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Synthesis and characterization of tailorable biodegradable thermoresponsive methacryloylamide polymers based on L-serine and L-threonine alkyl esters

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ABSTRACT

A series of monomers based on the methyl, ethyl, and isopropyl esters of N^{α}-(methacryloyl)-serine and -threonine were synthesized, and used in an AIBN-initiated radical polymerization reaction to yield polymers with an M_n ranging between 6.6 and 23.8 kDa. The newly synthesized polymers showed LCST behavior in aqueous solution that could be tailored by subtle variations of the hydrophobicity of the monomers to obtain a broad range of cloud points between 1.5 and >100°C. According to HPLC, the hydrolytic $t_{1/2}$-values (pH 7.4 at 37°C) of the monomers were found to be 5, 12, and 40 days of the methyl, ethyl, and isopropyl esters, respectively, while the hydrolysis rate of poly[N^{α}-(methacryloyl)-Ser-OMe] and poly[N^{α}-(methacryloyl)-Thr-OMe] was found to be significantly lower compared to the corresponding monomers. In order to obtain thermoresponsive nanoparticles, N^{α}-(methacryloyl)-Thr-OEt was polymerized with (PEG monomethyl ether 5000)$_2$-ABCPA as macroinitiator to yield an amphiphilic block co-polymer, poly[N^{α}-(methacryloyl)-Thr-OEt]-b-(PEG monomethyl ether 5000), which forms particles of 300 nm at a temperature higher than its cloud point of 24°C. Incubation at physiological conditions induced ester hydrolysis resulting in a destabilization of the particles making these particles suitable for drug delivery purposes.

1. Introduction

Thermoresponsive polymers with a lower critical solution temperature (LCST) are soluble in aqueous solution below a certain temperature referred to as the cloud point (CP), but precipitate above this temperature due to dehydration of the polymer chains [1]. Thermoresponsive polymers with a CP around physiological temperature are presently under investigation for biomedical and pharmaceutical applications and can be used for the design of thermosensitive drug delivery systems such as polymeric micelles [2–5] and hydrogels [6,7]. In an ideal polymeric micellar delivery system, the drug retains entrapped during transport through the bloodstream, and will be released only after reaching the site of action. These principally conflicting requirements can be approached by developing polymeric micelles that gradually become more hydrophilic resulting into a destabilization of the micelles and therefore in a controlled release of the entrapped drug. The incorporated biodegradability renders thermoresponsive polymers suitable for clinical applications.

One approach to induce biodegradability is the introduction of ester moieties in the polymeric side chains. Studies by Neradovic et al. [8,9] have shown that co-polymers consisting of *N*-isopropyl acrylamide (NIPAAm) and 2-hydroxyethyl methacryloyl monolactate (HEMA-monolactate) behave as thermoresponsive polymers with a tunable CP. By increasing the HEMA-monolactate content, the CP decreases, however, upon hydrolysis of the lactate ester, the CP gradually increases during time [10–12]. Another approach makes use of amino acid-based *N*-acryl amide-derived polymers. The thermoresponsive behavior of such polymers depends on the hydrophobic/hydrophilic balance of the polymer and in case of amino acid esters (e.g. alanine [13,14] and proline moieties [15–17]), hydrolysis of the ester results in partial biodegradability as well as in a shift of the hydrophobic/hydrophilic balance and thus in a variable CP during the time of incubation.

In the present study it was our aim, to design and synthesize a series of novel thermoresponsive polymers based on N^{α}-methacryloyl serine and threonine alkyl esters with tailorable CPs and

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degradation kinetics. Subtle variations of the hydrophobicity, either in the amino acid side chain (serine/threonine) or ester moiety (methyl/ethyl/isopropyl), lead to tailorable thermosensitive behavior. Since ester hydrolysis is strongly dependent on the alkyl chain, differences in hydrolysis rate of the methyl/ethyl/isopropyl ester will be used to control the degradation kinetics of the polymers.

2. Experimental

2.1. Materials and general procedures

2,2′-Azobis(isobutyronitrile) (AIBN) and serine (H-Ser-OH) were purchased from Acros Organics. Thionyl chloride, threonine (H-Thr-OH) and methacryloyl chloride were purchased from Fluka. Peptide synthesis grade *N*,*N*-dimethylformamide (DMF), MeOH, EtOH, 2-propanol and dioxane were obtained from Biosolve. Triethylamine was purchased from Merck. Solvents used for extractions and column chromatography were distilled prior to use. Thin layer chromatography (TLC) was performed on Merck silica gel 60 F-254 plates. Spots were visualized by UV-quenching, ninhydrin, *N*,*N*,*N*′,*N*′-tetramethyl-4,4′-diaminodiphenylmethane (TDM)/Cl_2 [18], $KMnO_4$. Silicycle silica 60 Å (particle size 41–63 μm) was used for column chromatography. ^{1}H NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer and chemical shifts are given in ppm (δ) relative to the internal reference tetramethylsilane (TMS) (0.00 ppm). ^{13}C NMR spectra were recorded at 75.5 MHz and chemical shifts are given in ppm (δ) relative to either $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.5 ppm). Analytical RP-HPLC runs were carried out on a Shimadzu automated HPLC system equipped with a UV/VIS detector operating at $\lambda = 214$ and 254 nm and using an Alltech Prosphere C18 column (250 × 4.6 mm, particle size: 5 μm, pore size: 300 Å) at a flow rate of 1 mL/min using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 min.

