

Novel Fast Degradable Thermosensitive Polymeric Micelles Based on PEG-*block*-poly(*N*-(2-hydroxyethyl)methacrylamide-oligolactates)

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The aim of this study was to design a thermosensitive polymeric micelle system with a relatively fast degradation time of around 1 day. These micelles are of interest for the (targeted) delivery of biologically active molecules. Therefore, *N*-(2-hydroxyethyl)methacrylamide-oligolactates (HEMAm–Lac_{*n*}) were synthesized and used as building blocks for biodegradable (block co) polymers. p(HEMAm–Lac₂) is a thermosensitive polymer with a cloud point (CP) of 22 °C which could be lowered by copolymerization with HEMAm–Lac₄. The block copolymer PEG-*b*-((80%HEMAm–Lac₂)-(20%HEMAm–Lac₄)) self-assembled into compact spherical micelles with an average size of 80 nm above the CP of the thermosensitive block (6 °C). Under physiological conditions (pH 7.4; 37 °C), the micelles started to swell after 4 h and were fully destabilized within 8 h due to hydrolysis of the lactate side chains. Rapidly degrading thermosensitive polymeric micelles based on PEG-*b*-((80%HEMAm–Lac₂)-(20%HEMAm–Lac₄)) have attractive features as a (targeted) drug carrier system for therapeutic applications.

Introduction

Amphiphilic block copolymers can self-assemble in aqueous solutions into micellar structures consisting of a hydrophilic shell and a hydrophobic core. Generally, micelles have a small size and their hydrophobic core can accommodate hydrophobic drugs such as phthalocyanines and paclitaxel.^{1,2} These properties make polymeric micelles excellent drug delivery vehicles. Carriers that are currently under investigation for drug delivery purposes are based on block copolymers of the hydrophilic poly(ethylene glycol) (PEG) and a variety of hydrophobic blocks including polylactide, polyglycolide, poly(lactide-*co*-glycolide), poly(propylene oxide) (Pluronic), poly(caprolactone), or poly(benzyl aspartate).³

The ideal pharmaceutical nanoparticle displays a high drug encapsulation efficiency, protects the incorporated drug against chemical or enzymatic degradation and is able to release its content in a controlled way, preferable upon arrival at its target site.^{4,5} Besides “simple” solubilization of the drug in the hydrophobic core, covalent binding of a drug (e.g., doxorubicin) onto the hydrophobic polymer segment via an enzymatically cleavable spacer⁶ and via acid sensitive linkers³ has also been studied to improve and control the drug loading and release properties of polymeric micelles. The hydrophilic block needs to be long enough to provide steric stabilization of the particles and at the same time to give the particle

“stealth” properties.^{7,8} After intravenous administration, these long circulating PEGylated colloidal particles (<200 nm) will extravasate and accumulate in tumors and other pathological areas referred to as the enhanced permeability and retention (EPR) effect.⁹

Special classes of polymers are those that exhibit thermosensitive behavior. Block copolymers composed of a hydrophilic block (PEG) and a thermosensitive block can form polymeric micelles above the cloud point temperature (CP) of the thermosensitive block. This has been demonstrated for PEG-PPO-PEG (Pluronic)^{10,11} and for PEG-*b*-p(*N*-isopropylacrylamide) (PEG-*b*-pNIPAAm).¹² Recently, biodegradable thermosensitive polymeric micelles based on PEG-*b*-(pNIPAAm-*co*-(*N*-(2-hydroxypropyl)methacrylamide-dilactate) (HPMAm–Lac₂)) and PEG-*b*-(pHPMAm–Lac₂) were described.^{13,14} These block copolymers formed small (±55 nm) uniform micelles above the CP of the thermosensitive block. Under physiological conditions, the hydrophobic lactate side chains of the pHPMAm–Lac₂ in the micelles are hydrolyzed. Concomitantly, the CP gradually increases and passes 37 °C, which is associated with destabilization of the micelles. This process is characterized by an initial swelling of the micelles during the first 60 h due to an increasing hydrophilicity of the core, followed by dissolution of the micelles after approximately one week of incubation.¹⁴

The circulation time of nanoparticles in blood, even in the case of PEGylated systems, is restricted in time. With a few exceptions, all long-circulating polymeric nanoparticles are cleared from the systemic circulation of experimental animals within the first 8–10 h after intravenous administra-

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tion.^{7,15} This implies that the ideal carrier is stable for this time and destabilizes upon arrival at its site of action with a concomitant release of the loaded drug. Consequently, there is a need for fast degrading systems, which completely destabilize within approximately 24 h.

The aim of this study was to design thermosensitive block copolymers that form stable micelles at 37 °C but which will destabilize within 1 day under physiological conditions. From previous research, it is known that the hydrolysis kinetics of (2-hydroxyethyl)methacrylate-dilactate (HEMA-Lac₂) is five times faster than that of HPMAM-Lac₂.¹⁶ This kinetic difference is explained by the faster hydrolysis of an ester of a primary alcohol in HEMA-Lac₂ versus a secondary alcohol in the HPMAM-Lac₂. However, it was shown that HEMA-Lac₂ was unappropriated as a (co)monomer. The corresponding degraded polymer (HEMA (co)polymer) is not hydrophilic enough to have a CP above body temperature, which is a prerequisite for the present application.¹⁷ It was therefore anticipated that a methacrylamide with a primary alcohol function, viz. *N*-(2-hydroxyethyl)methacrylamide (HEMAM), could be more suitable. This paper describes the synthesis and degradation kinetics of *N*-(2-hydroxyethyl)methacrylamide (HEMAM)-lactate derivatives as well as the synthesis and characterization of (co)polymers of these derivatives. Furthermore, amphiphilic and temperature sensitive block copolymers with poly(ethylene glycol) were synthesized. The micelle forming properties of these block copolymers were studied and subsequently the degradation kinetics of the micelles.

