Thermal Response of a PVCL–HA Conjugate

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ABSTRACT: The synthesis and self-assembling of a thermoresponsive conjugate of hyaluronic acid (HA) and poly(*N*-vinylcaprolactam) (PVCL) is reported. Both polymers were end functionalized: HA via reductive amination, thereby introducing an azide endgroup to the chain end, and PVCL via thioetherification to introduce a propargyl group. The two were coupled with a copper assisted "click" reaction into a bioconjugate composed of HA blocks with the molar mass 3,600 g mol^{-1} (1618 saccharide units) and PVCL blocks of 3,500 g mol^{-1} (~25 repeating units). The cloud point temperature measured by transmittance was 50–51 °C in water. The calorimetrically observed phase transition temperature of PVCL in the conjugate increased by 2 °C to 47.7 °C, whereas the enthalpy of the phase transition was unaffected by the conjugation. HA-PVCL conjugate self-assembles in water upon heating into monodisperse, colloidally stable, hollow spherical particles whose size may be tuned with the heating rate of the solution. Slow and fast heating resulted in vesicles with the hydrodynamic radii of 443 or 275 nm, respectively. The heating rate did not, however, affect the cloud point. Salt did not noticeably affect the size of the polymer particles, presumably because of interactions between the HA and PVCL blocks. © 2015 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2016**, *54*, 425–436

KEYWORDS: click chemistry; hyaluronic acid; poly(N-vinylcaprolactam); thermoresponsive; vesicle

INTRODUCTION Hyaluronic acid (HA) is an attractive candidate for targeting active substances to certain sites in the human body. Combination of HA with synthetic, biocompatible, and thermoresponsive poly(*N*-vinylcaprolactam) (PVCL) is of interest for applications as drug delivery. HA is ubiquitous in the human body, and its turnover ranges from hours to several days.[1] In the extra cellular matrix, several proteoglycans can be found that bind HA,[1] and HA plays an important role in stabilizing cartilage tissue.[1] The binding of HA to cell surface receptors can initiate endocytosis, catabolism, cellular proliferation, the binding of cells to other cells, the formation of specialized cell matrix structures, and can also influence cell motility.[1,2] Targeted conjugates of HA and hydrophobic drugs, such as paclitaxel, have been prepared, and it has been shown that these bioconjugates exhibit more prominent cell toxicity toward cancer cells than the free drug.[2] This is due to over expression of HA receptors in some cancer cells.

When constructing bioconjugates, polysaccharides may be functionalized by esterification or etherification of the hydroxyl or carboxylic acid groups present in the saccharide units. "Click" reactions, such as copper-assisted alkyneazide coupling[3–5] and thiol-ene chemistry,[6,7] have been utilized in conjugations. Such bioconjugates have brush-like structures or are cross-linked, however it is possible to prepare linear diblock copolymers via coupling reactions.[8,9] In the present case, reductive amination is an option that is selective to the reducing end of the carbohydrate chain. This reaction relies on the equilibrium between the hemiacetal and free aldehyde form of carbohydrates. The free aldehyde reacts with an added amine and the produced imine is quickly reduced into a more stable amine by a reducing agent, cyanoborohydride.[8,10,11] Oxime formation is another route in preparing diblock copolymers of polysaccharides, in which the aldehyde of the reducing end of the polysaccharide reacts with an aminooxy group.[12,13]

Additional Supporting Information may be found in the online version of this article.

Currently, HA is primarily produced by fermentation, however, the molecular weight is usually very high, up to millions of grams per mole.[14] Enzymatic degradation and fractionation may be used in order to produce shorter and fairly monodisperse fragments of HA, suitable for preparation of diblock conjugates.[9,11] Thermoresponsive conjugates of hyaluronic acid have been prepared by conjugating HA with poly(*N*-isopropylacrylamide) (PNIPAM),[5,7,15–17] poly(2-ethyl-2-oxazoline),[9] and poly(ethylene glycol) methyl ether methacrylate-co-2-(2-methoxyethoxy) ethyl methacrylate-co-poly(ethylene glycol) diacrylate (PEGMEMA–MEO_2MA–PEGDA).[7] These bioconjugates include brush-like copolymers with HA as the backbone,[5,15–17] cross-linked HA,[7] and linear diblock copolymers.[9] Linear diblock copolymers of HA have also been prepared with poly(ethylene glycol)[13,18] and poly(-lactic acid).[19]

Poly(*N*-vinylcaprolactam) is a neutral thermoresponsive polymer with a cloud point temperature of around 32 °C, and is regarded as a biocompatible one. Upon the hydrolysis of the amide side group, no amines are released, contrary to PNIPAM.[20] However, both PVCL and PNIPAM have been found to be well tolerated by cells at concentrations below 10 mg mL^{-1} and at temperatures below the cloud points of the polymers. Increasing the temperature to body temperature increased the cell toxicity of both polymers.[21] PVCL exhibits stronger interaction with macrophages both below and above the cloud point than PNIPAM.[22] In another study, bioconjugate beads of chitosan and PVCL were found to show a slow and steady release of drug molecules but also to be nontoxic to human endothelial cells.[23]

Several studies have shown that the thermoresponsiveness of PVCL is affected by diblock formation. It has been shown that the phase transition of PVCL is shifted to higher temperatures in diblock copolymers with polyethylene oxide (PEO),[24] poly(*N*-vinyl pyrrolidone); PVP[25,26], polyvinyl alcohol (PVOH)[27], or poly(dimethylaminoethylmethacrylate); PDMAEMA[28] as the other block. Double thermoresponsive diblock copolymers of PVCL have been prepared with PDMAEMA[28] and PNIPAM.[29] In the case of PDMAEMA, one or two phase transitions were observed depending on the pH of the solution. Lowering the pH of the solution increased the charge of the PDMAEMA block and thus the interactions between the PVCL and PDMAEMA were affected, but more importantly, the phase transitions were shifted far enough from each other as to be separately visible. In the case of PVCL-PNIPAM, as is also the case with a random copolymer of the two, only one transition was observed.[29] Cobalt-mediated polymerization has been used in preparing block copolymers of PVCL with poly(vinyl acetate); PVAc, which was later hydrolyzed to PVOH.[27] The PVCL-PVAc block copolymers form micelles at room temperature in aqueous solutions, due to their amphiphilic nature, and show only a slight change in size at elevated temperatures. Whereas the hydrophilic PVCL-PVOH produced large micelles at temperatures above the T_{CP} of PVCL. Hydrolysis of the hydrophobic PVAc to hydrophilic PVOH, increased the T_{CP} from 27 to 36–42 °C.[27] Using the same approach, di- and tri-block copolymers of PVCL and random copolymers consisting of *N*-vinylcaprolactam and *N*-vinylpyrrolidone have been reported, exhibiting two cloud points in aqueous solutions.[26]

In random copolymers of PVCL, with vinylacetate, *N*-methyl-*N*-vinylacetamide, vinyl pivalate, and *N*-vinylacetamide, the nature of the comonomer greatly affects the T_{CP} of the copolymer. Vinylacetate and *N*-methyl-*N*-vinylacetamide both increase the T_{CP} with increasing the fraction of the comonomer. Contrary to the more hydrophobic vinyl pivalate and *N*-vinylacetamide, which both decrease the T_{CP} with increasing fraction.[30]

Self-assembling bioconjugates are materials of interest in designing vehicles in drug delivery applications. In this paper, we present the preparation of a diblock bioconjugate consisting of PVCL and hyaluronic acid. HA, PVCL, and their conjugate were characterized by size exclusion chromatography (SEC), nuclear magnetic resonance (NMR), matrix-assisted laser desorption/ionizationtime of flight (MALDI-TOF), and infrared (IR) spectroscopy. The HA block (3,600 g mol^{-1}, 16 to 18 saccharide units) was obtained via enzymatic degradation and fractionation of high molecular weight HA, whereas *N*-vinylcaprolactam (3,500 g mol^{-1}, ~25 repeating units) was prepared by macromolecular design via interchange of xanthate (MADIX) polymerization. The thermoresponsive properties and dynamics of self-assembling of the bioconjugate were studied in detail by microcalometry and light scattering.

