between room and body temperature.[3,4] A general drawback of self-assembling hydrogels however is their limited mechanical strength and low stability due to swelling and subsequent dissolution of the polymers. To improve the gel strength and stability, the use of covalent cross-linking strategies,[5,6] among which photo-cross-linking, is well-known.[7−9] Recently, also click chemistry between azides and acetylenes is used to obtain well-defined network structures.[10−12] A drawback of these chemical cross-linking techniques is their need for catalysts, which can be toxic. Therefore, *in situ* gelation is usually not applicable for these kinds of hydrogels.

Michael addition reaction between thiol and acrylate or methacrylate groups[13,14] is a particularly suitable mechanism for *in situ* chemical cross-linking because it occurs at physiological conditions without the need for (toxic) catalysts.[15−18] Because of its advantageous properties, the Michael addition reaction has been successfully applied for the preparation of a number of hydrogels for biomedical applications,[17,19−25] as reviewed by Mather et al.[26]

Besides injectability and stability issues, also biocompatibility is an important property that should be taken into consideration for the design of hydrogels for biomedical purposes. A well-known strategy to ensure biocompatibility and cell adhesion and proliferation in the gels is the use of natural polymers such as polypeptides and polysaccharides. Hyaluronic acid (HA) is a natural polysaccharide, which can be found in synovial fluids, extracellular matrix, connective tissues and organs. HA is a linear

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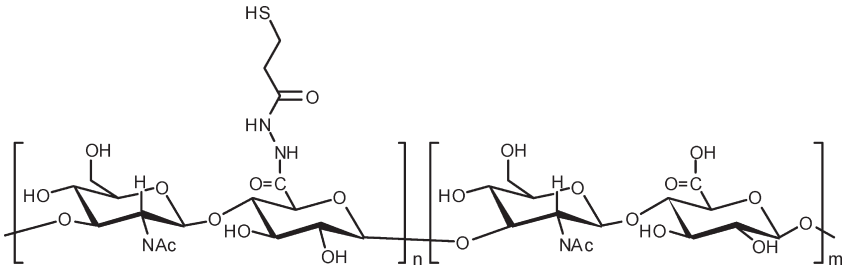
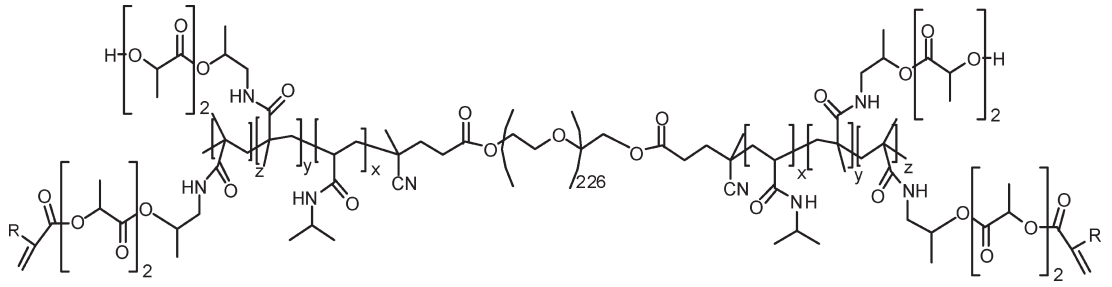
Scheme 1. Chemical Structure of Partially Thiolated Hyaluronic Acid (HA-SH)



Scheme 2. Chemical Structure of ABA-Triblock Copolymers Consisting of a PEG_{10000} B-Block and Random pNIPAm-$HPMAm_{lac2}$ A-Blocks (R = H or CH_3 for pNHPta and pNHPtma, Respectively)



polymer consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine moieties and is known for its favorable physical (e.g., viscosity, hydration) and biological (protein and cell interactions) properties.[27] In our approach, hyaluronic acid was derivatized with thiol moieties,[28−30] as shown in Scheme 1, and used as a curing agent for thermal gelling (meth)acrylate bearing polymer hydrogels.[30−33]

Cellesi et al. have described the simultaneous thermal gelling and Michael addition cross-linking of telechelic pluronic for the design of a synthetic substitute of alginate.[17] Tandem physically and chemically gelling systems based on poly(NIPAAm-*co*-cysteamine)[14] and on poly(NIPAAm-*co*-HEMA-acrylate)[34] and poly(NIPAAm-*co*-PEG-acrylate)[35] copolymers have been described by Vernon et al. More recently, Park et al. reported on injectable HA/Pluronic F127 composite tissue-adhesive hydrogels[31] and Wang et al. described the preparation of Michael addition cross-linked injectable thiol- and vinyl-modified poly(*N*-isopropylacrylamide) (PNIPAAm)-based copolymer hydrogels for controlled drug delivery.[36]

Recently, we applied a similar cross-linking approach to design a novel hydrogel composed of thermosensitive biodegradable polymers capable to cross-link via Michael addition reaction with thiolated hyaluronic acid. This hydrogel possesses an innovative combination of advantageous aspects, such as stability at the site of injection, due to the rapid thermal gelling upon injection, structural stability, due to Michael addition cross-linking, biocompatibility, and cell adhesion properties, due to the presence of hyaluronic acid, biodegradability and flexibility.