Materials

Methacryloyl chloride, α,α -azoisobutyronitrile (AIBN), 4-methoxyphenol (99%), lithium chloride (LiCl), mono-methyl ether of poly(ethylene glycol), M_w is 5000 g/mol (mPEG₅₀₀₀), and pyrene were obtained from Fluka Chemie AG (Buchs, Switzerland). L-Lactide ((3*S*-*cis*)-3,6-dimethyl-1,4-dioxane-2,5-dione, >99.5%) was obtained from Purac Biochem BV (Gorinchem, The Netherlands). Ethanolamine (>99%), stannous 2-ethyl hexanoate (approximately 95%) and deuterated dimethyl sulfoxide, (99.9%, DMSO-*d*₆) were obtained from Acros (Zwijndrecht, The Netherlands). The PEG₂-macroinitiator with mPEG₅₀₀₀ was prepared with 4,4-azobis(4-cyanopentanoic acid) (ABCPA) as described previously.¹³ Diethyl ether (analytical grade) and triethylamine were obtained from Merck (Darmstadt, Germany). Acetonitrile (HPLC-S, gradient grade), dichloromethane, dimethylformamide (DMF), and 1,4-dioxane were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). 1,4-Dioxane was purified by distillation prior to use. All buffers were filtered through 0.2 μ m filter (Schleicher & Schuell MicroScience GmbH, Dassel, Germany) prior to use. The other chemicals were used as received.

Methods

Synthesis of HEMAM and HEMAM-Lac_n. *N*-(2-Hydroxyethyl)methacrylamide (HEMAM) was synthesized essentially as described by Song et al.¹⁸ In brief, freshly distilled

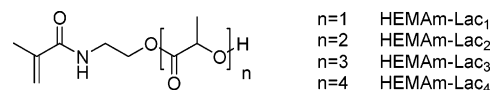


Figure 1. *N*-(2-Hydroxyethyl)methacrylamide (HEMAM)- oligolactates.

methacryloyl chloride (130 mL, 1.34 mol) dissolved in 250 mL of dichloromethane was added dropwise to an ice-cold solution of 2-aminoethanol (162 mL, 2.68 mol) and triethylamine (109 mL, 1.47 mole) in 250 mL of dichloromethane in 3 h under a nitrogen atmosphere. Subsequently, the mixture was stirred overnight at room temperature under a nitrogen atmosphere. The crude product was filtered and purified by flash chromatography using dichloromethane/methanol (85/15) as the eluent ($R_f = 0.45$).

The oligolactate derivatives of HEMAM were synthesized via the procedure as reported by Van Dijk-Wolthuis et al.¹⁹ Briefly, L-lactide (33.5 g; 0.233 mol) and HEMAM (20 g; 0.155 mol) were stirred at 110 °C until the lactide was molten. 4-Methoxyphenol (~0.1 mol % relative to HEMAM) was added as a radical scavenger. Subsequently, a catalytic amount of stannous 2-ethyl hexanoate (630 mg; 1 mol % relative to HEMAM) was added. The resulting mixture was stirred for 2 h and allowed to cool to room temperature. After dissolution of the product in 250 mL of water-acetonitrile (50:50), the HEMAM-oligolactate was fractionated with preparative chromatography essentially as described by Neradovic et al.¹⁶ In detail, the HPLC system (Waters Associates Inc., Milford, MA) consisted of a pump model 600, a variable wavelength absorbance detector model 2487, a sample manager model 2700, and a Xterra Prep MS C18 (10 μ m, 19 \times 250 mm i.d.) with a Xterra Prep MS C18 guard column (19 \times 10 mm) was used. The injection volume was 5 mL and the detection wavelength was 254 and 280 nm. A gradient was run using water/acetonitrile = 95:5 (w/w) (eluent A) and acetonitrile/water = 95:5 (w/w) (eluent B) from 0% B to 50% B in 35 min with a flow rate of 10.0 mL/min. Four fractions were collected, and after evaporation of the solvents, the identity of the obtained products, HEMAM mono-, di-, tri-, and tetralactate (further abbreviated as HEMAM-Lac₁, HEMAM-Lac₂, HEMAM-Lac₃, and HEMAM-Lac₄; see Figure 1), was established by NMR; the purity was assessed by HPLC (system as described below).

¹H NMR Spectroscopy. ¹H NMR spectra were recorded with a Gemini 300 MHz spectrometer (Varian Associates Inc., NMR Instruments, Palo Alto, CA). Spectra were obtained in DMSO-*d*₆. The central line of DMSO at 2.49 ppm was used as reference line.

Hydrolysis Kinetics of HEMAM-Oligolactates. The degradation studies of HEMAM-oligolactates were conducted according to the procedure as described by Neradovic et al.¹⁶ In brief, a 10 mM solution of HEMAM-oligolactate in DMSO was diluted 10 times with phosphate buffer pH 7.2 (100 mM) in a glass vial and the pH was adjusted to pH 7.4 with 4 M HCl. The resulting solutions of HEMAM-Lac₁, HEMAM-Lac₂, HEMAM-Lac₃, and HEMAM-Lac₄ were incubated in a water bath at 37 °C. At regular time intervals, samples of 300 μ L were withdrawn and 700 μ L of 1 M sodium acetate buffer (pH 3.8) was added to prevent further

hydrolysis. The samples were stored at 4 °C prior to HPLC analysis. The hydrolysis of HEMAm–Lac₃ and HEMAm–Lac₄ was also investigated in an acetonitrile–phosphate buffer pH 7.2 (100 mM) mixture (50:50 w/w) to compare the kinetic data with previous results.²⁰ HPLC analysis was carried out on a Waters system (Waters Associates Inc., Milford, MA) consisting of a pump model 600, an auto-injector model 717, a variable wavelength absorbance detector model 996, and an analytical reversed phase column LiChrosphere 100 RP-18 (5 μm, 125 × 4 mm i.d.) with a RP-18 guard column (4 × 4 mm) (Merck). The injection volume was 50 μL and the detection wavelength was 254 nm. After 5 min isocratic flow of water/acetonitrile = 95:5 (w/w), (eluent A), a gradient was run using 100% eluent A to 100% acetonitrile/water = 95:5 (w/w), (eluent B) in 30 min with a flow rate of 1.0 mL/min. The chromatograms were analyzed with Empower Software Version 1154 (Waters Associates Inc.). Calibration curves were generated for each monomer and for HEMAm with freshly prepared standard solutions in DMSO/pH 7.2 phosphate buffer (100 mM)/sodium acetate buffer pH 3.8 (1 M) (3:27:70) and were linear between 0.07 and 15 μM.

Synthesis of (co)-Polymers of HEMAm–Oligolactates. Homopolymers (HEMAm, HEMAm–Lac_n) and copolymers (mixtures of HEMAm–Lac₂ and HEMAm–Lac₄) were synthesized by free radical polymerization in airtight screw-cap glass vials. AIBN dissolved in 1,4-dioxane (ratio of monomers/initiator = 75–150 mol/mol) was added to 200 mg monomer dissolved in 1 mL of dioxane. After addition of the initiator, the solution was flushed with nitrogen for at least 10 min. Next, the solution was heated to 70 °C and stirred for 24 h. The polymers were precipitated by dropwise addition of the solution to an excess of diethyl ether. After centrifugation, the residue was dried overnight in a vacuum oven at 40 °C.