EXPERIMENTAL

Materials

Azoisobutyronitrile (Fluka) was recrystallized from methanol. 1,4-dioxane (VWR, analytical grade) and were distilled under reduced pressure prior use. N-vinylcaprolactam (Aldrich, 98%) was recrystallized from toluene prior use. Deuterated choloroform (Euriso-top), deuterium oxide (Euriso-top), dichloromethane (VWR, HPLC > 99.8%), diethyl ether (Sigma-Aldrich, > 99.8%), diethylene glycol (Sigma-Aldrich, 99%), dimethylformamide (DMF, Lab-Scan, HPLC > 99.8%), ethanol (96%, Altia), hyaluronic acid (2 MDa, Soliance, France), hyaluronidase from bovine testes (Sigma-Aldrich, Type I-S, lyophilized powder, 400–1000 units mg^{-1} solid), hydrochloric acid (VWR, 37%), magnesium sulfate (Alfa Aesar, anhydrous, 99.5%), methanesulfonyl chloride (Fluka, > 98%), methyl-2-bromopropionate (Aldrich, 98%), pentane (Aldrich, 99%), potassium ethyl xanthogenate (Aldrich, 96%), propargyl bromide (80 wt % in toluene, Sigma-Aldrich), sodium azide (Sigma-Aldrich, > 99%), sodium borohydride (Aldrich, > 96%), sodium carbonate(Sigma-Aldrich, 99.99%), sodium chloride (Fluka, 99.9%), sodium hydrogencarbonate (JT Baker, 99.7%), sodium hydroxide (Fluka, > 85%), sodium phosphate (1 H_2O, JT Baker), sodium sulfate (Fluka, anhydrous > 99%), sodium-hydrogen phosphate (12 H_2O, Merck), tetrahydrofuran (THF,

HPLC > 99.9%), triethylamine (Fluka, > 98%), and trimethylsilane (Fluka, NMR-grade) were used as received.

SYNTHESIS

Enzymatic Degradation of Hyaluronic Acid

The degradation of HA was done as reported by Yang et al.[9] In short, 5 g hyaluronic acid (2 MDa) was dissolved in 750 mL saline phosphate buffer (pH 7.2). Hyaluronidase of 160 mg was added and the solution was kept at 37 °C for 30 h. The enzymatic degradation was stopped by heating the solution to 100 °C for 5 min. A drastic change in viscosity of the solution was observed during the degradation process. The HA was then fractionated using ultrafiltration (10 k, 5 k, and 1 k MWCO). The fraction used was characterized by size exclusion chromatography (SEC) against pullulan standards using an aqueous solution of 0.1 M $NaNO_3$ with 3% acetonitrile as eluent in order to determine the polydispersity. The molecular weight of the fraction ($M_n = 3600$ g mol^{-1}, Supporting Information Fig. 1) was determined by matrix-assisted laser desorption/ionization (MALDI). The fractions obtained after dialysis and freeze drying: 500 mg with M > 10,000 g mol^{-1} and 2.5 g, M > 1,000 g mol^{-1}.

2-(2-Azidoethoxy)ethanamine

An amine and azide containing asymmetric linker (2-(2-azidoethoxy)ethanamine) was synthesized as reported by Schwabacher et al.[31] Reaction scheme is presented as Supporting Information (Fig. 2). Diethylene glycol (10.03 g, 0.094 mol) was dissolved in 110 mL THF and the solution was cooled on an ice bath. Methylsulfonylchloride of 18 mL (0.233 mol) was added to the cold solution. Triethylamine 32.5 mL (0.23 mol), diluted with 30 mL THF, was added drop wise to the reaction mixture. After 24 h the precipitate was filtered and the THF evaporated. The product was dissolved in a mixture of 80 mL DMF and 60 mL H_2O. The pH of the solution was adjusted to 8 with $NaHCO_3$. Sodium azide (12.88 g, 0.20 mol) was added and the solution was heated to 80 °C. After 22 h the solution was extracted with diethylether (6 × 60 mL). The combined ether phases were washed with brine. The ether phase was dried with Na_2SO_4 and evaporated. Crude *Product 1* (1-azido-2-(2-azidoethoxy)-ethane) of 12.46 g was obtained. ^{1}H NMR (500 MHz, $CDCl_3$, TMS, δ ppm): 1.80 (N_3–CH_2–) and 3.41(–O–CH_2–) with residual peaks 2.82 (DMF), 2.91(DMF), 7.95 (DMF), 1.14 (diethyl ether), 3.36 (diethyl ether), 3.63 (–O–C***H*****$_2$**–CH_2–O–SO_2Me), and 13.69 (–O–CH_2–C***H*****$_2$**–O–SO_2Me).

The crude product (5.43 g, 0.034 mol) was dissolved in 40 mL diethylether and 80 mL 1.2 M HCl was added, resulting in an emulsion. Triphenylphosphine (5.08 g, 0.019 mol), dissolved in 40 mL diethylether, was slowly added drop wise into the emulsion. After 20 h the precipitates were filtered and the ether was separated. The aqueous layer was washed with diethyl ether (4 × 60 mL). After traces of ether were evaporated from the aqueous layer, the pH was adjusted to 11 with sodium hydroxide and the solution was stored at 7 °C. After 24 h the solution was extracted with dichloromethane (5 × 50 mL). The dichloromethane was dried with Na_2SO_4. *Product 2* (2-(2-azidoethoxy)ethanamine) of 2.42 g was obtained after evaporation of the dichloromethane. ^{1}H NMR (500 MHz, $CDCl_3$, TMS, δ ppm): 1.60 (N_3–CH_2–), 3.36 (N–CH_2–), 3.50 (O–CH_2–), 3.63 (–O–CH_2–), 5.28 (–NH_2) and residual solvent peaks from DMF: 2.85 and 7.97.

Linking of 2-(2-Azidoethoxy)ethanamine to Hyaluronic Acid (Reaction 2)

103 mg hyaluronic acid ($M = 3{,}600$ g mol^{-1}) was dissolved in 12 mL borate buffer (pH 8.4, 0.1 M with 0.4 M NaCl) under N_2 flow. The 2-(2-azidoethoxy)ethanamine of 40 mg was added to the solution followed by 50 mg (0.796 mmol) of sodium cyanoborohydride and the solution was heated to 40 °C. After 9 days the product was purified by dialysis and the presence of azide was confirmed by IR (Supporting Information Fig. 3, absorbance band at 2112 cm^{-1}). Product of 100 mg was obtained.

Synthesis of MADIX-CTA, O-ethyl-S-(1-Methoxycarbonyl)ethyl-Dithiocarbonate

MADIX-CTA, O-ethyl-S-(1-methoxycarbonyl)ethyl-dithiocarbonate, was synthesized by adapting the synthesis method reported by Destarac et al.[32] Then 20.00 g of (0.12 mol) of methyl-2-bromopropionate was dissolved in 200 mL ethanol and cooled in an ice bath. Potassium ethyl xanthogenate 21.20 g (0.13 mol) was added slowly, during 30 min, into the cold reaction mixture. The reaction was stirred for 20 h at room temperature, after which it was extracted with 300 mL of pentane-diethylether mixture (2:1). The organic phase was washed with distilled water and dried with $MgSO_4$. After evaporation of the solvents, 14.90 g product was obtained. ^{1}H-NMR (300 MHz, $CDCl_3$, TMS, δ ppm): 1.42 (CH_2–C***H***$_3$), 1.56 (CH–C***H***$_3$), 3.76 (O–C***H***$_3$), 4.39 (O–C***H***$_2$–), and 4.62 (–C***H***–).

Synthesis of PVCL

The synthesis of PVCL was conducted as reported by Destarac et al.[33] *N*-VCL 5.00 g (0.036 mol), MADIX-CTA 0.052 g (0.25 mmol), and AIBN 0.0082 g (0.050 mmol) were dissolved in 10 g of dioxane and purged with N_2 for 40 min. The reaction flask was placed in an oil bath at 60 °C. After 22 h the reaction was stopped by quenching it with liquid nitrogen and product was precipitated twice into hexane. Polymer of 1.06 g was obtained. The molar mass was determined by SEC ($M_n = 3{,}500$ g mol^{-1}, Đ = 1.41).

Reduction of the Polymer End Group into a Thiol (PVCL-SH)

PVCL of 319 mg (0.09 mmol) was dissolved in 50 mL THF. Sodiumborohydride of 77.16 mg (2.03 mmol) was dissolved in 10 mL water and added to the PVCL solution. After 4 h the THF was evaporated and the polymer purified by dialysis against water and freeze dried, yielding 310 mg polymer (PVCLSH).

Propargyl Functionalized PVCL

PVCL-SH of 150 mg (0.04 mmol) was dissolved in 5 ml THF. Then 37 mg (0.35 mmol) sodium carbonate was dispersed

in the solution and 0.37 mL (0.36 mmol) propargyl bromide solution was added. The reaction was left over night at 40 °C. The polymer was precipitated into hexane twice from THF and then dialyzed and finally freeze dried. The presence of the propargyl group was confirmed by ^{13}C NMR (Supporting Information Fig. 4.). ^{1}H NMR (500 MHz, D_2O, TMS, δ ppm): 1.79, 2.37, 2.55, 3.33, 4.32, and 4.47. ^{13}C NMR (176 MHz, D_2O, TMS, δ ppm): 23.0, 23.3, 29.0, 29.3, 30.1, 37.6, 42.4, 46.1, 47.2, 68.5 (propargyl), and 176.2.