The thermosensitive polymers were designed with an ABA-triblock architecture consisting of a hydrophilic poly(ethylene glycol) (PEG) middle block and flanking thermosensitive blocks of random copolymer of *N*-isopropylacrylamide (NIPAm) and *N*-(2-hydroxypropyl) methacrylamide dilactate ($HPMAm_{lac2}$) in a NIPAm/$HPMAm_{lac2}$ ratio of 76/24, as shown in Scheme 2. pNIPAm is a nondegradable thermosensitive polymer with a cloud point of 32 °C.[37,38] When the hydrophobic $HPMAm_{lac2}$ (24 mol %) is copolymerized with NIPAm, the cloud point of the obtained copolymer decreases to 23 °C. Furthermore, the introduction of $HPMAm_{lac2}$ makes the polymer biodegradable, because under physiological conditions, the lactate side chains will be hydrolyzed in time, yielding a more hydrophilic polymer (random pNIPAm and poly(*N*-(2-hydroxypropyl) methacrylamide monolactate) with a cloud point above 37 °C.[39,40] Therefore, at body temperature the hydrolyzed polymers are expected to dissolve and can be excreted by renal filtration when the molecular weight is below the renal excretion treshold of 50 kDa.[41] To enable a Michael addition reaction between the triblock copolymers and the thiolated HA, acrylate and methacrylate groups were introduced in the side chains of the $HPMAm_{lac2}$ units.

In the present work, we describe the synthesis, characterization, and potential biomedical applications of this injectable *in situ* gelling, initially structurally stable but biodegradable hydrogel, prepared by tandem physical gelation and chemical stabilization of the themal gelling network by Michael addition cross-linking with thiolated hyaluronic acid.

Experimental Section

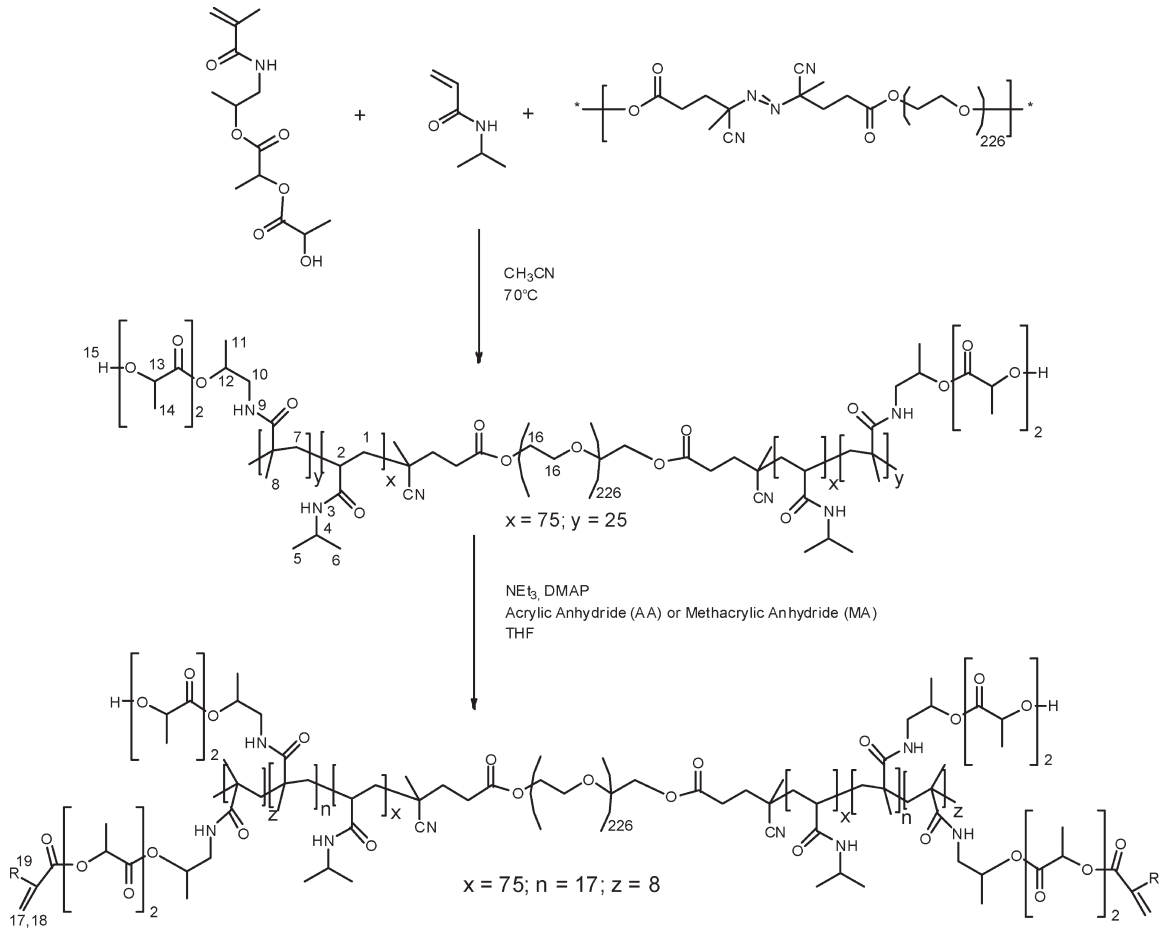
Materials. All commercial chemicals were obtained from Aldrich unless indicated otherwise and were used as received. L-Lactide was obtained from Purac Biochem BV (Gorinchem, The Netherlands). Low molecular weight hyaluronic acid (sodium hyaluronate) with a molecular weight of 35000 Da was supplied by Lifecore (Chaska, MN) Hydroxypropylmethacrylamide-dilactate (HPMAm-dilactate) was synthesized according to a previously reported method.[42] 3,3′-Dithiobis(propanoic dihydrazide) (DTP) was synthesized by the method described by Vercruysse et al.[43]