¹H NMR (DMSO, *d*₆): δ = 7.5 (b, CO–NH–CH₂), 5.5 (b, CH–OH), 5.0 (b, CO–CH(CH₃)–O), 4.1 (b, CO–CH(CH₃)–OH), 4.0 (b, CH₂–CH₂–O), 3.4 (b, NH–CH₂–CH₂), 1.4, (b, CO–CH–CH₃), 1.3 (b, HO–CH–CH₃), 1.0–0.6 (pHEMAm–Lac_n main chain protons).

The HEMAm–Lac₂/HEMAm–Lac₄ comonomer ratio (mol/mol) in the copolymer was determined by ¹H NMR from the ratio of the integral of the methine protons (*H*_m; δ = 5.0 ppm) to the alcoholic proton (*H*_{oh} δ = 5.5 ppm). The following equation was used:

$$\% \text{HEMAm-Lac}_4 = ((I_{H_m} - I_{H_{oh}})/2) \times 100\% \quad (1)$$

Synthesis of Poly(HEMAm–Lac_n)-*b*-PEG₅₀₀₀. Block copolymers with HEMAm–Lac_n as thermosensitive block and PEG as hydrophilic block were prepared by radical polymerization using PEG₂–ABCPA as macroinitiator (ratio of monomer/initiator = 150:1 mol/mol) according to the procedure as previously described for the synthesis of related block copolymers.^{13,14} The concentration of the starting materials (monomer plus macroinitiator) was 300 mg/mL in acetonitrile in airtight glass vials. The solution was flushed with nitrogen for at least 10 min, heated to 70 °C, and stirred for 24 h. Next, by dropwise addition of the solution to an excess of diethyl ether, the polymers were precipitated. After

centrifugation, the residue was dried overnight in a vacuum oven at 40 °C.

¹H NMR (DMSO, *d*₆): δ = 7.5 (b, CO–NH–CH₂), 5.5 (b, CH–OH), 5.0 (b, CO–CH(CH₃)–O), 4.1 (b, CO–CH(CH₃)–OH), 4.0 (b, CH₂–CH₂–O), 3.6 (b, PEG methylene protons, O–CH₂–CH₂), 3.4 (b, NH–CH₂–CH₂), 1.4, (b, CO–CH–CH₃), 1.3 (b, HO–CH–CH₃), 1.0–0.6 (pHEMAm–Lac_n main chain protons).

The number average molecular weight (*M*_n) of the thermosensitive block was determined by ¹H NMR as follows (in the situation of copolymers, an average molecular weight of the monomers was used):

$$M_n = M_{w_{ave}} (\text{HEMAm-Lac}_n) I_{\text{HEMAm-Lac}_n} / (I_{\text{PEG}}/454) \quad (2)$$

*I*_{HEMAm–Lac_n} is the value of the integral of the alcoholic proton of the HEMAm–Lac_n (*H*_{oh} δ = 5.5 ppm); *I*_{PEG} is the value of the integral of the PEG protons divided by average number of protons per one PEG₅₀₀₀ chain (=454).

Gel Permeation Chromatography (GPC). The molecular weights and their distribution of the different polymers were determined by GPC. Two serial Pgel 3 μm MIXED-D columns (Polymer Laboratories) were used with a Waters System (Waters Associates Inc., Milford, MA) with a differential refractometer model 410. Poly(ethylene glycol)s of defined molecular weights were used as standards. The eluent was DMF containing 10 mM LiCl.²¹ The samples were dissolved overnight at a concentration of 5 mg/mL in the eluent and filtered through a 0.45 μm filter prior to analysis. The flow rate was 0.7 mL/min and the column temperature was 40 °C. Peak areas were determined with Empower Software Version 1154 (Waters Associates Inc.).

Determination of the Cloud Point (CP) of (block co) Polymers. The CP of the synthesized (block co) polymers in aqueous solutions was determined with static light scattering using a Horiba Fluorolog fluorometer (650 nm, 90° angle).^{13,14,22} The polymers were dissolved overnight at 4 °C in ammonium acetate buffer (pH 5, 120 mM) at a concentration of 1 and 2 mg/mL for the copolymers and the block copolymers, respectively. The scattering intensity was measured every 0.2 °C during heating (heating rate approximately 1 °C/min) thereby stirring the solution in the cuvette. The onset on the *X* axis, obtained by extrapolation of the intensity–temperatures curves to intensity zero, was considered as the CP. The CP determinations were done at least two times and the deviations were smaller than 1 °C.

Micelle Formation. Micelles were formed via the quick heating procedure as described previously.¹⁴ In brief, the block copolymers were dissolved overnight at 4 °C at various concentrations in ammonium acetate buffer (pH 5, 120 mM). The solutions were incubated at 0 °C for at least 15 min in glass vials. Subsequently, the vials were rapidly heated from 0 to 50 °C by putting them into a water bath under vigorous stirring to form micelles. After 1 min of incubation at 50 °C, the mixtures were slowly cooled to room temperature and filtrated through a 0.22 μm filter.

Dynamic Light Scattering (DLS) and Static Light Scattering (SLS). Dynamic and static light scattering (DLS and SLS, respectively) were used to characterize the polymeric micelles. The equipment consisted of a Malvern CGS-3

multi-angle goniometer (Malvern Ltd., Malvern, U.K.) with He–Ne JDS Uniphase laser ($\lambda = 632.8$ nm, 22 mW output power), an optical fiber based detector, a digital LV/LSE–5003 correlator, and a temperature controller (Julabo Waterbath). Time correlation functions were analyzed using the ALV-60 \times 0 Software V.3.X provided by Malvern.

DLS provides the hydrodynamic radius using the Stokes–Einstein equation

$$R_h = (k_B T \cdot q^2) / (6\pi\eta\Gamma) \quad (3)$$

where k_B is the Boltzmann constant, q is the scattering vector, ($q = (4\pi n \sin(\theta/2)) / \lambda$, where n is the refractive index of the solution, θ is the scattering angle, and λ is the wavelength of the incident laser light), η is the solvent viscosity, and Γ is the decay rate.