Click Reaction of HA and PVCL

Propargyl-PVCL (77.0 mg, 0.022 mmol, $M_n = 3{,}500$ g mol^{-1}), HA with azide end group (75.3 mg, 0.025 mmol, $M_n = 3{,}600$ g mol^{-1}) and 25 mg (0.14 mmol) ascorbic acid were dissolved in 3 mL of borate buffer (pH 8.5 with 0.4 M NaCl) and 1 mL of water. Copper(I)chloride (24.75 mg, 0.25 mmol) was placed in a separate vial. Both vials were purged with N_2 for 30 min, after which the HA-polymer-solution was transferred into the vial with the CuCl. After 8 days, with continuous N_2 flow, the product was dialyzed against water and then ultrafiltrated using a 5000 MWCO membrane. After the filtration the sample was freeze dried. Product of 78.8 mg was obtained. SEC of the product gave one peak with Đ 1.3. The composition of the conjugate was determined, using the NMR peaks at 2.03 (3H, HA, 12) and 2.33 (2H, PVCL, P1) ppm, to be 51% HA and 49% PVCL. The NMR spectra are presented as figures 2 and 3. ^{1}H NMR (HA+PVCL-Click, 500 MHz, D_2O, TMS, δ ppm): *1.78 (broad),* 2.03, 2.33, 3.34, 3.57, 3.93, 4.32, and 4.48. ^{13}C NMR (HA+PVCL-Click, 176 MHz, D_2O, TMS, δ ppm): 22.06, 27.76, 28.95, 33.45, 36.54, 42.24, 47.22, 53.87, 60.04, 67.87, 71.90, 74.99, 79.49, 82.10, 99.90, 103.70, 174.89, and 178.21. ^{1}H NMR (HA, 500 MHz, D_2O, TMS, δ ppm): 2.06, 3.39, 3.53, 3.61, 3.75, 3.88, 3.94, 4.49, and 4.59.

INSTRUMENTS

Calorimetric analyses were conducted with a MicroCal VP DSC microcalorimeter. Solutions were degased at 5 °C prior the measurements. The 0.5 ml samples were run from 5 to 90 °C with 60 °C h^{-1}, repeating the heating and cooling cycle three times. The pre-equilibration time was 120 min at 5 °C before each heating cycle. The polymer concentrations were corrected by subtracting the amount of hyaluronic acid in the samples. The measured enthalpy values are given per repeating *N*-VCL unit.

The IR spectra were measured with a PerkinElmer Spectrum One spectrometer.

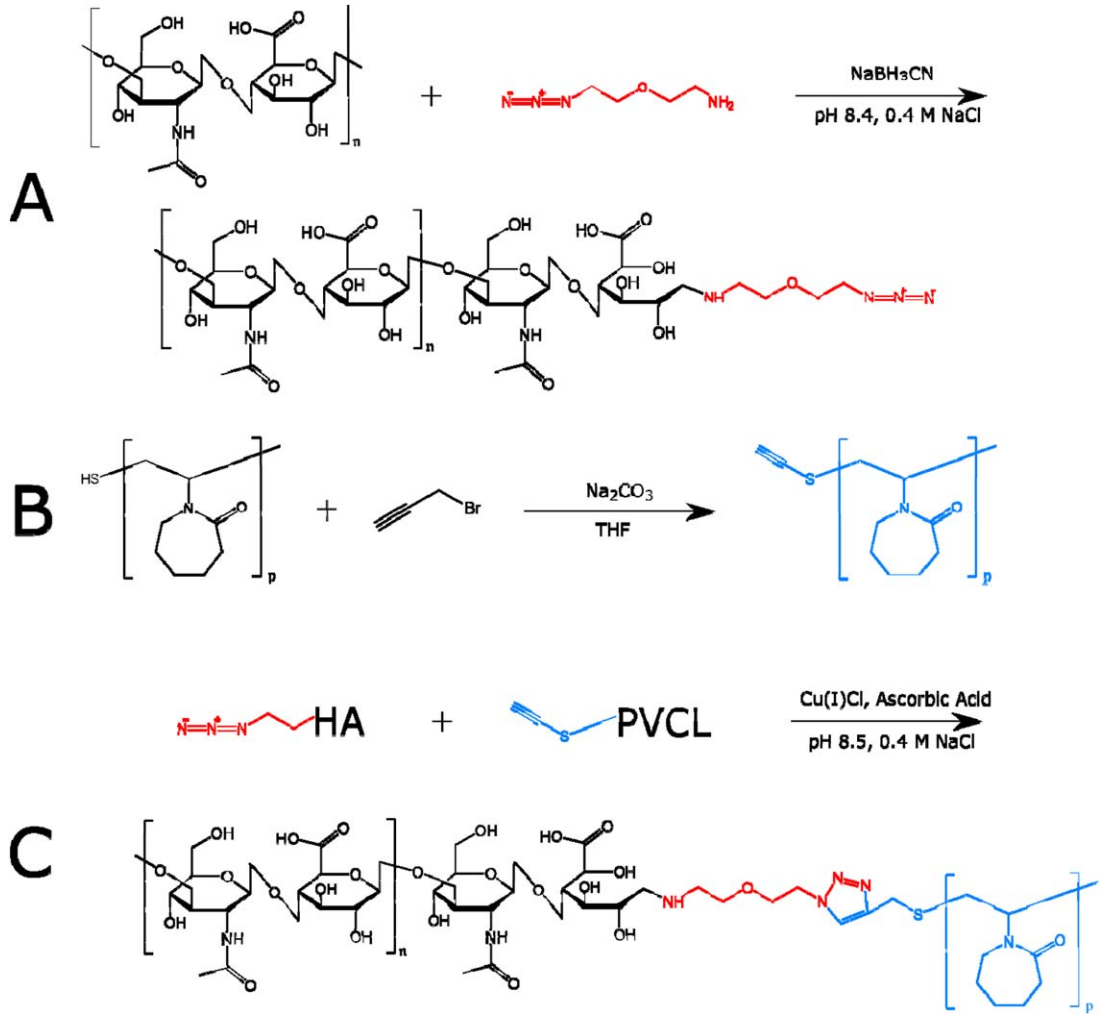
Light scattering (LS) experiments were conducted using a Brookhaven Instruments BI-200SM goniometer, a BIC-TurboCorr digital pseudo-cross-correlator, and a BI-CrossCorr detector, including two BIC-DS1 detectors; pseudo-cross-correlation functions of the scattered light intensity were collected with the self-beating method;[34] a Sapphire 488-100 CDRH laser from Coherent GmbH operating at $\lambda_o = 488$ nm and the power adjusting from 10 to 50 mW was used as a light source. Intensity of scattered light was collected in the angular range from 30 to 150°. LS studies were performed in the temperature range from 20 to 55 °C. Solutions were filtered through 0.45 μm CHROMAFIL® Xtra PVDF-45/25 directly into LS cells. Correlation functions of the intensity of scattered light, $G_2(t)$, were recorded simultaneously with the integral time-averaged intensities, $I_\theta \equiv I_q$, where q $(=(4\pi n_o \lambda_o^{-1})\sin\theta/2)$ is the scattering vector, n_o is the refractive index of the medium, and θ is the scattering angle. Methodological aspects of DLS and SLS can be found elsewhere.[35,36] In our LS experiments, bioconjugate solutions in saline water were heated from 20 up to 55 °C either using equilibrium (EH) or fast heating (FH). LS intensity measured at 45° scattering angle was a criterion of solution stability at fixed ambient temperature. The LS intensities at the scattering angles of 135°, 90°, and 45° were recorded at each temperature step simultaneously with the apparent hydrodynamic radius, R_h. Then the LS intensity and R_h were extrapolated to zero angle. Contrast between HA and PVCL in water below the phase separation temperature of PVCL should be similar, which allows the analysis of LS data with appropriate accuracy. Thus dn/dc of HA is 0.16–0.18 ml g^{-1},[37] whereas dn/dc of PVCL is 0.18–0.19 ml g^{-1}.[24]

NMR spectra were recorded with a 300 MHz ^{UNITY}Varian or 500 MHz Bruker Avance III spectrometer. Sample concentrations were 60 mg mL^{-1} in D_2O or $CDCl_3$ with tetramethylsilane as the reference.