^{1}H NMR Spectroscopy. Polymers dissolved in $CDCl_3$, DMSO-d_6 or D_2O were characterized on a Varian Mercury Plus 300 spectrometer. Chemical shifts were referred to the solvent peak (δ = 7.24 ppm for $CHCl_3$, 2.54 ppm for DMSO and 4.79 ppm for H_2O).

Gel Permeation Chromatography (GPC). The molecular weights of the polymers were determined by GPC using a Plgel 5 μm MIXED-D column (Polymer Laboratories) with a column temperature of 40 °C. DMF containing 10 mM LiCl was used as eluent with an elution rate of 0.7 mL/min, and the sample concentration was 5 mg/mL in the same eluent. Poly(ethylene glycols) with defined molecular weights were used as calibration standards.

Synthesis of Thermosensitive Triblock Copolymers. Thermosensitive ABA triblock polymers were prepared according to a previously described procedure (Scheme 3).[42] 4,4′-azobis(4-cyanopentanoic acid) (ABCPA, Fluka) was used to prepare a

Scheme 3. Synthesis Route of (Meth)acrylate Bearing ABA-Triblock Copolymers Consisting of a PEG_{10000} B-Block and Random pNIPAm-$HPMAm_{lac2}$ A-Blocks (R = H or CH_3 for pNHPta and pNHPtma, Respectively)



PEG_{10000}-ABCPA macroinitiator with PEG molecular weight of 10000. This initiator was used to copolymerize $HPMAm_{lac2}$ (25%) and NIPAm (75%) to obtain triblock copolymers with pHPMAmlac$_2$/NIPAm as outer blocks and PEG as midblock.

The OH side groups of $HPMAm_{lac2}$ were partially methacrylated or acrylated by the following procedure.[39] Triblock copolymer (1 g) was dissolved in 100 mL of dry THF under N_2 atmosphere. Dimethylaminopyridine (3 mg) and triethylamine (90 μL) were added at 0 °C. Methacrylic anhydride or acrylic anhydride was added in an anhydride/OH molar ratio of 0.5. The reaction mixture was subsequently stirred for 24 h at room temperature. Afterward, the polymers were diluted with water, dialyzed (membrane with a cutoff of 12−14 kDa) against water for 2 days and isolated by freeze-drying. The final thermosensitive triblock copolymers are abbreviated as pNHPtma (*p*oly*N*IPAm/*H*PMAm_{lac2}-*P*EG *t*riblock *m*eth*a*crylated) and pNHPta (*p*oly*N*-IPAm/*H*PMAm_{lac2}-*P*EG *t*riblock *a*crylated), respectively.

Before (*Meth*)*acrylation*. ^{1}H NMR ($CDCl_3$) δ 6.5 (H3, H9), 5.2−4.8 (H13), 4.45−4.25 (H12), 4.05−3.8 (H4), 3.7−3.5 (H16), 2.6−0.8 (other protons). The monomer ratio (mol/mol) in the block polymer was determined from the ratio of the integral of H4 of NIPAm/H12 of $HPMAm_{lac2}$. The M_n of the thermosensitive blocks was calculated by comparison of the NIPAm and $HPMAm_{lac2}$ integrals with the integral of PEG protons. The obtained ratio of NIPAm/$HPMAm_{lac2}$ was 74/26. The total mass of the thermosensitive blocks was 27 kDa (M_n of A−B−A triblock polymer = 13.5−10−13.5 kDa).

Cloud point: 23.1 °C for polymer dissolved in 120 mM ammonium acetate buffer pH 5 at a polymer concentration of 2 mg/mL by static light scattering at a wavelength of 650 nm. The cloud point was defined as the onset of increased scattering intensity.

After Methacrylation. ^{1}H NMR (DMSO-d_6): δ 7.35 (H3, H9), 6.15 and 5.80 (H17, H18), 5.4 (H15), 4.95 (H13), 4.2 (H13 next to OH), 3.8 (H4), 3.60 (H16), 3.3−0.6 (other protons). The degree of methacrylation (DM) was calculated from the ratio of the average intensity of the peaks at 6.15 and 5.80 and intensity of the peak at 5.4 ppm as follows: $((I_{6.15} + I_{5.8})/2)/((I_{6.15} + I_{5.8})/2 + I_{5.4}) \times 100\%$. The degree of methacrylation, defined as the percentage of OH groups derivatized by methacrylate moieties, was 28%.

GPC: M_w = 46 kDa; M_n = 22 kDa; PDI = 2.14.

Cloud point: 18.8 °C.