Scattering of the micellar solutions was measured at an angle of 90° at 25°C in an optical quality 8 mL borosilicate cell giving rise to the hydrodynamic diameter ($Z_{\text{ave}} = 2R_h$) of the micelles and their polydispersity (PD). The destabilization behavior of the polymeric micelles was studied at 2 mg/mL in phosphate buffer (pH 7.4, 100 mM). A cell with approximately 1 mL micellar solution was incubated in the DLS machine at 37°C and measured at regular time intervals.

The radius of gyration (R_g) and the aggregation number (N_{agg}) are two important physical parameters commonly used to describe the characteristics of (polymeric) micelles. The scattered intensities of the polymer solutions at different angles (between 30° and 150°) and concentrations were analyzed by the graphical method first reported by Zimm.²³ This method involves simultaneously the extrapolation of the scattering data to both zero angle and zero concentrations. The relationship between the concentration and the intensity of the scattered light is given by the following equation:

$$\frac{Kc}{R(q)} = \frac{1}{M_w} \left(1 + \frac{1}{3} R_g^2 q^2 \right) + 2A_2c \quad (4)$$

where c is the concentration, M_w is the weight average molecular weight of the micelles, R_g is the radius of gyration, A_2 is the second virial coefficient, and $R(q)$ is the excess Rayleigh ratio of the solute (excess intensity of scattered light at scattering vector q).

The optical constant K is defined as

$$K = \frac{4\pi^2}{N_A \lambda^4} n_T^2 \left(\frac{dn}{dc} \right)^2 \quad (5)$$

where n_{tol} is the refractive index of toluene (1.494), (dn/dc) the specific refractive index increment of the micellar solution, N_A is the Avogadro's constant. The parameter dn/dc was here estimated to be 0.1 based on previous experiments on PEG-*b*-(HPMAm–Lac₂).¹⁴ The absolute excess time-averaged scattered intensity, i.e., Rayleigh ratio $R(q)$ is expressed by the equation

$$R(q) = R_{\text{tol},90} \left(\frac{n}{n_{\text{tol}}} \right)^2 \frac{I - I_0}{I_{\text{tol}}} \sin(\theta) \quad (6)$$

where $R_{\text{tol},90}$ is the Rayleigh ratio of toluene at an angle of

90° and amounts $13.7 \times 10^{-6} \text{ cm}^{-1}$, n is the refractive index of the solvent, I , I_0 , and I_{tol} are the scattered intensities of the solution, solvent, and toluene, respectively, and θ is the measurement angle.

Critical Micelle Concentration (CMC). The CMC of the block copolymers was determined using pyrene as a fluorescent probe.²⁴ Micelles of the different block copolymers were formed as described above in 120 mM ammonium acetate buffer (pH 5.0) at a concentration of 2 mg/mL. The micellar solutions were cooled to room temperature and subsequently diluted with the same buffer yielding different polymer concentrations ranging from 1×10^{-5} to 1 mg/mL. Next, 15 μL of pyrene dissolved in acetone (concentration 1.8×10^{-4} M) was added to 4.5 mL of polymer solution (final pyrene concentration 6×10^{-7} M). Samples were incubated for 15 h at room temperature in the dark to allow equilibration of the samples. Prior to the measurements, the solutions were incubated at 37°C for at least 15 min. Fluorescence excitation spectra of pyrene were obtained using a Horiba Fluorolog fluorometer (90° angle). The excitation spectra were recorded at 37°C from 300–360 nm with the emission wavelength at 390 nm. The excitation and emission band slits were 4 and 2 nm, respectively. The intensity ratio of I_{338}/I_{333} was plotted against the polymer concentration to determine the CMC.

Cryogenic Transmission Electron Microscopy (Cryo-TEM). Cryo-TEM measurements were performed on 2 mg/mL micellar solutions in 120 mM ammonium acetate buffer (pH 5.0). The sample preparation was done in a temperature and humidity controlled chamber using a “Vitrobot”. A thin aqueous film of micellar solution was formed by blotting a 200 mesh copper grid covered with Quantifoil holey carbon foil (Micro Tools GmbH, Germany) at 22°C and at 100% relative humidity (glow discharged grid; 1 blot during 0.5 s). The thin film was rapidly vitrified by quickly plunging the grid into liquid ethane. The grids with the vitrified thin films were transferred into the microscope chamber using a GATAN 626 cryo-holder system. A Tecnai12 transmission electron microscope (Philips) operating at 120 kV was used with the specimen at -180°C and using low-dose imaging conditions to avoid melting of the vitrified film. Images were recorded on a SIS–CCD camera and processed with AnalySIS software. Images were taken of micelles captured in the thin vitrified liquid film in the holes of the carbon foil to image them as undisturbed as possible.

Results

Synthesis of HEMAm and HEMAm–Lac_n. *N*-(2-Hydroxyethyl)methacrylamide (HEMAm) was synthesized by the reaction of ethanolamine with methacryloyl chloride.¹⁸ After column chromatography, the product was obtained in a high yield (85%) as a light-yellow oil and a high purity (>95%, NMR). Oligolactate esters of *N*-(2-hydroxyethyl)methacrylamide (HEMAm-oligolactate) were obtained by ring-opening oligomerization of L-lactide, using HEMAm as the initiator and stannous 2-ethyl hexanoate as a catalyst.¹⁷ HEMAm–Lac₁ to HEMAm–Lac₄ (Figure 1) were obtained with high purity and good yield ($\sim 50\%$) after fractionation with a preparative HPLC system.

Table 1. Degradation Kinetics of HEMAm–Oligolactates and Related Structures at 37 °C

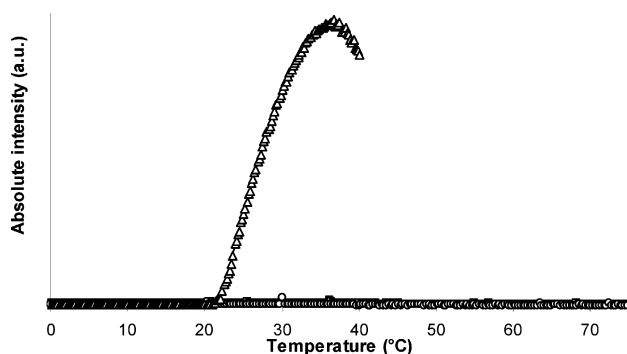
monomer	pH	cosolvent %	primary hydrolysis product	$t_{1/2}$ (h) ^a	ref
HEMAm–Lac ₁	7.4	10% DMSO	HEMAm	58	this work
HEMAm–Lac ₂	7.4	10% DMSO	HEMAm	5.6	this work
HEMAm–Lac ₃	7.4	10% DMSO	HEMAm–Lac ₁	0.63	this work
HEMAm–Lac ₄	7.4	10% DMSO	HEMAm–Lac ₂	0.49	this work
HEMAm–Lac ₃	7.4	50% ACN	HEMAm–Lac ₁	4.2	this work
HEMAm–Lac ₄	7.4	50% ACN	HEMAm–Lac ₂	3.4	this work
HEMA–Lac ₁	7.5	10% DMSO	HEMA	31	16
HEMA–Lac ₂	7.5	10% DMSO	HEMA	3	16
HPMAm–Lac ₁	7.5	10% DMSO	HPMAm	87	16
HPMAm–Lac ₂	7.5	10% DMSO	HPMAm	15	16
HPMAm–Lac ₇	7.4	50% ACN	HPMAm–Lac ₅	3.1	20
HPMAm–Lac ₁₂	7.4	50% ACN	HPMAm–Lac ₁₀	3.1	20

^a Experimental error is about 5%.