MALDI-TOF (matrix-assisted laser desorption/ionization–time of flight) analysis was conducted with a Bruker Microflex equipped with 337 nm N_2 laser, using 20 kV accelerating voltage in pressure 5.0×10^{-6} mbar. α-cyano-4-hydroxycinnamic acid (CHCA) was used as matrix.[38] CHCA solution of 10 mg mL^{-1} was prepared in methanol containing 0.1% TFA. The samples were prepared by dissolving 1 mg in 100 μL water and 2 μL was mixed (1:1) with the matrix solution. A blank of the CHCA matrix was also investigated.

Size exclusion chromatography (SEC) was used to determine the molar mass and polydispersity of the polymers. The apparatus using THF as eluent included the following instruments: Biotech model 2003 degasser, Waters 515 HPLC pump, Waters 717plus auto sampler, Viscotek 270 dual detector, Waters 2487 dual λ absorbance detector, Waters 2410 refractive index detector, and the OmnisecTM software from Viscotek. Styragel HR 1, 2, and 4 columns and a flow rate of 0.8 ml min^{-1} were used in the measurements. PMMA standards from PSS Polymer Standards Service GmbH were used for calibration. For aqueous SEC the instrumental set up was as follows. Water chromatograph equipped with precolumn (Waters, Ultrahyrdrogel 6 × 40 mm) and three columns (Waters Ultrahydrogel 2000, 250, and 120), HPLC pump (Waters 515), Autosampler (Waters 717plus), and a RI-detector (Waters 2410). The HA degradation products were characterized using an aqueous eluent of 0.1 M $NaNO_3$ with 3% acetonitrile and the diblock copolymer with a 1:1 mixture of dimethylsulfoxide and water.

Turbidity measurements: Transmittance as a function of temperature was measured with JASCO J-815 CD-spectrometer



SCHEME 1 Schematic representation of the procedure for preparing the HA-PVCL block copolymer: (A) End functionalization of HA with 2-(2-azidoethoxy)ethanamine by reductive amination. (B) End functionalization of PVCL with propargylbromide. (C) "Click" coupling of HA and PVCL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

equipped with PTC-423S/15 Peltier-type temperature control system. The transmittances of the samples were monitored at wavelength of 488 nm. Sample holder was heated from 10 to 80 °C with varying rates; the reported temperatures are those of the sample. After heating, the sample holder immediately was cooled back to 10 °C, still monitoring the sample temperature. Aqueous solutions of the bioconjugate of 0.1 mg/mL were investigated both in pure water and in saline solution (0.1 M NaCl).

RESULTS AND DISCUSSION

Synthesis of the Diblock Copolymer

The molecular weight for the HA was determined by MALDI-TOF ($M_n = 3{,}600$ g mol^{-1}, Supporting Information Fig. 1) and the molecular mass distribution was determined by SEC ($Đ = 1.63$). Thus, the HA fragment consists of 8 to 9 disaccharide units. The PVCL block is roughly 25 repeating units long ($M_n = 3{,}500$ g mol^{-1}, $Đ = 1.41$).

The formation of the conjugate (scheme 1) could be confirmed from SEC, NMR, and IR data (Supporting Information Fig. 5 and 6). After purification by dialysis and ultrafiltration, the SEC eluogram of the block copolymer shows no trace of free PVCL (Fig. 4). However, the presence of PVCL is observed in the NMR spectra. In the ^{1}H NMR (Fig. 1), the peaks at 2.33 ppm (PVCL $-CH_2-$ in the ring) and methyl group from the hyaluronic acid (2.06 ppm) were used to determine the composition of the conjugate. From integration of these peaks, the composition of the product was estimated to be 49% PVCL and 51% hyaluronic acid. According to the NMR spectra there are traces of free hyaluronic acid in the product. The excessive purification was followed by NMR (Supporting Information Fig. 6), where in the crude product an excess of PVCL was observed. From the NMR spectra it can be seen how free homopolymers of PVCL and HA are removed from the product. The free PVCL was removed by dialysis (3500 MWCO) and free HA was removed after the dialysis by ultrafiltration (5000 MWCO). It should be noted that during this long purification

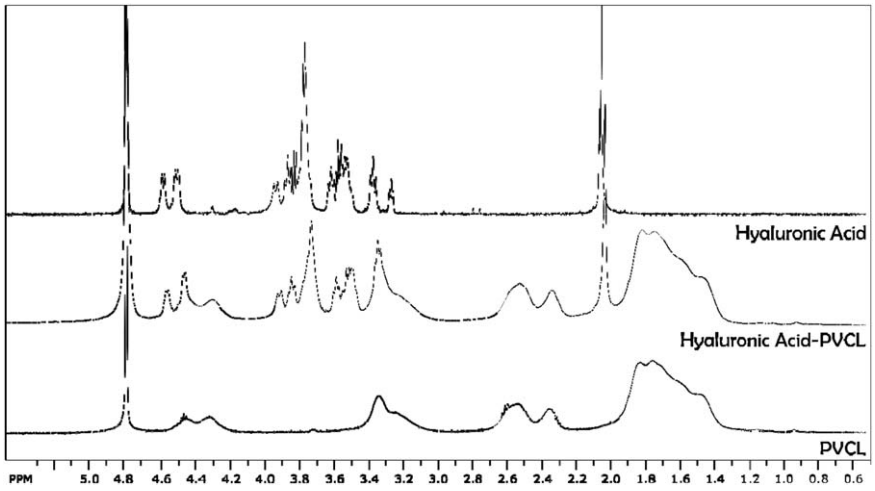


FIGURE 1 ^{1}H NMR spectra of hyaluronic acid (top), hyaluronic acid–PVCL conjugate (middle) and poly(*N*-vinylcaprolactam) (PVCL, bottom).

process, some degradation of the HA might occur. In this case, it would work in our advantage, since smaller fragments are removed more readily during the purification process.

The triazole ring is visible as a very weak signal at 7.14 ppm (Supporting Information Fig. 6). ^{13}C spectrum was also recorded to confirm the composition, however, the triazole ring could not be observed. In the IR spectrum, the characteristic absorption band for azide at 2112 cm^{-1} is diminished (Supporting Information Fig. 5.) while absorption bands for both polymers can be observed.

Microcalorimetric Observations

Conjugation of PVCL to HA increases its phase separation temperature, seen calorimetrically as a shift of both T_{onset} and T_{max} (Fig. 5). The enthalpies for the phase transition are similar for both free PVCL (3.8 kJ mol^{-1}) and for PVCL linked to HA (3.7 kJ mol^{-1}).

The phase transition temperature of PVCL is dependent on the molecular weight of the polymer. It has been shown that T_{max} increases from 33.1 to 43.6 °C when the molecular weight decreases from 1,500,000 g mol^{-1} to 21,000 g mol^{-1}.[39] Since the PVCL block is short, the phase transition is expected to occur at a temperature higher than 32 °C, and indeed, the T_{max} for the homopolymer is observed at 45.2 °C and for the diblock copolymer at 47.7 °C.

Hyaluronic acid is known to influence the phase transition of thermoresponsive polymers. Ohya et al. have studied the

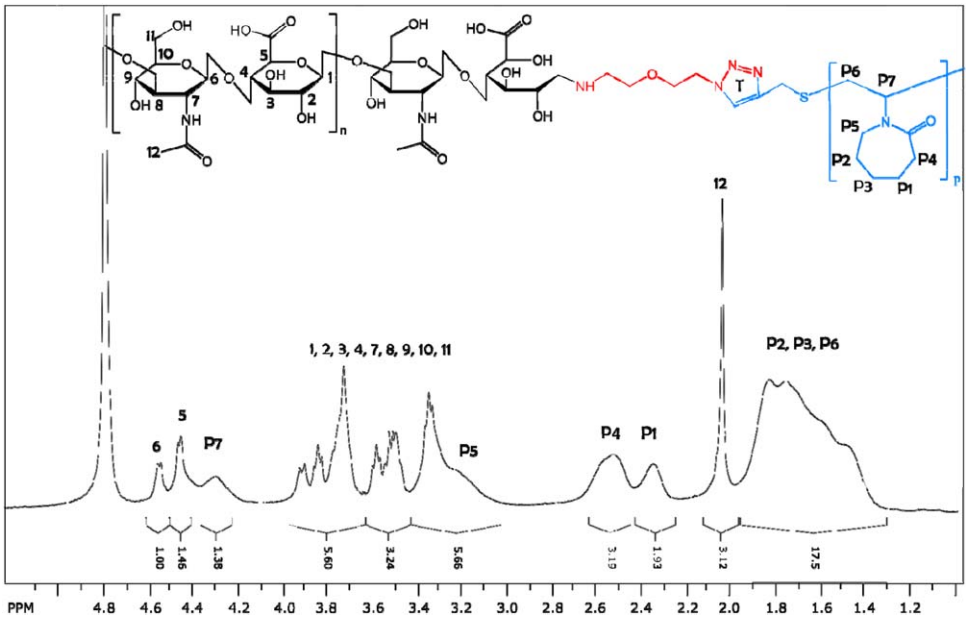


FIGURE 2 ^{1}H NMR spectrum of the hyaluronic acid–PVCL conjugate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