2.2. Characterization methods

2.2.1. Gel permeation chromatography (GPC)

GPC was performed on a Waters 2695 Controller equipped with a refractive index detector (model 2414). The analyses were run on a PLgel MIXED-D column (particle size: 5 μm) (Polymer Laboratories) at 40°C using 10 mM LiCl in DMF as the mobile phase at a flow rate of 0.7 mL/min. The polymers were dissolved in DMF (containing 10 mM LiCl) at a concentration of 5 mg/mL for at least 24 h before filtering them through a 0.45 μm filter prior analysis. The samples were analyzed and calibration was done using PEG as standards (M: 194-439,600 g/mol; Polymer Laboratories). Peak areas were determined with Empower Software Version 1154 (Waters Associates Inc.).

2.2.2. Cloud point (CP) determination

Cloud point measurements were preformed on a Shimadzu UV-2450 UV/VIS spectrophotometer. Polymer sample solutions were prepared by dissolving 1 mg of polymer per mL H_2O. The samples were placed in a cuvette, heated from 0°C to 95°C and then cooled to 0°C while measuring the absorption at 450 nm at a heating/cooling rate of 1°C/min. The cloud point was determined by extrapolating the slope of the absorption increase to zero absorption. Another series (compounds **10a**, **11**, and **22**) was measured in PBS-buffer (containing 0.15 M NaCl, 0.01 M $Na_2HPO_4.12H_2O$, 0.002 M $NaH_2PO_4.2H_2O$ at pH 7.4) and PBS-buffer containing 10% foetal calf serum. CP determinations in PBS-buffer were measured from 0°C to 40°C, as described above. PBS buffer alone became turbid above 40°C, and as a consequence of this, polymers with a CP >40°C (as determined in plain water) could not be analyzed.

2.2.3. Dynamic light scattering (DLS)

DLS was performed on a Malvern CG-3 multi-angle goniometer (Malvern Ltd., Malvern, U.K.) equipped with a 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-60X0 Software V.3.X provided by Malvern. Scattering of the micellar dispersions was measured at an angle of 90° in an optical quality 8 mL borosilicate cell. A cell with approximately 1 mL micellar dispersion (1 mg/mL) was incubated in the DLS apparatus and measured at regular time intervals between 17 and 25°C.

2.2.4. Differential scanning calorimetry (DSC)

DSC was carried out on a Q1000 differential scanning calorimeter (TA Instruments). For the DSC measurement, the samples were heated (10°C/min) from room temperature to 150°C. Then, these samples were cooled to 10°C and subsequently heated (10°C/min) for a second time to 150°C.

2.3. Synthesis

2.3.1. Monomer synthesis

*2.3.1.1. HCl.H-Ser-OMe (**3a**).* In a typical experiment, MeOH (200 mL) was cooled on ice and $SOCl_2$ (18 mL, 250 mmol) was added dropwise. After the addition was complete, H-Ser-OH (**1**) (5.25 g, 50 mmol) was added as a single portion and the reaction mixture was stirred 1 h at 0°C followed by 16 h at room temperature. Then, the reaction mixture was concentrated to an oil, which was triturated with ice-cold diethyl ether to give methyl ester **3a** as a white solid in 84% yield (6.6 g). $R_f = 0.87$ ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.74 (s, 3H, OCH_3), 3.83 (s, 2H, βCH_2), 4.09 (m, 1H, αCH), 5.64 (broad s, 1H, OH), 8.60 (br s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 53.1, 54.3, 59.8, 168.8.

*2.3.1.2. HCl.H-Ser-OEt (**3b**).* R_f = 0.87 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.24 (*t* (*J* 7.0 Hz), 3H, OCH_2CH_3), 3.83 (s, 2H, βCH_2), 4.07 (*t* (*J* 3.4 Hz), 1H, αCH), 4.20 (m, 2H, OCH_2CH_3), 5.62 (broad s, 1H, OH), 8.55 (broad s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 13.9, 54.3, 59.5, 61.7, 168.0.

*2.3.1.3. HCl.H-Ser-OiPr (**3c**).* R_f = 0.89 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.24 (d (*J* 2.8 Hz), 6H, $OCH(CH_3)_2$), 3.81 (s, 2H, βCH_2), 4.02 (m, 1H, αCH), 4.99 (m, 1H, $OCH(CH_3)_2$), 5.61 (broad s, 1H, OH), 8.52 (broad s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 21.4, 54.4, 59.5, 69.5, 167.5.

*2.3.1.4. HCl.H-Thr-OMe (**4a**).* R_f = 0.92 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.20 (d (*J* 6.3 Hz), 3H, γCH_3), 3.75 (s, 3H, OCH_3), 3.93 (d (*J* 3.9 Hz), 1H, βCH), 4.13 (m, 1H, αCH), 5.65 (broad d (*J* 5.0 Hz), 1H, OH), 8.40 (broad s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 20.0, 52.7, 57.9, 65.0, 168.7.

*2.3.1.5. HCl.H-Thr-OEt (**4b**).* R_f = 0.88 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.21 (d (*J* 6.6 Hz), 3H, γCH_3), 1.24 (*t* (*J* 7.0 Hz), 3H, OCH_2CH_3), 3.90 (m, 1H, βCH), 4.13 (m, 1H, αCH), 4.21 (q (*J* 7.2 Hz), 2H, OCH_2CH_3), 8.39 (broad s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 13.9, 20.0, 57.8, 61.7, 65.0, 168.1.