Degradation Kinetics of HEMAm–Oligolactates. The monodisperse HEMAm–oligolactates (Figure 1) hydrolyzed when incubated at pH 7.4 and at 37 °C and followed pseudo-first-order kinetics with HEMAm and lactic acid as final degradation products. The concentrations of HEMAm–Lac₁ to HEMAm–Lac₄ were determined by HPLC. From the concentration versus time plots, the half-lives ($t_{1/2}$) were determined (Table 1). The buffer solution used for the degradation studies also contained DMSO (10%) to solubilize the oligolactates. Therefore, the half-lives in pure water are expected to be less (approximately halved) than the reported values, as discussed by Neradovic.¹⁶ Since the formation of methacrylic acid was not detected, it is concluded that the amide bond in HEMAm (–oligolactates) is highly stable under the selected conditions. Table 1 shows that the half-lives of HEMAm–Lac₁ and HEMAm–Lac₂ are 58 and 5.6 h, respectively. The about 10 times faster degradation rate of the dilactate as compared to the monolactate was also observed for HEMA–lactates¹⁶ and can be explained by intramolecular transesterification, also known as “back-biting”.²⁵ The possibility of two lactic acid units to form an intermediate six-membered ring structure increases the hydrolysis rate. HEMAm–Lac₁ is not able to form a ring intermediate and is accordingly hydrolyzed with a slower rate.

At similar conditions (pH 7.5, 10% DMSO), the half-lives of the methacrylate analogues of HEMAm–lactates, i.e., (2-hydroxyethyl)methacrylate (HEMA) mono- and dilactate, were 31 and 3 h, respectively¹⁶ indicating that HEMAm–Lac₁ and HEMAm–Lac₂ are slightly more stable (half-lives 58 and 5.6 h, respectively). In contrast, the half-lives of HPMAm–Lac₁ and HPMAm–Lac₂ are 87 and 15 h, respectively. This higher stability of esters of secondary alcohols (HPMAm–Lac_n) compared to esters of primary alcohols (HEMA–Lac_n and HEMAm–Lac_n) can be ascribed to steric effects. It has been reported that the presence of increasingly branched aliphatic groups has a retarding influence on the alkaline catalyzed hydrolysis of esters.²⁵

HEMAm derivatives with three and four lactic acid units (HEMAm–Lac₃ and HEMAm–Lac₄) displayed faster hydrolysis kinetics than HEMAm–Lac_{1–2}. Again, this fast degradation is caused by the back biting mechanism. The HPLC analysis clearly showed that HEMAm–Lac_n is primarily converted into HEMAm–Lac_{n–2} as an intermediate product that is ultimately converted into HEMAm. To

**Figure 2.** Scattering behavior of p(HEMAm) (□), p(HEMAm–Lac₁) (○) and p(HEMAm–Lac₂) (△).**Table 2.** Characteristics of the Homopolymers of HEMAm–Lac_n

monomer	ratio M]/[I]	yield (%)	M_w (GPC)	M_w/M_n	CP (°C)
HEMAm	150:1	92	24000	3.7	>75
HEMAm–Lac ₁	150:1	71	53000	3.1	>75
HEMAm–Lac ₂	100:1	81	68000	3.0	22
HEMAm–Lac ₃	75:1	83	24000	3.3	< 0
HEMAm–Lac ₄	100:1	76	59000	2.6	< 0

compare our results with the half-lives obtained for the previously reported HPMAm–Lac₇ and HPMAm–Lac₁₂,²⁰ the degradation experiments were also carried out with acetonitrile as cosolvent. The half-lives of both HPMAm–Lac₇ and HPMAm–Lac₁₂ were 3.1 h, which are close to those of HEMAm–Lac₃ and HEMAm–Lac₄ (4.2 and 3.4 h, respectively). Taken together, the degradation data of the monomers showed that, as anticipated, the hydrolysis of HEMAm–Lac_n into HEMAm is faster than the time scale wherein HPMAm–Lac_n is converted into HPMAm.

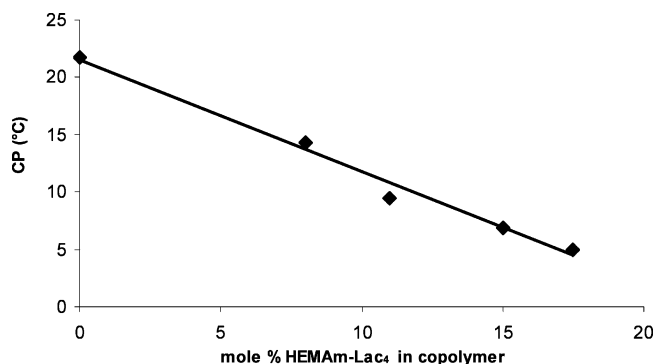
Homopolymers of HEMAm–Lac_n: Synthesis and Characterization. Free radical polymerization of HEMAm–Lac_n in dioxane yielded the corresponding polymers. Table 2 summarizes their properties.

pHEMAm and the pHEMAm–oligolactates were obtained in high yields (around 80%) under the selected polymerization conditions. The molecular weights were between 24 000 and 68 000 g/mol with a polydispersity (M_w/M_n) of about 3, which is standard for polymers synthesized by free radical polymerization. The thermosensitive properties of the polymers were investigated by static light scattering. To prevent hydrolysis of the lactic acid side chains, the polymers were dissolved in a pH 5 buffer at a concentration of 1 mg/mL. p(HEMAm–Lac₃) and p(HEMAm–Lac₄) did not dissolve at 0 °C, suggesting that the cloud point of these polymers is below this temperature. The scattering behavior of p(HEMAm), p(HEMAm–Lac₁), and p(HEMAm–Lac₂) solutions is displayed in Figure 2.