After Acrylation. ^{1}H NMR (DMSO-d_6) δ 7.35 (H3, H9), 6.2, 6.1, 6.0 (H17, H18, H19), 5.4 (H15), 4.95 (H13), 4.2 (H13 next to OH), 3.8 (H4), 3.60 (H16), 3.3−0.6 (other protons). The degree of acrylation (DA) was calculated from the ratio of the average intensity of the peaks at 6.2, 6.1, and 6.0 and intensity of the peak at 5.4 ppm as follows: $((I_{6.2} + I_{6.1} + I_{6.0})/3)/((I_{6.2} + I_{6.1} + I_{6.0})/3 + I_{5.4}) \times 100\%$. The degree of acrylation, defined as the percentage of OH groups derivatized by acrylate moieties, was 32%.

GPC: M_w = 57 kDa; M_n = 23 kDa; PDI = 2.5.

Cloud point: 16.0 °C.

Synthesis of Thiolated Hyaluronic Acid (HA-SH). Thiolated hyaluronic acid was synthesized according to the procedure described by Shu et al.[28] Briefly, 0.5 g of sodium hyaluronate (M_n = 37.9 kDa, PDI = 1.27, measured by GPC) was dissolved in water and 482 mg (1.70 mmol) of DTP was added while stirring. The pH was adjusted to 4.75 with 1 M HCl and subsequently 388 mg of 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC, 2.02 mmol) was added while keeping the pH at 4.75. The solution was stirred at room temperature for 48 h and the reaction was stopped by increasing the pH to pH 7 with 1 M NaOH. Then, 2.0 g of dithiothreitol (DTT) was added

and the pH was raised to 8.5. The reaction mixture was stirred for another 24 h and subsequently acidified to pH 3.5 with 1 M HCl. The mixture was purified by dialysis (M_w cutoff = 3500 Da) against dilute HCl (pH 3.5) containing 100 mM NaCl and finally against water at 4 °C. The final product was obtained as a white powder after lyophilization. The degree of substitution (DS), defined as the number of DTP residues per 100 disaccharide units, was determined by ^{1}H NMR and detection of free thiols by Ellman's method.

^{1}H NMR (D_2O): δ 4.6−3.3 protons of hyaluronic acid), 2.85 (CH_2−SH), 2.70 (CH_2CH_2SH), 2.00 ($NHCOCH_3$). DS: 54%.

Thiol content, defined as percentage of disaccharide units of HA derivatized with free thiol groups determined by Ellman's method: 50%, These two different methods showed good agreement between the DS values, within experimental error. The DS is indicated as a mean value of 52%.

GPC: M_n = 39.8 kDa; PDI = 1.26.

Viscotek. A viscotek GPC was used for characterization of hyaluronic acid and thiolated hyaluronic acid. A TDA 302 with triple detector array, column, grade GMPWxL no.H3371, and detector, GPC max VE 2001 GPC solvent sample module were used. As a standard, PEO with a concentration of 5 mg/mL was used and also the samples had a concentration of 5 mg/mL. The eluent was ammonium acetate buffer pH 5 (concentration 120 mM).

Preparation of the Placebo and Bradykinin Loaded Hydrogels. Gels of a volume of 200 μL were prepared in cylindrical shaped glass vials (diameter of 5 mm) as follows. pNHPtma or pNHPta was dissolved in 100 μL of PBS buffer pH 7.4 (8.2 g/L NaCl; 3.1 g/L NaH_2PO_4 12 H_2O; 0.3 g/L NaH_2PO_4, supplemented with 0.02% NaN_3). The samples were gently mixed using a needle and stored at 4 °C for 1 h to allow the complete dissolution of the polymer. Separately, HA-SH was dissolved in 100 μL of the same buffer at room temperature for 1 h, mixed using a needle. Upon complete dissolution, the HA-SH solution was mixed with the pNHPtma or pNHPta solutions at room temperature. The final concentration of the polymers was 20 and 5.4 wt % for pNHPt(m)a and HA-SH, respectively. The aforementioned concentrations corresponded to a ratio between thiol and (meth)acrylate groups of 1/1. Upon homogeneous mixing of the two polymer solutions, the samples were incubated at 37 °C for 1 h, unless indicated otherwise.

BK loaded hydrogels were prepared according to a slightly different procedure. PNHPtma was dissolved in 60 μL of PBS buffer at 4 °C, next 40 μL of a peptide solution (100 mg/mL) was added to the pNHPtma or pNHPta solution prior addition of the HA-SH solution. The peptide concentration was 2 wt %. The final polymer concentration was 20 and 5.4 wt % or 9 and 2.4 wt %, for pNHPtma and HA-SH, respectively, equal to a thiol/(meth)acrylate ratio of 1/1. Upon mixing the (meth)acrylated and thiolated polymer solutions, the gels were incubated at 37 °C for 1 h to allow the Michael addition reaction.

Rheology. An AR-G2 rheometer equipped with a Peltier plate. A 20 mm 1° steel cone−plate geometry was used for both the temperature- and the time-sweep rheological experiments. A frequency of 1 Hz and a strain of 1% was used in all experiments. The temperature-sweep ranged from 5 °C until 45 °C with a heating or cooling rate of 1 °C/min and the time-sweeps were performed during 4 h at 37 °C. For both time- and temperature-sweep experiments a solvent trap was used to prevent evaporation.