*2.3.1.6. HCl.H-Thr-OiPr (**4c**).* R_f = 0.92 ($CHCl_3$/MeOH/25% NH_4OH 8:4:1.5 v/v/v). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.26 (m, 9H, OCH $(CH_3)_2$ (6H)/γCH_3 (3H)), 3.83 (d (*J* 4.13), 1H, αCH), 4.11 (br s, 1H,

βCH), 5.01 (m, 1H, OC*H*(CH_3)$_2$), 5.64 (broad d (*J* 4.4 Hz), 1H, OH), 8.35 (broad s, 3H, NH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 20.1, 21.3, 21.5, 57.9, 65.2, 69.5, 167.6.

2.3.1.7. N$^{\alpha}$-(Methacryloyl)-serine methyl ester (MA-Ser-OMe, ***5a****).* In a typical experiment, to a vigorously stirred and ice-cold solution of HCl.H-Ser-OMe (**3a**) (6.7 g, 43 mmol) in dioxane/H_2O 1:1 v/v (300 mL), methacryloyl chloride (4.2 mL, 43 mmol) was added dropwise and the pH of the reaction mixture was kept between 8 and 9 by the addition of Et_3N (18 mL, 129 mmol). After the addition was complete, the reaction mixture was stirred for 16 h at room temperature. Then, the solvents were removed by evaporation and the residue was dissolved in Et_2O. The remaining solids were removed by filtration and washed with Et_2O. The combined filtrate was concentrated in vacuo and the residue was purified by column chromatography (hexane/Et_2O 1:1 v/v → Et_2O) to give MA-Ser-OMe (**5a**) as a colorless oil in 33% yield (2.5 g). R_f = 0.19 (Et_2O). R_t = 12.98 min (*C*18). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.99 (s, 3H, CH_3), 3.05 (broad s, 1H, OH), 3.80 (s, 3H, OCH_3), 3.96 (m, 2H, βCH_2), 4.71 (m, 1H, αCH), 5.42 (s, 1H, H_2C=(1H)), 5.81 (s, 1H, H_2C=(1H)), 6.82 (broad d (*J* 6.3 Hz), 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 18.7, 52.9, 55.1, 62.8, 121.3, 139.4, 169.3, 171.4.

2.3.1.8. N$^{\alpha}$-(Methacryloyl)-serine ethyl ester (MA-Ser-OEt, ***5b****).* R_f = 0.31 (Et_2O). R_t = 15.37 min (*C*18). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.31 (*t* (*J* 7.0), 3H, OCH_2CH_3), 1.99 (s, 3H, CH_3), 3.98 (m, 2H, βCH_2), 4.26 (q (*J* 6.5), 2H, OCH_2CH_3), 4.69 (m, 1H, αCH), 5.42 (s, 1H, H_2C=(1H)), 5.82 (s, 1H, H_2C=(1H)), 6.84 (broad d (*J* 6.1 Hz), 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.7, 18.0, 54.5, 61.4, 62.4, 120.5, 138.8, 168.4, 170.2.

2.3.1.9. N$^{\alpha}$-(Methacryloyl)-serine isopropyl ester (MA-Ser-OiPr, ***5c****).* R_f = 0.47 (Et_2O). R_t = 17.70 min (*C*18). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.89 (dd (J_{ax} 2.2, J_{bx} 6.3 Hz), 6H, OCH(CH_3)$_2$), 2.00 (m, 3H, CH_3), 3.04 (broad s, 1H, OH), 3.97 (d (*J* 3.9 Hz), 2H, βCH_2), 4.64 (m, 1H, αCH), 5.10 (septet (*J* 6.3 Hz), 1H, OC*H*(CH_3)$_2$), 5.41 (s, 1H, H_2C=(1H)), 5.82 (s, 1H, H_2C=(1H)), 6.82 (broad d (*J* 6.1 Hz), 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 18.7, 21.9, 55.4, 63.9, 70.1, 121.0, 139.4, 168.9, 170.2.

2.3.1.10. N$^{\alpha}$-(Methacryloyl)-threonine methyl ester (MA-Thr-OMe, ***6a****).* R_f = 0.28 (Et_2O). R_t = 14.92 min (*C*18). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.24 (d (*J* 6.6 Hz), 3H, γCH_3), 2.01 (*t* (*J* 1.2 Hz), 3H, CH_3), 2.29 (broad s, 1H, OH), 3.79 (s, 3H, OCH_3), 4.39 (m, 1H, βCH), 4.66 (m, 1H, αCH), 5.42 (s, 1H, H_2C=(1H)), 5.81 (s, 1H, H_2C=(1H)), 6.60 (broad d, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 18.8, 20.2, 52.9, 57.5, 68.3, 120.8, 139.7, 169.0, 171.8.

2.3.1.11. N$^{\alpha}$-(Methacryloyl)-threonine ethyl ester (MA-Thr-OEt, ***6b****).* R_f = 0.47 (Et_2O). R_t = 17.10 min (*C*18). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.26 (m, 6H, OCH_2CH_3 (3H)/γCH_3 (3H)), 2.01 (*t* (*J* 0.8 Hz), 3H, CH_3), 2.24 (broad s, 1H, OH), 4.24 (q (*J* 7.2 Hz), 2H, OCH_2CH_3), 4.37 (broad d (*J* 3.0 Hz), 1H, αCH), 4.64 (m, 1H, βCH), 5.41 (s, 1H, H_2C=(1H)), 5.80 (s, 1H, H_2C=(1H)), 6.57 (broad d (*J* 7.7 Hz), 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 14.0, 18.5, 20.0, 57.7, 61.5, 67.7, 120.5, 139.4, 169.2, 170.9.