p(HEMAm) and p(HEMAm–Lac₁) were well soluble in water and did not show any scattering up to 75 °C. An aqueous solution of p(HEMAm–Lac₂) showed a sharp increase in scattering intensity when the temperature was increased above 22 °C, demonstrating that this polymer has temperature sensitive properties. Figure 2 shows that the scattering intensity decreases above 36 °C. The polymer chains start to become insoluble due to dehydration at the CP and collapse into loose aggregates. This is visualized by

Table 3. Characteristics of Copolymers $p(\text{HEMAM-Lac}_2)\text{-}(\text{HEMAM-Lac}_4)$

% HEMAM-Lac ₄ in the feed	yield (%)	% HEMAM-Lac ₄ incorporated ^a	M_w (GPC)	M_w/M_n	CP (°C)
6	79	8	71000	3.0	14.5
12	76	11	62000	2.8	9.5
15	88	15	71000	2.6	7.0
18	77	18	68000	3.0	5.0
24	76	23	61000	2.8	< 0

^a Derived from ¹H NMR.**Figure 3.** CP (°C) of copolymers of $p(\text{HEMAM-Lac}_2)\text{-}(\text{HEMAM-Lac}_4)$ as a function of the mole percentage of HEMAM-Lac₄.

the fast increase in scattering intensity. The further increase in temperature results in full dehydration with concomitant strong intra- and intermolecular interactions and the formation of more compact aggregates that scatter less than the loose aggregates. Recently, it was found that the related polymers $p(\text{HPMAM-Lac}_1)$ and $p(\text{HPMAM-Lac}_2)$ had a CP of 63 °C and 10 °C, respectively.²¹ HPMAM contains an extra methyl group, which makes it slightly more hydrophobic than HEMAM. In line herewith, $p(\text{HPMAM-Lac}_2)$ exhibits a lower CP than $p(\text{HEMA-Lac}_2)$.

Copolymers $p(\text{HEMAM-Lac}_2)\text{-}(\text{HEMAM-Lac}_4)$. For practical reasons, it was aimed to obtain a polymer with a cloud point below room temperature. As reported above, the homopolymer of HEMAM-Lac₂ displayed its CP at 22 °C. To lower the CP of $p(\text{HEMA-Lac}_2)$, copolymers of HEMAM-Lac₂ with the more hydrophobic monomer HEMAM-Lac₄ were synthesized (Table 3).

Table 3 shows that the yields and molecular weights were comparable with those of the homopolymers (Table 2). The copolymer composition corresponded with the feed ratio. Light scattering measurements of aqueous solutions of these copolymers indeed showed that the CP of the copolymers linearly decreased with the amount of hydrophobic HEMAM-Lac₄ that was incorporated (Figure 3).

From this figure, it can be extrapolated that copolymers with more than 22% of HEMAM-Lac₄ are not soluble in water at 0°. Indeed, the copolymer with 24% HEMAM-Lac₄ did not dissolve in water at 0 °C (Table 3).

Block Copolymers of PEG and HEMAM-Oligolactates. Block copolymers of PEG and HEMAM-oligolactates were synthesized by free radical polymerization using a PEG₅₀₀₀-substituted azomacroinitiator (Figure 4) as reported previously for the synthesis of related block copolymers.^{13,14}

A block copolymer of PEG and HEMAM-Lac₂ as well as a block copolymer with 20% HEMAM-Lac₄ and 80%

HEMAM-Lac₂ in the thermosensitive block were synthesized (Table 4).

Block copolymers were obtained in a high yield. ¹H NMR showed that the amount of HEMAM-Lac₄ incorporated in PEG- b -((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) was equal to the feed ratio (21%). Micelles were formed during heating of an ice-cold polymer solution at the cloud point (CP) of the thermosensitive part of the block copolymer, which is 6 °C for PEG- b -((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)). Previous research has shown that the smallest and relatively monodisperse micelles are formed by applying the so-called quick heating method.¹⁴ The rapid heating from 0–50 °C results in full dehydration of the thermosensitive blocks and promotes the formation of a compact hydrophobic micellar core. The particle size and particle size distributions are displayed in Table 4. The incorporation of 20% HEMAM-Lac₄ in the thermosensitive block did not only decrease the CP from 22 to 6 °C, but also caused a significant decrease in particle size (from 124 to 80 nm). Obviously, the longer hydrophobic lactic acid side chains increased the hydrophobic interactions and created a more compact micellar core. This observation is consistent with previous results from Soga et al.¹⁴

The morphology of PEG- b -((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) micelles was studied with Cryo-TEM. Figure 5, a representative microphotograph, shows the spherical shape of the micelles as well as their narrow particle size distribution. It should be mentioned that only the core is visible as the density of the PEG is too low.

The Cryo-TEM image shows that the radius of the hydrophobic core (R) of the micelles was between 10 and 23 nm, which corresponds to radius of gyration (R_g) ranging between 8 and 18 nm (using equation $R = \sqrt{(5/3)R_g}$). Neutron scattering experiments for related block copolymers (PEG₅₀₀₀- b -((HPMAM-Lac₂))) showed that the R_g of PEG₅₀₀₀ is approximately 10 nm,²⁷ making the values of R_g of the PEG- b -((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) micelles between 18 and 28 nm, which are in the range of the R_g measured by SLS ($R_g = 31.4$ nm; see section below).

The CMC was determined with pyrene as a fluorescent probe.²⁴ The CMC was determined from the plot of the excitation intensity ratio I_{338}/I_{333} as a function of the concentration of the block copolymer (Figure 6).

PEG- b -((HEMA-Lac₂)) had a CMC of 0.4 mg/mL (Table 4), whereas PEG- b -((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) formed micelles above a concentration of 0.08 mg/mL (Figure 6). This 5-fold lower CMC for the latter system is likely because of stronger hydrophobic interactions in the core. In comparison, PEG- b -((HPMAM-Lac₂)) had a CMC of 0.03 mg/mL.¹⁴ Again, due to the extra methyl group, HPMAM-Lac₂ is slightly more hydrophobic than HEMAM-Lac₂, which results in a lower CMC.

Using the quick heating procedure, micelles were prepared from various polymer concentrations above the CMC (0.2–20 mg/mL; Figure 7).