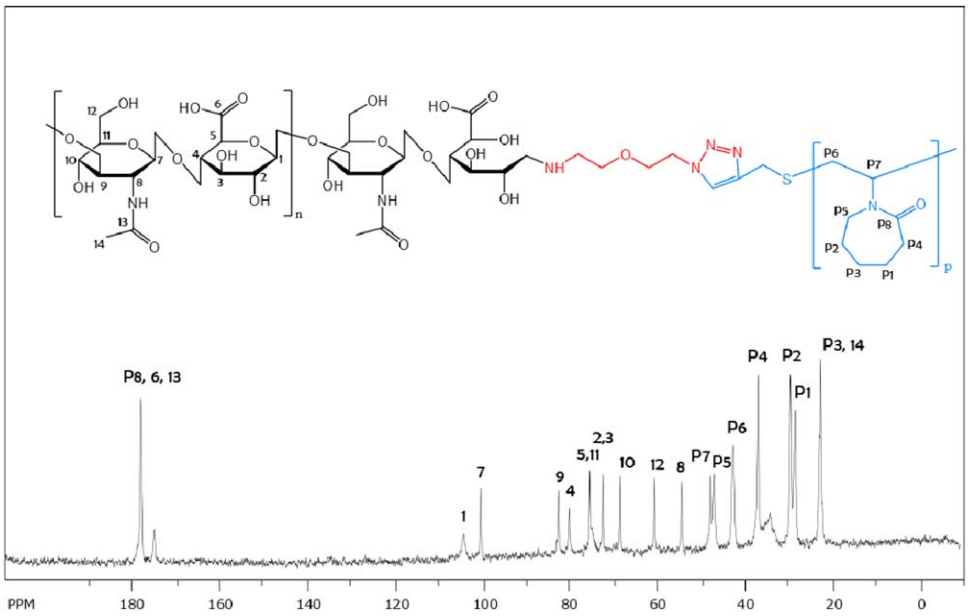


FIGURE 3 ^{13}C NMR spectrum of the hyaluronic acid–PVCL conjugate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

thermoresponsive behavior of PNIPAM (5,000–84,000 g mol^{-1}) grafted to HA (500–1.500 kg mol^{-1}) by turbidimetry. They report that the T_{onset} of the decrease in transmittance is shifted in the presence of HA. For pure PNIPAM the cloud point is observed at 32 °C, whereas in the presence of HA it is 33.5 to 34.4 °C, depending on the composition of the bioconjugates.[17] Mortisen et al. have studied the thermoresponsive behavior of PNIPAM (10,700–48,200 g mol^{-1}) grafted to HA (1,185 kg mol^{-1}). In their differential scanning calorimetric (DSC) measurements they found that the maximum of the phase transition peak shifts upon grafting. For the shortest grafts (10,700 g mol^{-1}) T_{max} increased from 29.2 to 30.2 °C. For PNIPAM grafts with higher molecular weights (26,800 and 48,200 g mol^{-1}) the transition occurred at lower temperatures, that is T_{max} decreased from 30.6 to 29.5 °C (26,800 g mol^{-1}).[5] Both the HA to PNIPAM ratio and the molecular weight of PNIPAM affect the change in the phase transition temperatures upon grafting to HA. However, in both cases the phase transitions for short PNIPAM chains shifted to higher temperatures.

Kirsh et al. found that the cloud point of PVCL shifts to higher temperatures in the presence of solutes with carbonyl or amide groups, and that the effect increases upon increasing the amount of the solute. The same was observed in the presence of glycols.[40] On the other hand, Yanul et al.[41] observed that the presence of polyethylene oxide (PEO) in the solution decreases the cloud point of PVCL. Laukkanen et al. grafted PVCL with alkyl modified PEO. They showed that with increasing grafting density the phase transition

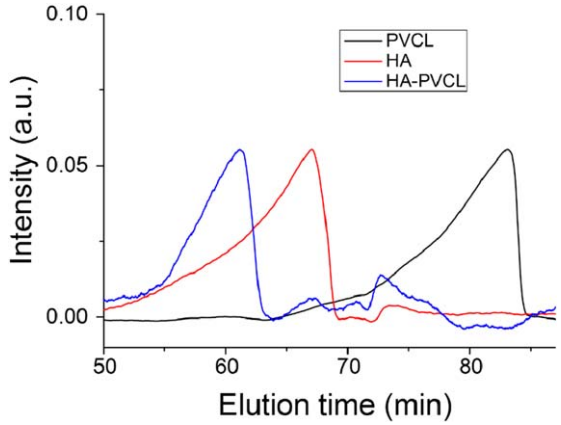


FIGURE 4 SEC trace of the HA-PVCL conjugate (blue) compared with the traces for HA (red) and PVCL (black). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

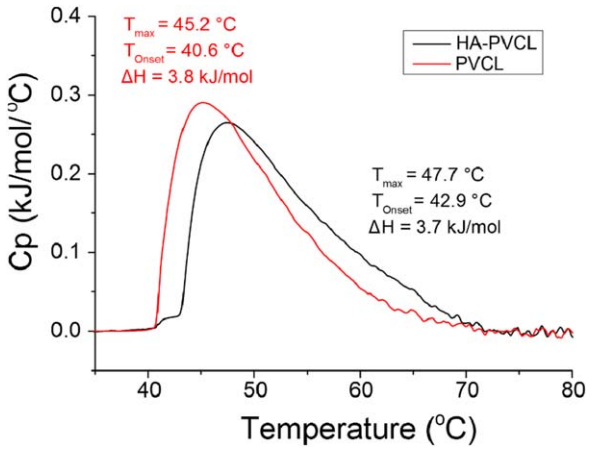


FIGURE 5 Micro calorimetric data of the HA-PVCL bioconjugate and PVCL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

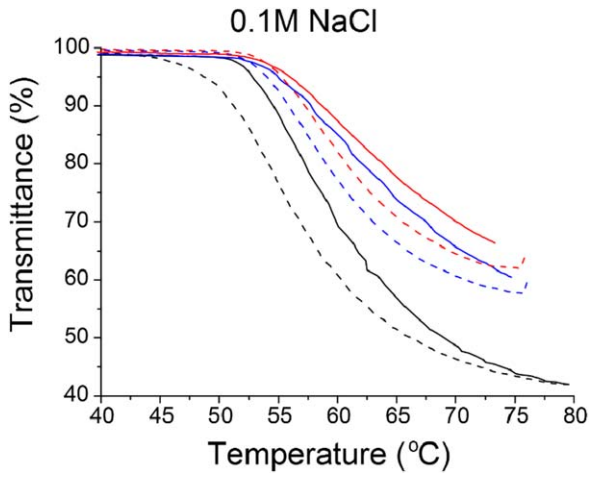


FIGURE 6 Turbidity measurements performed on 0.1 mg mL^{-1} HA-PVCL dissolved in aqueous 0.1 M NaCl. Heating (solid lines) and cooling (dashed lines) rate was varied: 1 (black), 5 (blue), and 10 (red) °C min^{-1}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

temprature of PVCL increases, that is, the T_{max} shifted from 34.9 to 38.2 °C. The enthalpy of the phase transition is also affected by the graft copolymerization, decreasing with increasing PEO content from 32 to 21 J g^{-1}.[24] When a PVCL-*b*-PEO block copolymer is attached to the surface of a silica particle the phase transition shifts to lower temperatures, that is, from 36.5 to 35.4 °C. In this case the grafting to the silica surface has a major effect on the enthalpy of transition, increasing it from 1.94 to 5.40 kJ mol^{-1}.[42] More recently, it was shown that when PVCL was connected to PDMAEMA either one or two transitions were observed, depending on the pH. The phase separation was monitored by turbidometry and calorimetry. However, in all cases, PDMAEMA