2.3.1.12. N$^{\alpha}$-(Methacryloyl)-threonine isopropyl ester (MA-Thr-OiPr, ***6c****).* R_f = 0.63 (Et_2O). R_t = 19.33 min (*C*18). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.26 (m, 9H, OCH(CH_3)$_2$ (6H)/γCH_3 (3H)), 2.01 (m, 3H, CH_3), 2.12 (broad s, 1H, OH), 4.36 (m, 1H, αCH), 4.62 (m, 1H, βCH), 5.10 (septet (*J* 6.2 Hz), 1H, OC*H*(CH_3)$_2$), 5.41 (s, 1H, H_2C=(1H)), 5.80 (s, 1H, H_2C=(1H)), 6.55 (broad d (*J* 8.0 Hz), 1H, NH,). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 18.7, 20.2, 21.8, 57.8, 68.1, 69.5, 120.5, 139.6, 169.2, 170.6.

2.3.2. Polymerization reactions

2.3.2.1. Homopolymer synthesis. The monomer (0.5 g) and AIBN (monomer/initiator (M/I) molar ratio was 100/1) were dissolved in dry DMF (5 mL) and the solution was degassed by three freeze-pump-thaw cycles. The reaction mixture was stirred for 48 h at 70°C after which the solvent was removed in vacuo and the residue was dissolved in MeOH (5 mL) and precipitated with ice-cold Et_2O (40 mL). The precipitate was collected by centrifugation (3000 rpm, 5 min) and the solvent was decanted. After drying in vacuo, the polymers were obtained as white solids in a yield of 82–96%.

2.3.2.2. Co-polymer synthesis. To an equimolar mixture of co-monomer$_1$ and co-monomer$_2$ (1.5 mmol), AIBN (5 mg, 30 μmol) was added and the solid compounds were dissolved in dry DMF (5 mL) and the obtained solution was degassed by three freeze-pump-thaw cycles. The reaction mixture was stirred for 48 h at 70°C after which the solvent was removed in vacuo and the residue was dissolved in $CHCl_3$ (5 mL) and precipitated with ice-cold hexane (40 mL). The precipitate was collected by centrifugation (3000 rpm, 5 min) and the solvent was decanted. After drying in vacuo, the co-polymers were obtained as white solids in a yield of 32–95%.

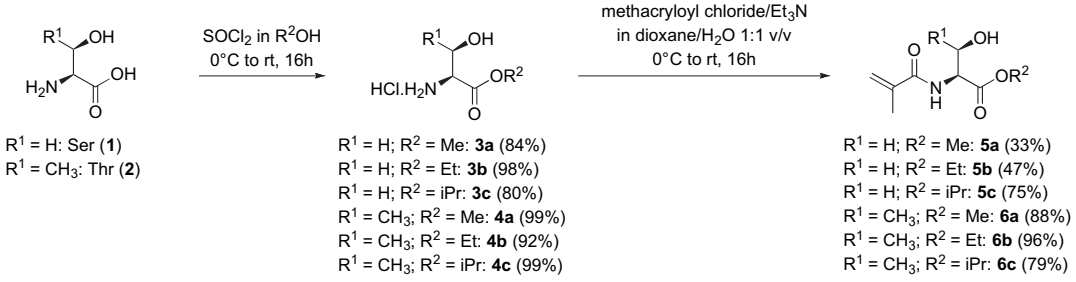
2.4. Degradation studies

2.4.1. Degradation of the monomers

The monomers **5a**–**c** and **6a**–**c** (15 mg) were dissolved in 100 mM sodium phosphate buffer pH 7.4 (5 mL) and incubated at 37°C; during the experiment the pH was checked to ensure the same start- and end-value. At regular time intervals a sample (100 μL) was drawn and immediately quenched with ice-cold 1 M sodium acetate buffer pH 3.8 (200 μL) and stored at 4°C prior to HPLC analysis. The $t_{1/2}$-values were determined by plotting the ln(AUC) versus time (AUC: area under the curve).

2.4.2. Degradation of the polymers

The homopolymers **7** and **8** (5 mg) were dissolved in a D_2O/sodium phosphate buffer (100 mM, pH 7.4, 5 mL) and incubated at 37°C. The degradation of the polymer was followed by ^{1}H NMR spectroscopy by monitoring the decrease of the COOC*H*$_3$ peak (δ:

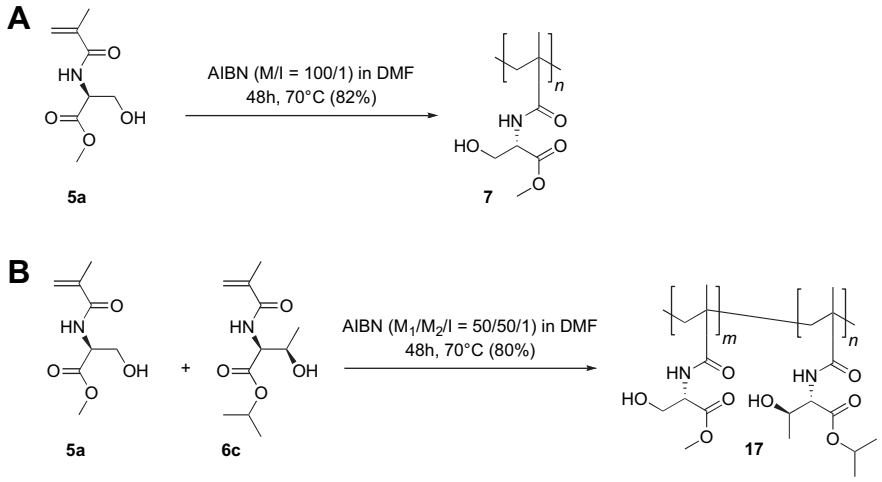


R^1 = H: Ser (**1**)
R^1 = CH_3: Thr (**2**)