Soga et al. described that rapid dehydration of the thermosensitive segments takes place and thereby well-defined core-shell structures were formed via the quick heating procedure.¹⁴ Figure 7 shows that relatively large and

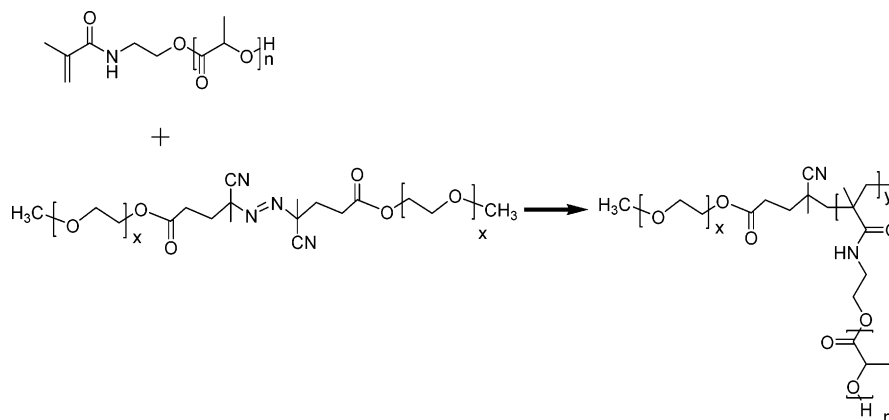


Figure 4. Synthesis of block copolymers PEG-*b*-(HEMAm-Lac_n) via PEG₅₀₀₀-azomacroinitiator.

Table 4. Characteristics of PEG-*b*-(HEMAm-Lac_n) Block Copolymers

block copolymer	yield (%)	M_n (GPC)	M_w/M_n	M_n Lac _n block (NMR)	CMT (°C) ^a	CMC (mg/mL)	micelle size (Z_{Ave}) ^a	micelle PD ^a
PEG- <i>b</i> -(HEMAm-Lac ₂)	69	28000	1.5	10800	22	0.4	124	0.2
PEG- <i>b</i> -((80%HEMAm-Lac ₂)-(20%HEMAm-Lac ₄))	85	24000	1.6	8700	6	0.08	80	0.1

^a 2 mg/mL solution.

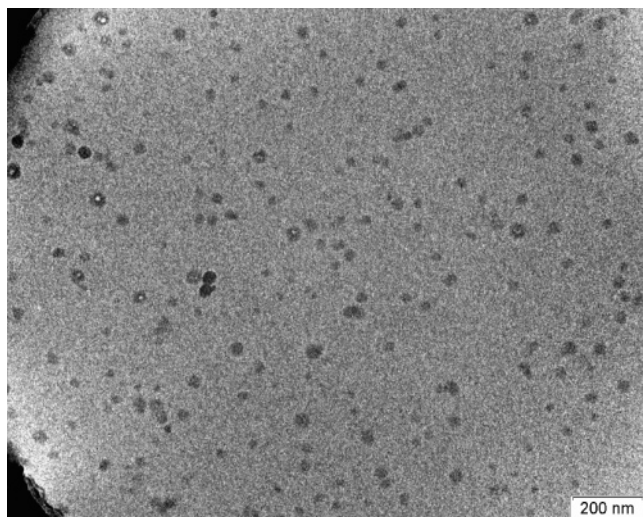


Figure 5. Cryo-TEM image of a 2% PEG-*b*-((80%HEMAm-Lac₂)-(20%HEMAm-Lac₄)) micellar solution.

polydisperse micelles are formed at concentrations below 0.5 mg/mL, which is close to the CMC. In the concentration range 0.5–10 mg/mL, the particle sizes of the micelles were relatively small (80 nm) and their polydispersity low (0.1–0.2). Above this concentration, the size and PD of the micelles slightly increases, which can be ascribed to a higher probability of interpolymer aggregation at higher polymer concentrations.²⁸

Static Light Scattering Analysis of Polymeric Micelles of PEG-*b*-((80%HEMAm-Lac₂)-(20%HEMAm-Lac₄)). Three micellar solutions of PEG-*b*-((80%HEMAm-Lac₂)-(20%HEMAm-Lac₄)) copolymers with concentrations 0.6, 0.8 and 1 mg/mL in pH 5 buffer were measured by SLS. By extrapolating the scattered intensities to zero concentration and by plotting ($Kc/R(q)$) against q^2 , both M_w and R_g were obtained from the “y” intercept to zero scattering angle and from the slope, respectively.²³

For PEG-*b*-((80%HEMAm-Lac₂)-(20%HEMAm-Lac₄)) micelles, the M_w was $27.0 \pm 1.7 \times 10^6$ g/mol and R_g was

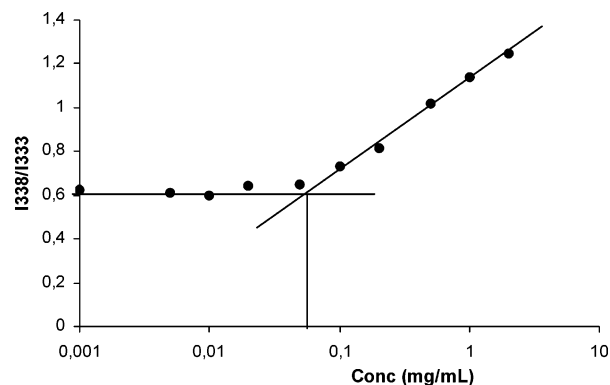


Figure 6. I_{338}/I_{333} ratio for pyrene as a function of the concentrations of PEG-*b*-((80%HEMAm-Lac₂)-(20%HEMAm-Lac₄)) in 120 mM acetate pH 5 buffer. The CMC was taken from the intersections of the horizontal line at low polymer concentrations with the tangent of the curve at high polymer concentrations.

equal to 31.4 ± 5.0 nm. The ratio R_g/R_h can be used to examine the morphology of the self-assembled micelles. With an R_h of 40 ± 2 nm (Table 4), an R_g/R_h of 0.785 ± 0.131 was calculated. Recently, an R_g/R_h ratio of 0.855 was reported for a core-shell structure;²⁹ for another core-shell structure a much lower value of R_g/R_h (0.66) was found and ascribed to a thick hydrated PEG shell.³⁰ The R_g/R_h value for PEG-*b*-((80%HEMAm-Lac₂)-(20%HEMAm-Lac₄)) micelles is between the values mentioned above, suggesting a core-shell structure for this system.