Swelling and Degradation Studies. A 0.9 mL aliquot of PBS buffer at pH 7.4 containing 0.02% NaN_3 was added on top of empty gels (W_0), prepared according to the described procedure and incubated at 37 °C for 1 h before starting the swelling experiments. At regular intervals the weight of the gel was measured (W_t) upon removal of the excess of buffer to calculate the swelling ratio (SR = W_t/W_0), defined as the ratio between the weight of the gel at different time points and the initial gel weight (W_0). After each measurement 0.9 mL of fresh buffer was added and the vials stored again in the water bath at 37 °C.

(Meth)acrylate Conversion Measurements. The gels, prepared as described above, were incubated at 37 °C for 0.5, 1, 4, 24, and 50 h and subsequently degraded in 4 mL of NaOH 0.02 M at 37 °C for 30 min. Upon complete degradation of the gel, the solution was neutralized by adding 0.5 mL of acetic acid 2 M. Degraded pNHPt(m)a was used as control. For quantification and detection of methacrylic acid a Waters Acquity UPLC trade system was used with a BEH C18 1.7 μm, 2.1 × 50 mm column, equipped with a UV detector operating at 210 nm. The eluent used was 95/5/0.1% H_2O/acetonitrile/trifluoroacetic acid (TFAA).[5,9] Acrylic acid was quantified using the above-mentioned UPLC system equipped with a HSS T3 1.8 μm, 2.1 × 50 mm column. The UV detection was done at 210 nm and H_2O acidified with 0.1% TFAA was used as eluent. For both acrylic and methacrylic acid a flow rate of 0.5 mL/min and a column temperature of 50 °C were used.

Bradykinin Release Studies. *In vitro* peptide release from Michael type cross-linked gels was studied using bradykinin (M_w = 1.1 kDa) as a model peptide. Gels were prepared according to the described procedure and incubated at 37 °C for 1 h before starting the release experiments. Next, 0.9 mL of PBS buffer pH 7.4 was applied on top of the gels and the vials were incubated in a shaking water bath at 37 °C. Samples of 0.15 mL were taken in time and replaced by an equal volume of fresh buffer. The concentration of bradykinin in the different samples was determined by using an Acquity UPLC trade with a BEH C18 1.7 μm, 2.1 × 50 mm column. An eluent gradient, from 30 to 70% of eluent A was used, where eluent A was 95/5/0.1% H_2O/acetonitrile/TFAA and eluent B was 100/0.1% acetonitrile/TFAA. The injection volumes of the samples were 5 μL, the flow rate was 0.25 mL/min, and detection was performed at 280 nm.

The release mechanism was studied by fitting the experimental release data to the Ritger−Peppas equation:[44,45]

$$M^t/M^\infty = kt^n$$

M^t/M^∞ represents the fractional release of the loaded peptide, k is a kinetic constant, t is the release time, and n is the diffusional exponent that can be related to the release mechanism of the entrapped molecules. If n = 0.5, the release is governed by Fickian diffusion. If n = 1, the molecules are released by surface erosion, while both mechanisms play a role in the release if n has a value between 0.5 and 1.

The peptide diffusion coefficients were calculated by the early time approximation equation of Fick's second law:[44]

$$M^t/M^\infty = 4(Dt/\pi\delta^2)^{1/2}$$

where M^t/M^∞ represents the fractional release of the entrapped peptide, D is the diffusion coefficient, t is the release time and δ is the diffusional distance, equal to the thickness of the gel.

Results and Discussion

Gelation Kinetics and Rheological Properties. pNHPt(m)a triblock copolymers showed a degree of (meth)acrylation (DM or DA), defined as the percentage of OH-groups that were derivatized in the polymer, of 28% and 32%, respectively, as determined by ^{1}H NMR. The (meth)acrylation rendered the polymers more hydrophobic and the cloud point dropped from 23 °C before (meth)acrylation to 19 and 16 °C for methacrylated and acrylated polymers, respectively.

HA-SH had a degree of thiolation of 52%, defined as percentage of disaccharide units of HA derivatized with free thiol groups, as determined by ^{1}H NMR.

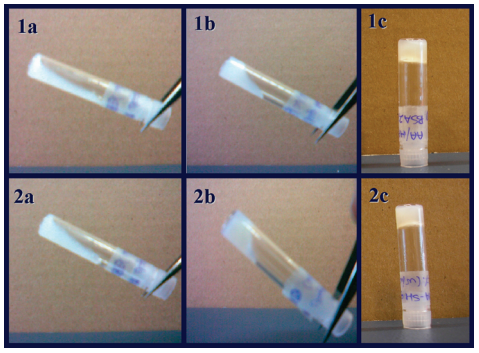


Figure 1. Pictures of polymer solutions/gels (polymer concentration: 20 wt % pNHPt(m)a, 5.4 wt % HA-SH; ratio thiol/(meth)acrylate groups = 1/1) during Michael addition reaction at 37 °C for 0.5 h (**a**), 1 h (**b**), and 4 h (**c**) for HA-SH and pNHPtma (**1**) and pNHPta (**2**), respectively.