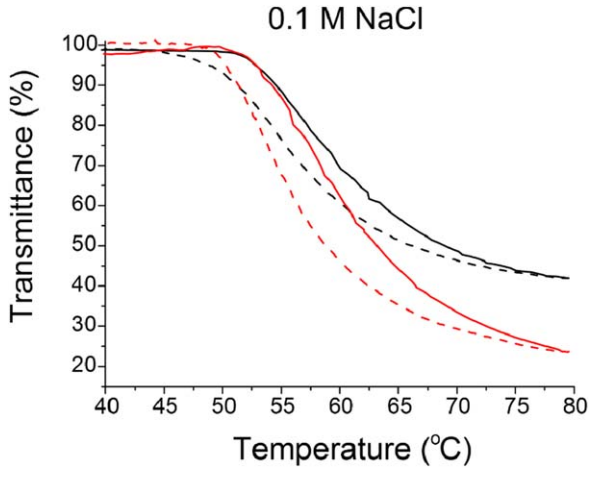


FIGURE 7 Turbidity measurements performed on 0.1 mg mL^{-1} HA-PVCL (black) and PVCL (red) dissolved in aqueous 0.1 M NaCl, with heating (solid lines) and cooling (dashed lines) rates of 1 °C min^{-1}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

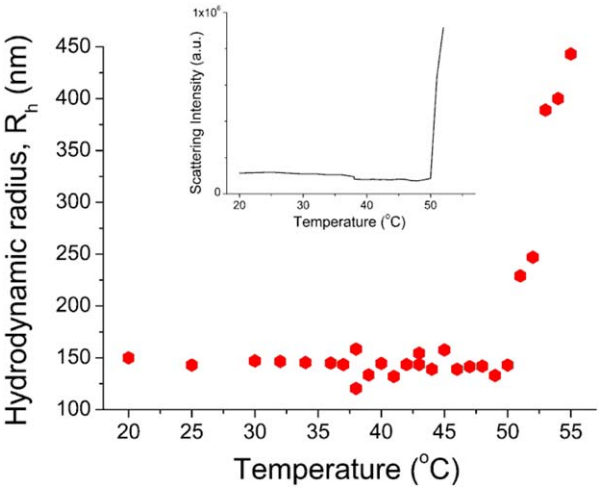


FIGURE 8 Equilibrium heating: hydrodynamic radius of the bioconjugate as function of temperature (extrapolated to 0° angle). In the inset, the scattering intensity at a 45° angle is presented as function of temperature. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

increased the T_c of PVCL.[28] Also, the water-soluble poly(*N*-vinyl pyrrolidone) has been observed to increase the cloud point of PVCL in PVCLPVP diblock copolymers.[25]

Hou and Wu studied diblock copolymers of PVCL and PNIPAM. Only one cloud point was observed, but by using FTIR measurements, it was possible to resolve two transitions. The values obtained for T_c with the two different methods differ noticeably; FTIR gives lower values than the turbidity measurement. In this case, the PVCL homopolymer and the PVCL block in the copolymer had different chain lengths.[29]

The shifts observed in the present study in the T_{onset} and T_{max} for the HA-PVCL conjugate are well in line with the reported observations for PVCL and PNIPAM. Interestingly though, in this case the HA has no effect on the enthalpy of the transition.

Turbidity Studies

Turbidimetric studies were conducted in aqueous solutions with and without NaCl (0.1 M) figures 6 and 7. The heating rate was varied in the measurements, that is, the samples were heated either 1, 5, or 10 °Cmin^{-1}. The polymer concentration c_p was 0.1 mg mL^{-1}. The solvents and polymer solutions were degassed at 5 °C. Fresh samples were used for each measurement, to minimize the effect of possible degradation of the HA block at elevated temperatures.

In saline solutions the cloud point (T_{cp} = 51 °C, Fig. 6) is not affected by the heating rate, however, the heating rate has an overall effect on the transition. The transition is more gradual and is still incomplete at 80 °C with the higher heating rates (5 and 10 °Cmin^{-1}). For the slowest heating/cooling rate studied (1 °Cmin^{-1}), transmittance reaches its lowest value and the observed hysteresis manifests a slow response of HA-PVCL. The observation shows that the conjugate has enough time to self-organize in response to the slow heating,

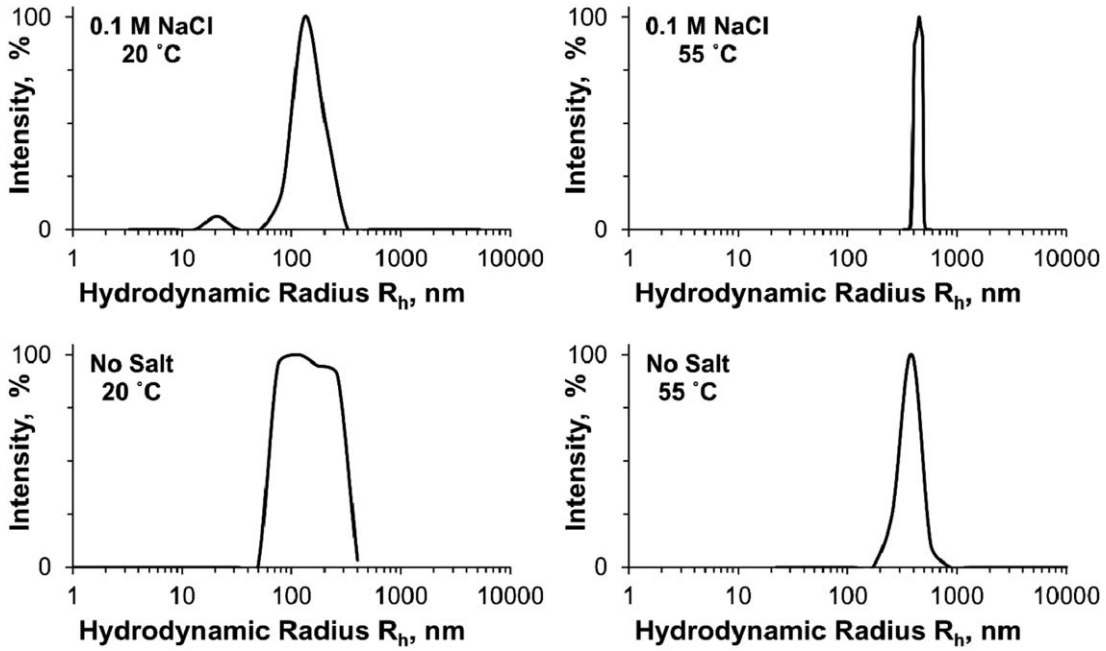


FIGURE 9 Sizes measured at an angle of 45° at 20 °C (left) and after equilibrium heating at 55 °C (right). Top, the size distributions of HA-PVCL in 0.1 M NaCl. At 20 °C the size of the size measured is 132 nm, whereas upon heating to 55 °C the size increases to 450 nm. Below, the size distributions of HA-PVCL in water. At 20 °C, the hydrodynamic radius measured is 92 to 225 nm, whereas upon heating to 55 °C, the size increases to 395 nm.

which consequently slows the resolubilization upon cooling. In aqueous solution without NaCl the cloud point is observed at practically the same temperatures as for the saline solution (Supporting Information Figs. 9 and 10) The hysteresis upon a heatingcooling cycle is, however, smaller in the salt-free solution.

Light Scattering with Equilibrium Heating (EH) in 0.1 M NaCl

In the EH procedure, the diblock copolymer solution (0.1 mg mL^{-1}) was heated in 1 °C steps. After each increase in temperature, the intensity of light scattered at 45° angle was measured to follow the equilibration of the solution. Upon heating from 20 up to 50 °C, the intensity of scattered light decreases slightly in accordance with decreasing mean peak value of the hydrodynamic radius (R_h). The scattering intensity increases strongly between 50 and 51 °C. Thus the T_{cp} can be determined to be 51 °C, which is in line with the turbidimetric measurements (Fig. 8).

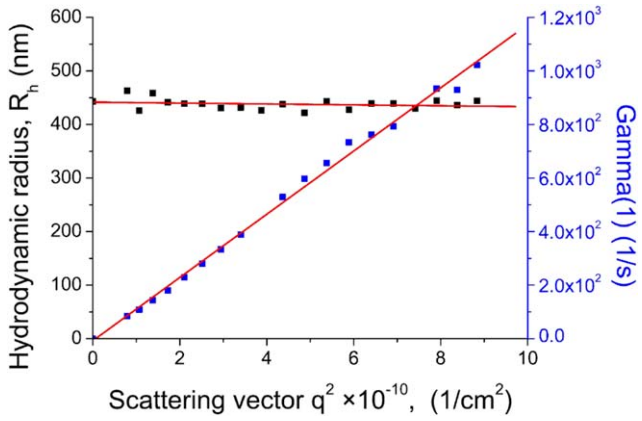


FIGURE 10 Equilibrium heating: R_h versus scattering vector at 55 °C, showing the linear fit in the extrapolation of the R_h to 0° angle (black) and the relaxation rate plotted against the scattering vector (blue). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

At 20 °C, HA-PVCL forms aggregate with $R_h = 145–150$ nm (Fig. 9). It is known that high molar mass PVCL may form loose aggregates at room temperature,[24] whereas HA as a polyelectrolyte swells in water. The existence of loose structures is supported by the relatively weak scattering by the polymer solutions. The angular dependence at 20 and 55 °C are shown in Supporting Information Figure 12.