R^1 = H; R^2 = Me: **3a** (84%)
R^1 = H; R^2 = Et: **3b** (98%)
R^1 = H; R^2 = iPr: **3c** (80%)
R^1 = CH_3; R^2 = Me: **4a** (99%)
R^1 = CH_3; R^2 = Et: **4b** (92%)
R^1 = CH_3; R^2 = iPr: **4c** (99%)

R^1 = H; R^2 = Me: **5a** (33%)
R^1 = H; R^2 = Et: **5b** (47%)
R^1 = H; R^2 = iPr: **5c** (75%)
R^1 = CH_3; R^2 = Me: **6a** (88%)
R^1 = CH_3; R^2 = Et: **6b** (96%)
R^1 = CH_3; R^2 = iPr: **6c** (79%)

Scheme 1. Synthesis of the monomers **5a**–**c** (serine-based) and **6a**–**c** (threonine-based).



Scheme 2. Synthesis of the polymers. A. Homopolymer based on **5a** (entry 1, Table 1). B. Co-polymer based on **5a** and **6c** (entry 13, Table 1).

3.64 ppm) and the increase of the CH_3OD peak (δ: 3.20 ppm) during time. The *H*DO peak ((δ: 4.64 ppm) was used as an internal reference.

2.5. Nanoparticles of a thermoresponsive amphiphilic block co-polymer

2.5.1. Synthesis of poly(MA-Thr-OEt)-b-(PEG monomethyl ether 5000) (*26*)

N^{α}-(methacryloyl)-Thr-OEt **6b** (493 mg, 2.30 mmol) and (PEG monomethyl ether 5000)$_2$-ABCPA **25** [19] (238 mg 0.023 mmol) were dissolved in dry DMF (5 mL) and the solution was subjected to three freeze-pump-thaw cycles for degassing and the reaction mixture was stirred at 70°C for 72 h. Then, the solvent was removed under reduced pressure and the residue was dissolved in $CHCl_3$ (5 mL) and the polymer was precipitated with ice-cold hexane (40 mL). The precipitate was collected by centrifugation (3000 rpm, 5 min) and the solvent was decanted. After drying in vacuo, the amphiphilic co-polymer was obtained as a white solid in 63% yield (0.46 g).

2.5.2. Particle preparation and particle destabilization studies of poly(MA-Thr-OEt)-b-(PEG monomethyl ether 5000)

A sample of poly(MA-Thr-OEt)-b-(PEG monomethyl ether 5000) co-polymer (3 mg) was dissolved in ice-cold sodium phosphate buffer (100 mM, pH 7.4, 3 mL) containing 0.02% NaN_3. The solution was stirred and rapidly heated until the temperature was above CP. Then, the polymer solution was incubated at 37°C (pH 7.4) and the particle size and mean count rate was determined at regular time intervals by dynamic light scattering.

Table 1
Characterization of the polymers.[a]

Entry	Compound	Polymer [b]	Yield %	M_n [c]kDa	M_w [c]kDa	PDI [d]	CP [e]°C	CP[f]°C	calcd CP [g]°C	T_g°C
1	**7**	poly(MA-Ser-OMe)	82	15.7	29.7	1.9	>100	–	–	>150
2	**8**	poly(MA-Thr-OMe)	90	12.3	22.4	1.8	64.5	–	–	83
3	**9**	poly(MA-Ser-OEt)	96	14.4	25.0	1.7	49.5	–	–	101
4	**10a**	poly(MA-Thr-OEt)	84	12.6	20.2	1.7	24.0	18.5 (18.5)	–	89
5	**10b**	poly(MA-Thr-OEt) [h]	81	6.6	12.1	1.8	24.5	–	–	83
6	**10c**	poly(MA-Thr-OEt)[i]	78	23.8	46.7	2.0	19.5	–	–	92
7	**11**	poly(MA-Ser-OiPr)	90	13.8	26.9	1.9	6.5	3.5 (2.5)	–	91
8	**12**	poly(MA-Thr-OiPr)	88	9.8	16.7	1.7	1.5	–	–	92
9	**13**	poly(MA-Ser-OMe-co-MA-Thr-OMe)	88	16.2	26.2	1.6	83.0	–	>82	113
10	**14**	poly(MA-Ser-OMe-co-MA-Ser-OEt)	50	13.0	22.6	1.7	81.0	–	>75	80
11	**15**	poly(MA-Ser-OMe-co-MA-Thr-OEt)	83	14.6	26.3	1.8	46.0	–	>62	94
12	**16**	poly(MA-Ser-OMe-co-MA-Ser-OiPr)	65	23.4	45.3	1.9	32.0	–	>53	100
13	**17**	poly(MA-Ser-OMe-co-MA-Thr-OiPr)	80	13.2	23.6	1.8	26.0	–	>50	92
14	**18**	poly(MA-Ser-OiPr-co-MA-Thr-OMe)	91	13.3	25.0	1.9	25.0	–	35	88
15	**19**	poly(MA-Ser-OiPr-co-MA-Ser-OEt)	78	13.7	25.3	1.8	24.8	–	28	88
16	**20**	poly(MA-Thr-OiPr-co-MA-Ser-OEt)	89	14.0	22.7	1.6	17.0	–	33	83
17	**21**	poly(MA-Thr-OiPr-co-MA-Thr-OMe)	95	18.2	33.4	1.8	14.0	–	25	92
18	**22**	poly(MA-Ser-OiPr-co-MA-Thr-OEt)	75	16.3	30.0	1.7	14.0	10 (10)	14	93
19	**23**	poly(MA-Thr-OiPr-co-MA-Thr-OEt)	88	17.8	29.8	1.7	10.5	–	13	89
20	**24**	poly(MA-Thr-OiPr-co-MA-Ser-OiPr)	32	15.2	24.8	1.6	6.0	–	4	80