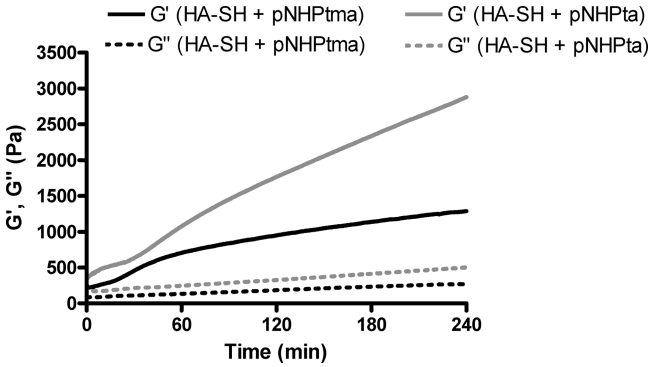


Figure 2. Storage (G') and loss (G'') moduli at 37 °C as a function of time directly after mixing of HA-SH with pNHPtma and pNHPta, respectively. Polymer concentration: 20 wt % pNHPtma or pNHPta +5.4 wt % HA-SH.

The hydrogels were prepared by mixing a pNHPtma or pNHPta solution in PBS buffer pH 7.4 with a HA-SH solution in the same buffer at 37 °C. The concentration was 20 wt % and 5.4 wt % for pNHPt(m)a and HA-SH, respectively corresponding to a molar ratio thiol/(meth)-acrylate groups of 1/1. Figure 1 shows photographs of pNHPtma (**1**) and pNHPta (**2**) mixed with HA-SH incubated at 37 °C at different time points. The progressive conversion of (meth)acrylate and thiol groups into chemical cross-links by Michael addition reaction resulted in a notable increase in viscosity. After 0.5 h (**1a**, **2a**) white opalescent viscous solutions were obtained; after 1 h (**1b**, **2b**) a further increase in viscosity was observed and within 4 h (**1c**, **2c**) stable hydrogels were formed. As expected, acrylated gels (**2a**−**c**) showed slightly faster gelation kinetics than the methacrylated systems (**1a**−**c**).

Rheological characterization of the mixed system was performed at 37 °C as shown in Figure 2. Already at time = 0, the formation of a physical network was observed in both the acrylate and methacrylate modified polymer formulations. This behavior is ascribed to the thermosensitive properties of pNHPt(m)a polymers, that self-assemble at body temperature by hydrophobic interaction of the pNIPAm/pHPMAm$_{lac2}$ chains. The formation of a self-assembled hydrogel upon injection is particularly beneficial for its intended biomedical application, as it would give immediate stability of the network upon administration. In time, a gradual increase of storage and loss moduli (G' and G'', respectively) was observed, confirming that the structure of the gels was progressively stabilized by the formation of chemical cross-links.

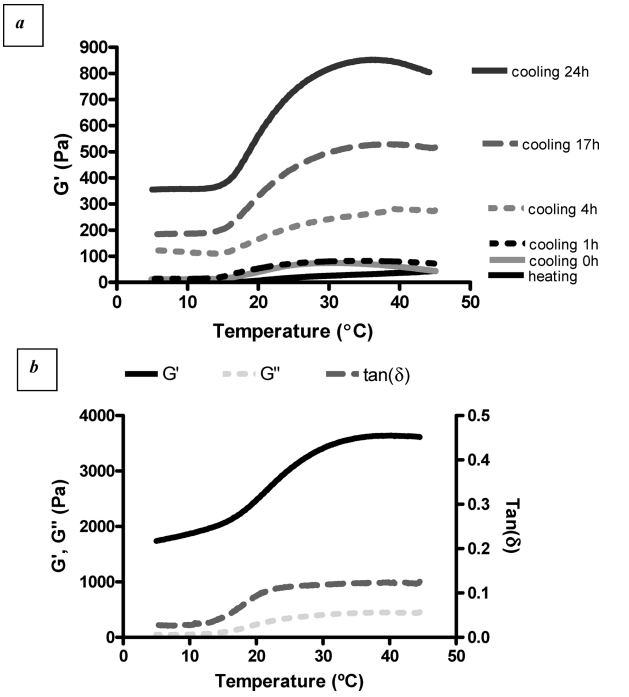


Figure 3. (a) Storage modulus(G') of HA-SH + pNHPtma gels during heating cycles (heating rate 1 °C/min) directly after mixing of HA-SH with pNHPtma and during cooling cycles after Michael addition reaction for 0, 1, 4, 17, and 24 h. (b) Tan δ, storage (G') and loss (G'') moduli of HA-SH + pNHPtma gels during a cooling cycle upon quantitative conversion of methacrylate groups. (Polymer concentration: 20 wt % pNHPtma +5.4 wt % HA-SH).

The acrylated system displayed higher values of G' and G'' as compared to the methacrylated one, indicating the formation of a gel of higher cross-link density after 4 h. This observation is likely due to the faster reaction kinetics of acrylate groups with thiol groups, as compared to the methacrylate modified polymers.

Temperature sweep analysis of HA-SH + pNHPtma gels during the Michael addition curing were performed. Figure 3a shows that upon mixing of HA-SH with pNHPtma and increase of the temperature from 4 to 45 °C, a slight and reversible increase of G' was observed. When the temperature is higher than the cloud point of the polymer, the thermosensitive chains self-assemble, leading to the formation of hydrophobic domains.[46] At increasing incubation time at 37 °C, higher values of G' were reached, as the Michael addition reaction occurred between methacrylate and thiol groups within the hydrophobic domains, resulting in a more stable and irreversible network. The G' values during the cooling cycles at different time points demonstrated the formation of the covalent cross-links, as at temperatures below the cloud point viscoelastic networks were still present and increasing values of G' at 5 °C were observed at increasing curing time. It was further shown that thermal and chemical gelation mechanisms act synergistically to enhance the mechanical properties of the gels.