Both the scattering intensity and the size of the particles, formed above T_{cp} (51 °C), increase upon further heating. At 55 °C, $R_h = 443$ nm. The dissymmetry (z) in the intensity of scattered light measured at 45 and 135° scattering angle, $z = I(135°)/I(45°)$, is 10. Assuming a spherical conformation of the particles, $z = 10$ corresponds to a sphere with its radius of gyration $R_g = 139$ nm.[43] Such combination of geometrical parameters is not possible for a hard sphere. Therefore, the angular dependence of the intensity of light scattered from solutions at 55 °C was analyzed simultaneously with R_h measurements. The solution scatters strongly and primarily forward, which is typical for large particles. However, the LS intensity is not as high by its absolute value as one may expect judging by the particle size and

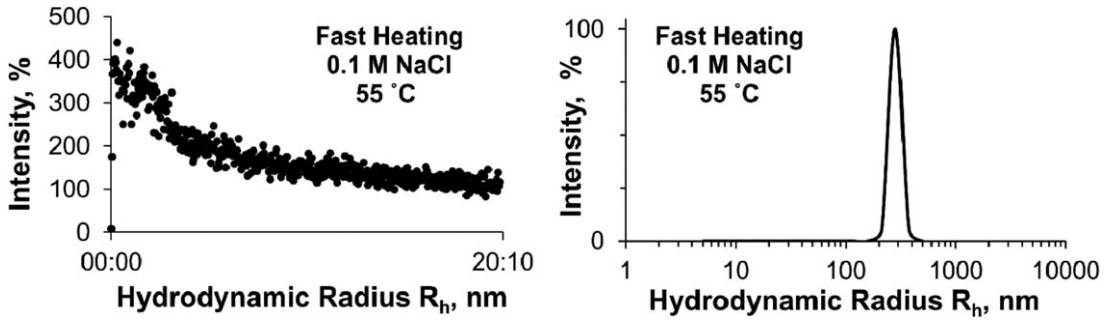


FIGURE 11 Fast heating: On the left, scattering intensity as function of time, showing the slow reorganization of the forming particles. Right, size distribution of HA-PVCL in 0.1 M NaCl at 55 °C, measured at 45° angle. The average hydrodynamic radius of the aggregates is in the order of 280 nm.

thermodynamically poor condition. As a result, at the angles above 90°, light scattered forward and reflected back by several quartz surfaces of the goniometer overcomes weak back scattering and makes data analysis impossible (e.g., Kratky plot). Distributions of the hydrodynamic radius clearly are bi- and multi-modal above 120° scattering angle, whereas below 90°, the distributions are monomodal and narrow. The second-order cumulants analysis shows that the relaxation rate decreases linearly with decreasing the scattering vector below 90° and goes through zero, thus representing translational diffusion (Fig. 10).

Therefore, R_g was calculated in the angular range of 30 to 90°. The second-order polynomial fit to the data in Zimm's presentation[43] gives $R_g = 478$ nm. Rough estimation of the molar mass of the aggregates (assuming the increment of refractive index $dn/dc_p = 0.18$ cm^3 g^{-1}) suggests packing density of polymeric material within such particles to be 4 mg mL^{-1}. This value is too low for a hard sphere. Taking into account the weak scattering form the particles, their low density, and the shape parameter $R_g/R_h \approx 1.1$, our LS results suggest that the shape of the particles formed at 55 °C upon equilibrium heating is a hollow sphere. For comparison, it has been reported that a PVCL (330,000 g mol^{-1}, 0.1 mg mL^{-1}) has $R_h = 127$ nm, $R_g = 54$ nm, $R_g/R_h = 0.43$, and the mesoglobules have a density of 650 mg mL^{-1} at 50 °C in aqueous solution.[44]

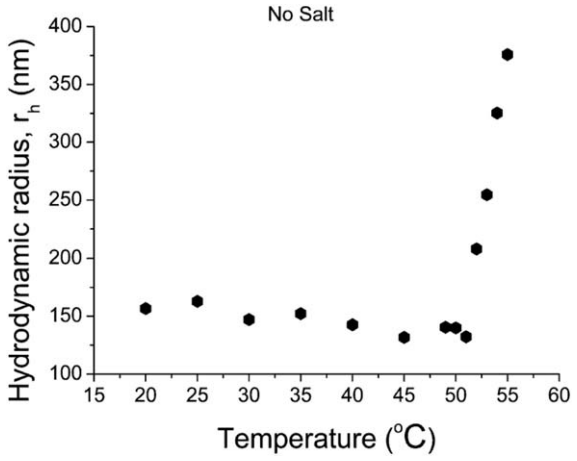


FIGURE 12 Equilibrium heating in pure water: hydrodynamic radius of the bioconjugate as function of temperature (45° angle).

Fast Heating, FH in 0.1 M NaCl

In the FH procedure, a polymer solution (0.1 mg mL^{-1}) was first equilibrated at 20 °C and then immersed directly to the LS goniometer preheated at 55 °C. As follows from our LS, calorimetric, and turbidity studies, 55 °C is within the transition range, that is the phase transition of the PVCL block is not over at this temperature and the formed particles have freedom to reorganize. This process is slow and the sample was left to stabilize for 20 h (Fig. 11).

Angular dependence of the LS intensity was analyzed at 55 °C simultaneously with R_h measurements and R_g was calculated in the angular range of 30–90° using the Zimm analysis.[43] Accordingly at zero scattering angle (Supporting Information Fig. 10), $R_h = 275$ nm, $R_g = 270$ nm, packing density of the particles is 7 mg mL^{-1}, and the shape parameter is $R_g/R_h \approx 1.0$. These parameters support a vesicle-like model for the bioconjugate particles.

One can note that FH procedure yields particles of smaller size in comparison to EH procedure (Figs. 9 and 11). This has been observed previously for homopolymers of PVCL, PNIPAM, and poly(vinyl methyl ether).[44] However, kinetics of the bioconjugate is much slower. Once the particles are formed, they do not grow with time to adopt the size of the EH particles. Evidently, the phase-separated PVCL is hindered by tangling HA blocks. Possibly, slow equilibration originates from reorganization inside the particles.

Light Scattering with Equilibrium Heating (EH) in Aqueous Solution Without NaCl

In aqueous solutions without salt, the size distributions of the block copolymers observed at 20 °C are broader than those in the saline solution (Fig. 9). The size distribution becomes narrower at 55 °C, however the average radius of the obtained particles is smaller than in the presence of salt.

The size of the aggregates remains fairly stable until around 50 °C, above which the size increased rapidly (Fig. 12). The size of the block copolymers at 20 °C (~150 nm) are of the same order with and without NaCl in the solution. This shows that the solubility of the block copolymer is affected to a lesser degree by the presence of salt.

PVCL can complex ionic substances, as has been shown with surfactants. Cetylpyridinium chloride and sodium dodecyl sulfate have been shown to bind and affect the size of PVCL coils or aggregates in aqueous solutions. At low concentrations (< 1:1) the surfactants decrease the size of the aggregates, whereas at higher concentrations (> 1:1) the size was observed to increase, showing the binding of the surfactants to the PVCL.[45] Similar behavior was observed with PVCL-PDMAEMA block copolymer, where at room temperature the polymer was observed to form loose associates, due to inter molecular interactions between the PDMAEMA and PVCL blocks.[28]

The dynamic light scattering studies of HA-PVCL show that, independent of salt, the polymers form inter molecular loose associates with broad size distributions, in aqueous solutions at room temperature (Fig. 9). Upon heating, the interactions are overcome by hydrophobic interactions during the phase transition of the PVCL block, resulting in more compact particles. Knowing that the HA-PVCL polymers interact with each other sheds further light to the static light scattering studies, which suggest that the self-assembled particles obtained at 55 °C are hollow. As the HA-PVCL forms loose associates at low temperatures, that are later squeezed into particles; one would expect some of the HA blocks to end up inside the particles and some to form a corona on the outside. The inside of the vesicular aggregate would be significantly hydrated by the HA and be less dense than the collapsed PVCL.