[a] The polymers are listed based on the CP-value.
[b] Polymerization reactions were performed at a molar M/I ratio of 100/1. The co-polymer ratios (1:1 w/w) could not be verified by ^{1}H NMR due to overlapping signals.
[c] M_n and M_w were determined by GPC with 10 mM LiCl in DMF as eluent and PEG as standards for calibration.
[d] PDI: polydispersity index (M_w/M_n).
[e] CP: cloud point, measured in H_2O.
[f] CP measured in PBS-buffer (PBS-buffer with 10% foetal calf serum).
[g] calcd CP: calculated cloud point: ($CP_{homopolymer\ 1}$ + $CP_{homopolymer\ 2}$)/2.
[h] M/I = 50/1.
[i] M/I = 200/1.

Table 2
Half-life times ($t_{1/2}$) of monomers **5a**–**c** and **6a**–**c**.[a]

Entry	Monomer	$t_{1/2}$ (days)[b]
1	MA-Ser-OMe **5a**	5.0 ± 0.6
2	MA-Ser-OEt **5b**	11.0 ± 0.1
3	MA-Ser-OiPr **5c**	40.5 ± 0.5
4	MA-Thr-OMe **6a**	5.4 ± 0.4
5	MA-Thr-OEt **6b**	11.8 ± 0.8
6	MA-Thr-OiPr **6c**	33.7 ± 4.5

[a] Reaction conditions: pH 7.4 at 37°C.
[b] Average ± SD.

3. Results and discussion

3.1. Synthesis of the monomers

The monomers **5a**–**c** and **6a**–**c** were synthesized via a two-step approach as shown in Scheme 1. In the first step, the aimed esters (**3**, **4**) were synthesized by reacting L-serine (**1**) or L-threonine (**2**) with the corresponding alcohol in the presence of thionyl chloride in good to excellent yield (80–99%) [20]. In the second step, the amino acid esters were N-terminally acylated by treatment with methacryloyl chloride under Schotten–Baumann conditions in the presence of Et_3N as base. After purification by column chromatography, the monomers **5a**–**c** and **6a**–**c** were characterized by NMR and analyzed by HPLC (purity >98%) and were obtained in 33–88% yield. The monomers **5a**–**c** and **6a**–**c** were stored at −20°C prior to use.

3.2. Synthesis of the polymers

The monomers were polymerized via a free radical polymerization, mediated with 2,2′-azobis(isobutyronitrile) (AIBN) as radical initiator. The polymerization reactions were run at 70°C during 48 h in dry, O_2-free, DMF with a molar monomer/initiator (M/I) ratio of 100/1. A series of homopolymers (**7**–**12**) and a set of co-polymers (**13**–**24**) were synthesized in good to very high yield (82–96%), as shown in Scheme 2 and Table 1. The polymers were obtained as white solids and characterized by GPC, which gave M_n-values ranging between 6.6 and 23.8 kDa with a PDI between 1.6 and 2.0.

3.2.1. Cloud point determinations

The homopolymers **7**–**12** (entries 1–8, Table 1) were found to have cloud points (CP) ranging from 1.5 to >100°C, which was in direct correlation with the overall hydrophobicity (methyl versus ethyl versus isopropyl, and serine versus threonine) of the polymers, since a lower CP was found with an increased hydrophobicity of the polymer. This property offered us an opportunity for tailoring the CP by synthesizing a series of co-polymers **13**–**24** (entries 9–20, Table 1) with a 1:1 co-monomer molar ratio to arrive at a CP between those of the homopolymers.

Since it is known that PBS may have an influence on the CP-value, three compounds (**10a**, **11**, and **22**) were measured in PBS-buffer and PBS-buffer containing 10% foetal calf serum, and the CP-value was determined (Table 1). It was found that PBS had a measurable, however, a rather small influence on the CP which was in accordance with the data from Soga et al. [12]

The newly synthesized polymers were also characterized by determination of their glass transition temperature (T_g, Table 1). These values fall in the range between 80 and 113°C, with one exception: poly(MA-Ser-OMe) (compound **7**, entry 1), which had a T_g of >150°C. We did not observe a clear correlation between structure of the polymers or their physicochemical properties (e.g. hydrophobicity or CP-values) with the measured T_g.