After 50 h the hydrogels displayed remarkable values of G' (3.6 kPa) and tan(δ) values below 0.2 at 37 °C, as shown in Figure 3b, and no further increase of the storage modulus in time was observed, indicating that full conversion of methacrylate and thiol groups was achieved.

The hydrogel methacrylate conversion was further investigated by the indirect method of the quantification of the unreacted methacrylate groups and compared to the acrylate conversion in order to gain insight into the gelation kinetics.

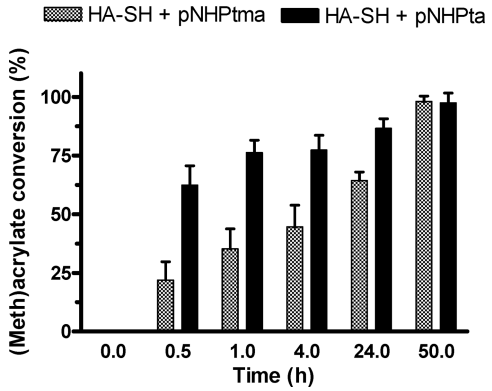


Figure 4. (Meth)acrylate conversion in time (polymer concentration: 20 wt % pNHPt(m)a + 5.4 wt % HA-SH; ratio thiol/(meth)acrylate groups = 1/1). Data are shown as mean ± standard deviation (n = 3).

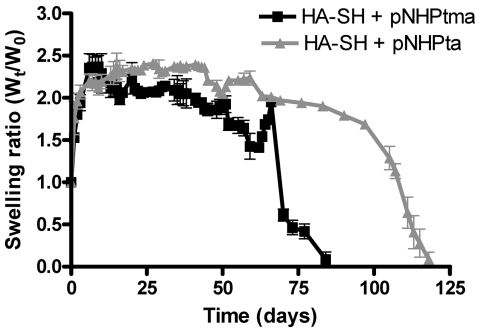


Figure 5. Swelling ratio (= gel weight at time t (W_t)/ initial gel weight (W_0)) of polymer gels (polymer concentration: 20 wt % pNHPt(m)a + 5.4 wt % HA-SH; ratio thiol/(meth)acrylate groups = 1/1) for HA-SH and pNHPt(m)a, respectively. Data are shown as mean ± standard deviation (n = 2).

Figure 4 shows that the (meth)acrylate groups were converted in time, until reaching quantitative conversion after 50 h incubation. This observation implies that the Michael addition reaction was the only cross-linking pathway involved in the gelation process, as the ratio between thiols and (meth)acrylates is 1:1, and the formation of disulfide bonds due to auto-oxidation of thiols was negligible. The Michael addition was confirmed to be the only cross-linking pathway by rheological time-sweep experiments of HA-SH solutions 5.4 wt % at 37 °C, that showed no increase in G' values in time, indicating negligible disulfide bond formation (Supporting Information, Figure 1SI). The acrylate groups showed higher conversion into chemical cross-links by Michael addition as compared to the methacrylates at corresponding time-points. For instance, a conversion of approximately 60 and 20% after 0.5 h was shown by acrylated and methacrylated polymers, respectively.

(Meth)acrylate conversion experiments further showed that the Michael addition followed relatively slow kinetics, in particular in case of methacrylate derivatized hydrogels. The slow kinetics are most likely caused by the limited accessibility of the (meth)acrylate moieties for Michael addition reaction with thiol derivatized hydrophilic hyaluronic acid, as the methacrylate groups are situated in phase-separated hydrophobic domains of thermosensitive blocks at 37 °C.[47] At 37 °C, indeed, the thermosensitive pNIPAm/pHPMAm$_{lac2}$ chains are in a dynamic equilibrium between the relaxed and the self-assembled state. The difference in cross-linking rate between acrylates and methacrylates can be attributed to the higher reactivity of acrylate groups toward Michael addition reaction with thiols due to less steric hindrance as compared to methacrylates as well as to the less hydrophobic character of acrylate groups that makes the accessibility of HA-SH easier.

Swelling and Degradation of Hydrogels. The swelling and degradation kinetics of hydrogels at 37 °C (Figure 5) show that during incubation with PBS buffer pH 7.4 the pNHPtma/HA-SH and pNHPta/HA-SH gels absorbed water, resulting in swelling ratio (SR) values up to 2.5 in approximately 6 and 15 days and subsequently the gels degraded completely in 84 and 118 days, respectively. When the lactate groups are cleaved, the polymer becomes more hydrophilic and consequently will absorb more water, resulting in an increased swelling ratio during the first days. The degradation of the hydrogels is caused by the hydrolysis of the esters between PEG and thermo blocks as well as between lactate and methacrylate groups.[39,42,46]

Rheological data (Figure 2), showing that the acrylate gels had higher cross-link density than the methacrylate gels, explain the slower degradation of the former gels. The high reactivity of the acrylate groups toward thiol groups might represent a drawback with respect to the biomedical applications these hydrogels are designed for. In the presence of cells or proteins, acrylate moieties might be more reactive toward thiols or amines present in cysteine or lysine residues of proteins, peptides encapsulated in the hydrogels network or present on the cell membranes, leading to cytotoxicity and/or chemical immobilization of the active in the gel. Further, acrylic monomers have been reported to be more toxic than their methacrylic analogues.[48,49] It has also been shown that binding of proteins, such as BSA, to hydrogel precursors through Michael addition reaction might occur.[50] Therefore, further characterization studies were focused on methacrylated gels, which are envisioned to show a better cytocompatibility and to be more protein and peptide friendly.