CONCLUSIONS

A bioconjugate with a well-defined diblock copolymer structure, consisting of hyaluronic acid and PVCL was produced by utilizing reductive amination of the HA end group and copper-assisted alkyne-azide "click" reaction. The phase transition of PVCL is affected by its molar mass and thus for short almost oligomeric PVCL block ($M_n = 3{,}500$ g mol^{-1}) the phase transition occurs at relatively high temperatures, 45.2 °C. The enthalpy of the phase transition of PVCL is unaffected by the bioconjugation. However, the phase transition is shifted by 2 °C to higher temperatures, compared to the PVCL homopolymer. In agreement with previous reports, the phase transition of the PVLC is affected by the presence of the second block. That is, the presence of a hydrophilic block increases the cloud point temperature of PVCL. However, the phase transition occurs independently of the second block since no change in the enthalpy of the phase transition is observed. Surprisingly, the phase transition of the PVCL block is also independent of the presence of salt, as is shown by the turbidimetric studies, which may be due to the presence of the HA or the low molecular weight of the PVCL.

Above the cloud point of the PVCL block, the bioconjugate self-assembles into colloidally stable particles and the structure of the particles was investigated by light scattering. The light scattering studies indicate that at room temperature the bioconjugate forms loose associates. Above the cloud point the molecules self-assemble into hollow spherical particles with a hydrodynamic radius of 443 nm. The heating rate has an effect on the size of the formed particles. Fast heating resulted in smaller particles with hydrodynamic radius of 275 nm. Turbidimetric studies show that the cloud point of the bioconjugate is unaffected by the heating rate. The turbidimetric studies also show a hysteresis when heating and cooling the solutions, this indicating that the phase separated particles have time to organize during their formation. This organization process can also be seen in the light scattering data, when the solution is heated fast to 55 °C, since the intensity of scattered light takes up to 20 h to stabilize.

REFERENCES AND NOTES

1 J. R. E. Fraser, T. C. Laurent, U. B. G. Laurent, *J. Intern. Med*. **1997**, *242*, 27–33.

2 H. Lee, K. Lee, T. G. Park, *Bioconjugate Chem*. **2008**, *19*, 1319–1325.

3 G. Testa, C. Di Meo, S. Nardecchia, D. Capitani, L. Mannina, R. Lamanna, A. Barbetta, M. Dentini, *Int. J. Pharm*. **2009**, *378*, 86–92.

4 G. Huerta-Angeles, D. Šmejkalová, D. Chládková, T. Ehlová, R. Buffa, V. Velebný, *Carbohydr. Polym*. **2011**, *84*, 1293–1300.

5 D. Mortisen, M. Peroglio, M. Alini, D. Eglin, *Biomacromolecules* **2010**, *11*, 1261–1272.

6 J. Mergy, A. Fournier, E. Hachet, R. Auzély-Velty, *J. Polym. Sci., Part A: Polym. Chem*. **2012**, *50*, 4019–4028.

7 Y. Dong, A. O. Saeed, W. Hassan, C. Keigher, Y. Zheng, H. Tai, A. Pandit, W. Wang, *Macromol. Rapid Commun*. **2012**, *33*, 120–126.

8 C. Schatz, S. Lecommandoux, *Macromol. Rapid Commun*. **2010**, *31*, 1664–1684.

9 Y. Yang, K. Kataoka, F. M. Winnik, *Macromolecules* **2005**, *38*, 2043–2046.

10 R. F. Borch, M. D. Bernstein, H. D. Durst, *J. Am. Chem. Soc*. **1971**, *93*, 2897–2904.

11 S. Asayama, M. Nogawa, Y. Takei, T. Akaike, A. Maruyama, *Bioconjugate Chem*. **1998**, *9*, 476–481.

12 E. M. Sletten, C. R. Bertozzi, *Angew. Chem. Int. Ed*. **2009**, *48*, 6974–6998.

13 R. Novoa-Carballal, A. H. E. Müller, *Chem. Commun*. **2012**, *48*, 3781–3783.

14 L. Liu, Y. Liu, J. Li, G. Du, J. Chen, *Microb. Cell Fact*. **2011**, *10*, 99

15 M. D'Este, M. Alini, D. Eglin, *Carbohydr. Polym*. **2012**, *90*, 1378–1385.

16 H. Tan, C. M. Ramirez, N. Miljkovic, H. Li, J. P. Rubin, K. G. Marra, *Biomaterials* **2009**, *30*, 6844–6853.

17 S. Ohya, Y. Nakayama, T. Matsuda, *Biomacromolecules* **2001**, *2*, 856–863.

18 R. Novoa-Carballal, D. V. Pergushov, A. H. E. Müller, *Soft Matter* **2013**, *9*, 4297–4303.

19 C. C. Liu, K. Y. Chang, Y. J. Wang, *J. Polym. Res*. **2010**, *17*, 459–469.

20 A. C. W. Lau, C. Wu, *Macromolecules* **1999**, *32*, 581–584.

21 H. Vihola, A. Laukkanen, L. Valtola, H. Tenhu, J. Hirvonen, *Biomaterials* **2005**, *26*, 3055–3064.

22 H. Vihola, A. K. Marttila, J. S. Pakkanen, M. Andersson, A. Laukkanen, A. M. Kaukonen, H. Tenhu, J. Hirvonen, *Int. J. Pharm*. **2007**, *343*, 238–246.

23 M. Prabaharan, J. J. Grailer, D. A. Steeber, S. Gong, *Macromol. Biosci*. **2008**, *8*, 843–851.

24 A. Laukkanen, L. Valtola, F. M. Winnik, H. Tenhu. *Polym. J*. **2005**, *46*, 7055–7065.

25 X. Liang, V. Kozlovskaya, C. P. Cox, Y. Wang, M. Saeed, E. Kharlampieva, *J. Polym. Sci., Part A Polym. Chem*. **2014**, *52*, 2725–2737.

26 A. Kermagoret, K. Mathieu, J. M. Thomassin, C. A. Fustin, R. Duchêne, C. Jérôme, C. Detrembleur, A. Debuigne, *Polym. Chem*. **2014**, *5*, 6534–6544.

27 M. Hurtgen, J. Liu, A. Debuigne, C. Jerome, C. Detrembleur, *J. Polym. Sci. Part A Polym. Chem*. **2012**, *50*, 400–408.

28 M. Karesoja, E. Karjalainen, S. Hietala, H. Tenhu, *J. Phys. Chem. B* **2014**, *118*, 10776–10784.

29 L. Hou, P. Wu, *Soft Matter* **2015**, *11*, 2771–2781.

30 A. Kermagoret, C. A. Fustin, M. Bourguignon, C. Detrembleur, C. Jérôme, A. Debuigne, *Polym. Chem*. **2013**, *4*, 2575–2583.

31 A. W. Schwabacher, J. W. Lane, M. W. Schiesher, K. M. Leigh, C. W. Johnson, *J. Org. Chem*. **1998**, *63*, 1727–1729.

32 M. Destarac, C. Brochon, J. M. Catala, A. Wilczewska, S. Z. Zard, *Macromol. Chem. Phys*. **2002**, *203*, 2281–2289.

33 M. Beija, J. D. Marty, M. Destarac, *Chem. Commun*. **2011**, *47*, 2826–2828.

34 B. Chu, Laser Light Scattering: Basic Principles and Practice, Academic Press: Boston, **1991**.

35 W. Schärtl, Light Scattering From Polymer Solutions and Nanoparticle Dispersions, Springer Verlag: Berlin, Heidelberg, **2007**.

36 W. Brown, Dynamic Light Scattering: The Method and Some Application, Claredon Press: Oxford, **1993**.

37 S. Hokputsa, K. Jumel, C. Alexander, S. E. Harding, *Carbohydr. Polym*. **2003**, *52*, 111–117.

38 S. Sakai, K. Hirano, H. Toyoda, R. J. Linhardt, T. Toida, *Anal. Chim. Acta*. **2007**, *593*, 207–213.

39 A. Laukkanen, L. Valtola, F. M. Winnik, H. Tenhu, *Macromolecules* **2004**, *37*, 2268–2274.

40 Y. Kirsh, N. Yanul, Y. Popkov, *Eur. Polym. J*. **2002**, *38*, 403–406.

41 N. A. Yanul, Y. E. Kirsh, S. Verbrugghe, E. J. Goethals, F. E. Du Prez, *Macromol. Chem. Phys*. **2001**, *202*, 1700–1709.

42 M. Karesoja, J. McKee, E. Karjalainen, S. Hietala, L. Bergman, M. Linden, H. Tenhu, *J. Polym. Sci., Part A Polym. Chem*. **2013**, *51*, 5012–5020.

43 P. Kratochvil, In Light Scattering From Polymer Solution, M.B. Huglin), Ed.; Academic Press: London, New York, **1972**; pp 333–384.

44 V. Aseyev, S. Hietala, A. Laukkanen, M. Nuopponen, O. Confortini, F. E. Du Prez, H. Tenhu, *Polymer J*. **2005**, *46*, 7118–7131.

45 E. E. Makhaeva, H. Tenhu, A. R. Khokhlov, *Macromolecules* **1998**, *31*, 6112–6118.