As described in the literature for poly(*N*-isopropyl acrylamide) (PNIPAAM) [21] and poly(2-n-propyl-2-oxazoline) (PnPropOx) [22], the CP decreased with an increasing molecular weight. Therefore, this effect was also studied with poly(MA-Thr-OEt) since the effect of different M/I ratios (50/1, 100/1, and 200/1) on M_n in relation to CP was investigated. As can be seen from Table 1 (entries 4–6), the influence of M_n on CP was rather small in case of M/I 50/1 and M/I 100/1, respectively (24.5 and 24°C), however, in case of M/I 200/1 the CP was significantly lower (19.5°C).

3.3. Degradation studies

3.3.1. Degradation of the monomers

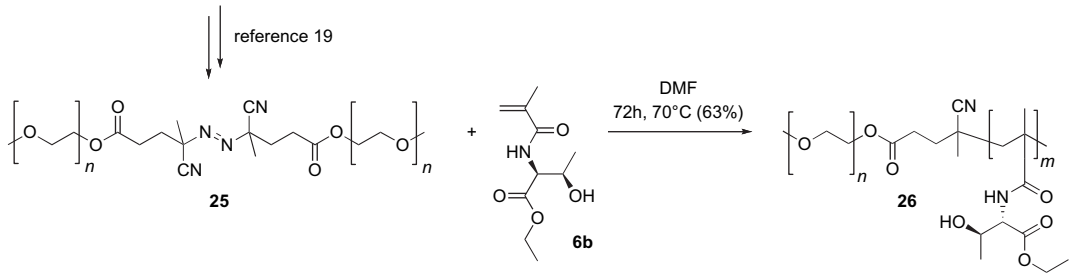
To obtain insight into the chemical stability of the polymers, the degradation of the monomers under physiological conditions (pH 7.4, 37°C) was studied and expressed in half-life time ($t_{1/2}$) values (Table 2). In this series, the hydrolysis of the esters followed the order: methyl > ethyl > isopropyl, irrespective of the amino acid residue. Apparently, the hydrolysis rate is independent of the overall hydrophobicity, in contrast to the cloud points of the corresponding monomers (Table 1), and followed the leaving group character of the alkyl moiety.

3.3.2. Degradation of the polymers

The rate of ester hydrolysis was studied with polymers **7** and **8** by means of ^{1}H NMR spectroscopy. For this purpose the relative intensity of the $COOCH_3$ peak (δ: 3.64 ppm) was monitored during two weeks. The intensity of this peak was correlated to βCH_2 Ser and γCH_3 Thr in case of **7** and **8**, respectively. It was found that the hydrolysis of both polymers was rather slow compared to the individual monomers, since the conversion after 14 days was ~25 and ~12% for **7** and **8**, respectively. This reduction in hydrolysis rate may be explained by the hydrophobic nature of the polymer main-chain, in which solvation of the side chains is reduced and thereby slowing down hydrolysis of the ester bond [19].

3.4. Thermoresponsive polymeric micelles

To study the applicability of the serine- and threonine-based methacryloyl polymers as thermoresponsive polymeric micelles, a thermoresponsive amphiphilic block co-polymer was synthesized



Scheme 3. Synthesis of poly[N$^{\alpha}$-(methacryloyl)-Thr-OEt]-b-(PEG monomethyl ether 5000) **26** as a thermoresponsive amphiphilic block co-polymer.

Table 3
Characterization of poly[N$^{\alpha}$-(methacryloyl)-Thr-OEt]-b-(PEG monomethyl ether 5000) **26** and its precursor **25**.

Polymer	Yield (%)	M_n [a]kDa	M_w kDa	M_w/M_n (PDI)	M_n Thr-b [b](kDa)	Z_{ave} [c]nm	PD [c]
25	46	11.1	12.2	1.1	–	–	–
26	94	13.0	22.7	1.7	6.4	216	0.06

[a] M_n and M_w were determined by GPC with 10 mM LiCl in DMF as eluent and PEG-based standards for calibration.
[b] M_n of the N$^{\alpha}$-(methacryloyl)-Thr-OEt block was determined by ^{1}H NMR.
[c] Z-average diameter and polydispersity (PD) as determined by DLS at 1 mg/mL dispersion in H_2O at 25°C.

(Scheme 3). Based on a literature procedure according to Neradovic et al. [19], the macroinitiator (PEG monomethyl ether 5000)$_2$-ABCPA **25** was prepared and used in combination with N$^{\alpha}$-(methacryloyl)-Thr-OEt (**6b**) in a radical polymerization reaction to obtain poly[N$^{\alpha}$-(methacryloyl)-Thr-OEt]-b-(PEG monomethyl ether 5000) **26** in 63% yield. At an M/I ratio of 100/1 the isolated polymer had an average of 30 repeating units of N$^{\alpha}$-(methacryloyl)-Thr-OEt as judged by ^{1}H NMR analysis (Table 3).

Based on Dynamic Light Scattering (DLS) measurements, particles were formed when a solution of poly[N$^{\alpha}$-(methacryloyl)-Thr-OEt]-b-(PEG monomethyl ether 5000) in H_2O (1 mg/mL) was slowly heated from 0 to 25°C, since the clear solution became turbid at 22°C resulting in a high particle count rate (Fig. 1). The measured particle size (Table 3) was rather high, which indicated that polymer aggregates (larger aggregates consisting of smaller polymeric aggregates) rather than core-shell micelles were formed, most likely due to the slow heating of the polymer solution, as previously described by Neradovic et al. [8,9] Cooling of the dispersion resulted in a decrease of particle count rate, and at 20°C almost no particles could be detected, indicating that the amphiphilic block co-polymer was completely soluble. The critical aggregation temperature between 20 and 25°C corresponded very well with the cloud point of 24°C of the homopolymer, poly[N$^{\alpha}$-(methacryloyl)-Thr-OEt] **10a** (Table 1).