Peptide Release Behavior of Hydrogels. The suitability of the HA-SH/pNHPtma gels for the delivery of peptides was studied. To this end, bradykinin (BK) was selected as a model peptide since its molecular weight (1.1 kDa) is representative for a wide range of therapeutic peptides. A continuous release of the peptide from gels of 9 and 20 wt % pNHPtma was observed (Figure 6a). BK was quantitatively released from hydrogels of 9 wt % polymer concentration in 120 h, while the hydrogel of higher polymer content showed slower release kinetics with a cumulative release of approximately 77% in 220 h. The incomplete recovery of bradykinin from the hydrogel of higher polymer concentration can be explained by the higher density of hydrophobic domains formed by self-assembled pNIPAm/pHPMAm$_{lac2}$ at 20 wt % initial solid content. When the density of the hydrophobic domains is high, the peptide will partition both into hydrophobic and hydrophilic domains.[47] The release of the peptide from the hydrophobic compartments probably requires significant degradation of the network, that occurs on a longer time scale (see swelling/degradation studies) than the one considered in the release experiments. Because of the progressive dilution of the release medium during the experiment, quantification of the peptide was impossible after 500 h because its concentration reached the detection limit of the analytical technique used. From the release profiles, it can be concluded that the polymer concentration tailors not only the cross-linking density and pore size of the network but consequently also the release kinetics of the peptide.

Figure 6b shows that the cumulative release scaled linearly with the square root of time, up to a cumulative release of 83 and 75%, for pNHPtma and pNHPta, respectively. This first order kinetics means that the release is diffusion governed and implies that the pores in the hydrogel network are bigger

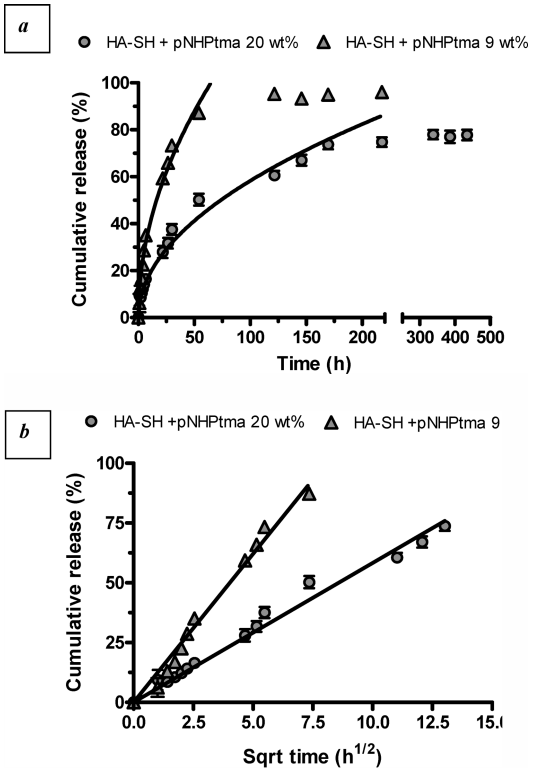


Figure 6. Bradykinin cumulative release from pNHPtma/HA-SH gels of 20 and 9 wt % pNHPtma after Michael addition reaction for 1 h. Release kinetics are shown as a function of time (a) and as a function of the square root of time (b). Data are shown as mean ± standard deviation (n = 3)..

than the hydrodynamic diameter of the peptide.[9,44,45] BK diffusion coefficients, calculated by the early time approximation equation of Fick's second law, were 22.7 ± 1.5 and 11.2 ± 1.9 $\mu m^2/s$ for gels of 9 and 20 wt % pNHPtma content, respectively. These values correspond to a 8 to 16-fold decrease in peptide mobility when compared to the diffusion coefficient of BK in water at 37 °C, as extrapolated from diffusion coefficients in water of other proteins[51,52] and demonstrated the suitability of the studied hydrogel as diffusion controlled delivery system.

Concluding Remarks

In conclusion, we have shown that the combination of thermosensitive gelling and Michael addition cross-linking is an attractive approach for the preparation of injectable, mechanically stable and biodegradable hydrogels with favorable properties for the delivery of peptides.

Acrylated thermosensitive polymers showed higher reactivity toward thiol groups, yielding gels with faster gel formation kinetics and higher mechanical strength. The degradability of the system is ensured by the presence of hydrolytically sensitive ester bonds within the thermosensitive polymer and enzymatically degradable bonds of the hyaluronic acid. The suitability of the methacrylated gels as diffusion controlled delivery system for peptides was demonstrated and the tailorability of the release kinetics by the gel characteristics was assessed.

Supporting Information Available: Figures showing and text discussing rheological data of HA-SH and pNHPtma separately as well as elastic moduli of HA-SH/pNHPT(m)a gels. This material is available free of charge via the Internet at http://pubs.acs.org.

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