The aggregation number of the micelle was calculated using the equation $N_{agg} = M_w/M_{n,o}$, in which $M_{n,o}$ is the molar mass of the single copolymer chain obtained from ¹H NMR measurements (e.g., the M_n for PEG-*b*-((80%HEMAm-Lac₂)-(20%HEMAm-Lac₄)) is 13700) and M_w is the weight average molecular weight of the micelle obtained from SLS. For the PEG-*b*-((80%HEMAm-Lac₂)-(20%HEMAm-Lac₄)) micelles, N_{agg} is 1970.

The density of the micelle was calculated: $\rho_{mic} = M_w(mic)/N_a V$, where N_a is Avogadro's number and V is the average

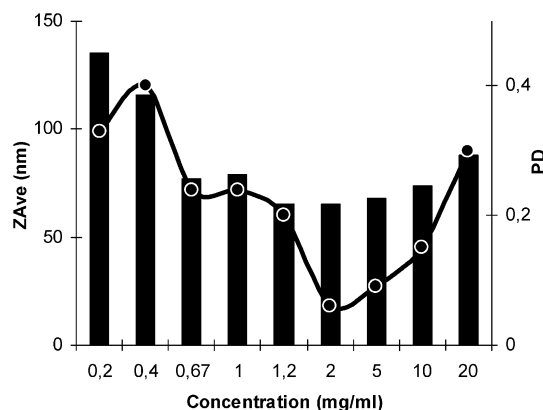


Figure 7. Size (bars) and polydispersity (dots) of PEG-*b*-((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) micelles solutions versus the block copolymer concentration. The measurements were done in duplo and experimental error was less than 10 percent.

volume of micelles (V was calculated via R_h). The surface area of the micellar shell available per PEG chain (S/N_{agg}) was calculated by dividing S (surface area of the shell of micelles calculated based on R_h) by N_{agg} . For the PEG-*b*-((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) micelles, the ρ_{mic} and S/N_{agg} are 0.167 g/cm³ and 10.2 nm², respectively. PEG₅₀₀₀-*b*-(HPMAM-Lac₂) (M_n is 11 900) micelles gave comparable results with a ρ_{mic} of 0.16 g/cm³ and S/N_{agg} 12.7 nm.¹⁴ The small surface area per PEG chain (i.e., higher grafting density) indicates that the micelles have a compact structure when compared to other micellar systems, e.g., PEG-PLA copolymers.³¹

The distance between PEG chains on the surface of nanoparticles (d) is critical to avoid adsorption of plasma proteins. For instance, it has been reported that a decrease in the distance between PEG chains on the surface of polystyrene from 6.2 to 5.1 nm drastically decreases the adsorption of apolipoproteins up to 90%.³² The distance d can be calculated via $\sqrt{(4S)/\pi}$. For the PEG-*b*-((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) micelles, it was calculated that the distance between neighboring PEG chains is 3.6 nm which will likely prevent adsorption of serum proteins.

Destabilization of the Micelles. Micelles prepared from PEG-*b*-((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) were incubated in a phosphate buffer of pH 7.4 and at 37 °C while following their size in time by DLS (Figure 8). The micelles were also incubated at pH 5 to slow hydrolysis.

Figure 8 shows that the micelles were stable for at least 18 h at pH 5. At pH 7.4 and 37 °C, the particle size hardly changed during the first 4 h, followed by a swelling phase until 6 h. After that period, the micelles started to dissolve as seen by the measured scattering intensity that first increased (during 4 till 6 h of incubation) and then dropped until below detectable levels (after 8 h). Previously, Soga et al. showed that PEG-*b*-(HPMAM-Lac₂) micelles dissolved after approximately one week incubation at the same (physiological) conditions.¹⁴ It was shown above (Table 1) that HEMAM-oligolactates hydrolyzed faster than the corresponding HPMAM-oligolactate monomers. In line with these results, polymeric micelles based on PEG-*b*-((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) are far more hydro-

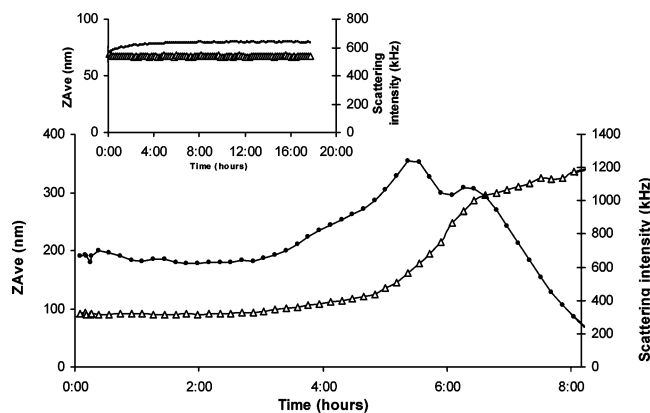


Figure 8. Size (Δ) and scattering intensity (\bullet) of a 2 mg/mL PEG-*b*-((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) micellar solution versus time at pH 7.4 and 37 °C. The insert shows the size (Δ) and scattering intensity (\bullet) of micelles incubated at pH 5 and 37 °C.

lytically sensitive than the related HPMAM system and fully destabilized within 8 h.

Conclusion

This study shows that, as anticipated, *N*-(2-hydroxyethyl)-methacrylamide-oligolactates (HEMAM-Lac_{*n*}) are more rapidly hydrolyzed than *N*-(2-hydroxypropyl)methacrylamide-oligolactates (HPMAM-Lac_{*n*}). Thermosensitive (block co)polymers of HEMAM-oligolactates were synthesized in high yields by free radical polymerization. It appeared that the cloud point was influenced by the composition of the HEMAM-oligolactates copolymer. Furthermore, the hydrophobicity of the thermosensitive block p(HEMAM-Lac₂) did not only influence the CP, but also the CMC and the micellar particle size. Incorporation of 20% of HEMAM-Lac₄ in the thermosensitive block resulted in a low CP (6 °C), a low CMC (0.08 mg/mL) and small micelles (80 nm). Static light scattering measurements and Cryo-TEM pictures showed that the micelles have a compact core with a concomitant high density of PEG chains on the surface that are a prerequisite to prevent the adsorption of serum proteins. Under physiological pH and temperature, micelles based on PEG-*b*-((80%HEMAM-Lac₂)-(20%HEMAM-Lac₄)) started to swell after 4 h and were fully destabilized after 8 h. This unique destabilization profile might be advantageous for *in vivo* use because the observed induction period is just long enough to allow accumulation of the micelles after intravenous administration at their site of action, e.g., a tumor via the EPR effect. The drug loading capacity and release behavior of these novel thermosensitive fast degrading polymeric micelles is currently under investigation.

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