The polymer poly[N$^{\alpha}$-(methacryloyl)-Thr-OEt]-b-(PEG monomethyl ether 5000) was dissolved in an aqueous buffer (either pH 5.0 or 7.4) and the solution was incubated at 37°C after particle formation by heating the polymer solution. The stability of the particles was analyzed by DLS as function of the incubation time. During each transfer of the sample from the incubation bath to the DLS machine at the measuring time points, the sample was allowed to cool down and subsequently slowly heated in the DLS machine, which explained, as already mentioned above, the rather high particle size (Z_{ave}: 300 nm and PD: 0.1–0.15). Since ester hydrolysis is rather slow

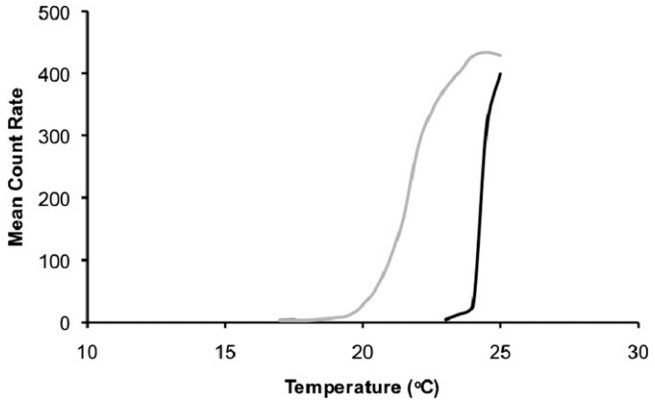


Fig. 1. Mean count rate of poly[N$^{\alpha}$-(methacryloyl)-Thr-OEt]-b-(PEG monomethyl ether 5000) in H_2O (1 mg/mL) upon heating (black line) and cooling (grey line).

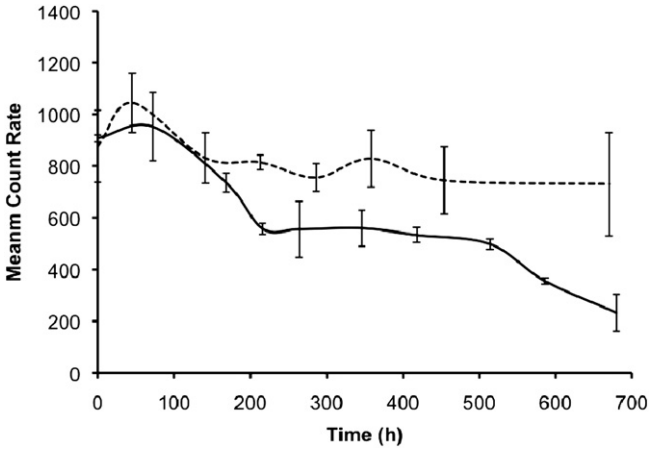


Fig. 2. Stability of particles formed by poly[N$^{\alpha}$-(methacryloyl)-Thr-OEt]-b-(PEG monomethyl ether 5000) incubated at 37°C and pH 5.0 (dashed line) and 7.4 (solid line), respectively. The data are shown as average ± SD, based on three independent determinations.

at pH 5.0, the mean count rate was almost constant over a period of 700 h, indicating that at this pH the particles were stable (Fig. 2). Moreover, the diameter and PD of the particles remained constant during the time of incubation. This was in sharp contrast with the particles that were incubated at pH 7.4. At this pH, ester hydrolysis induced an increase of the hydrophilic character of the polymer which resulted in a destabilization of the particles and thus in a gradual decrease of the mean count rate and an increase in the PD from 0.1 at $t = 0$ to 0.5 at $t = 700$ h. Furthermore, the half-life time ($t_{1/2}$) of the particles could be estimated to be approximately 400 h (16.7 days) as the mean count rate was 50% of its starting value, while the monomer N$^{\alpha}$-(methacryloyl)-Thr-OEt had a similar half-life time ($t_{1/2}$) of 11.8 days (Table 2). Again, the slower degradation kinetics of the polymer as compared to that of the monomer can be ascribed to dehydration of the thermosensitive block above its CP.

4. Conclusions

A series of N$^{\alpha}$-methacryloyl amino acid alkyl esters was synthesized and used for the preparation of a particular set of homo- and co-polymers. It turned out that the cloud point of these polymers could be tailored by variation of the hydrophobicity of the monomer. Furthermore, the leaving group character of the ester moiety was a major determinant of the half-life time of the monomers in aqueous solution, while the polymers were hydrolyzed at least with a four-fold lower rate compared to the respective monomer. Functionalization with a hydrophilic PEG-chain resulted in a co-polymer, poly[N$^{\alpha}$-(methacryloyl)-Thr-OEt]-b-(PEG monomethyl ether 5000), which was soluble in aqueous buffer below its cloud point, while nanoparticles were formed above 21°C. Incubation of these microparticles at physiological conditions resulted in a gradual decrease in particle amount, since ester hydrolysis induced hydrophilicity and thus increasing the CP which resulted in destabilization of the microparticles. The independently tunable LCST and degradation behavior of the newly designed amino acid-based polymers may find application in the controlled release of bioactive molecules.

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