Stimulus Responsive Behavior of Elastin-Based Side Chain Polymers

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ABSTRACT: Linear poly(VPGVG) has been extensively studied over the years as a model for the structural protein elastin. Elastin is characterized by a lower critical solution temperature (LCST). This LCST can be influenced by several factors, mainly molecular weight, concentration, and pH. An ABA type block copolymer was synthesized using atom transfer radical polymerization (ATRP), in which the VPGVG sequence is in the side chain of the A block and the B block is poly(ethylene glycol) (PEG). The LCST behavior of this series of elastin based side chain block copolymers was investigated by changing the degree of polymerization, polymer concentration, and pH. The effects of these parameters on the LCST of the side chain block copolymers were similar when compared with linear poly(VPGVG). The aggregates which were formed above the transition temperature were investigated using light scattering and cryo-SEM techniques.

Introduction

Elastin is one of the most important classes of natural structural proteins,[1,2] as it is responsible for the elasticity of mammalian tissue, making it crucial for the functioning of skin, ligaments, arteries, and lung tissue.[3,4] The structure of tropoelastin, the un-cross-linked precursor protein of mammalian elastin, is one of the most studied and well-characterized types of elastin[5−9] and has been found to have VPGVG (V = valine, P = proline, and G = glycine) as its most prominent amino acid sequence.[10,11] Poly(VPGVG) has been shown to undergo a transition from random coil to β spiral as it is heated.[12] This unusual transition from an unordered structure to an entropically less favored ordered conformation is caused by hydrophobic dehydration.[13,14] This means that as elastin is heated its bound water is expelled, leading to a more hydrophobic protein.[15] This results in the formation of a β-spiral in which the hydrophobic side chains of the valines are interacting with each other and are shielded from the aqueous environment.[16,17] The release of water compensates for the loss in conformational freedom of the protein.[18] The change in structure and hydrophobicity is accompanied by aggregation and precipitation of the elastin molecules. Elastin therefore exhibits an inverse transition temperature or lower critical solution temperature (LCST).[19]

One aspect that makes elastin a very versatile material is that the LCST can be fine-tuned by changing the hydrophobicity of the pentapeptide repeat. By replacing the second valine by any other amino acid, except proline, the difference in polarity of the side chains leads to different LCST's. Pioneering work by Urry has shown that virtually any phase transition temperature can be obtained by the correct composition of elastin.[1] Furthermore, the LCST behavior of linear poly(VPGVG) can also be influenced by other parameters, such as molecular weight,[20,21] concentration,[19,20] and, when amino acids with acidic or basic side chains are introduced, pH.[22]

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In a recent paper we have described the preparation of a new class of elastin hybrid polymers. Inspired by the work of Reiersen and Reese,[20,23] who showed that one single repeat of VPGVG also undergoes the structural transition from random coil to type II β-turn, we investigated whether the transition found in linear VPGVG was also introduced into a triblock copolymer in which the pentapeptide units were incorporated in the polymer side chains.[24] By polymerizing a methacrylate-functionalized VPGVG monomer from a bifunctional poly(ethylene glycol) initiator via atom transfer radical polymerization (ATRP), the desired structure was prepared. We found that the VPGVG sequence in the side chain of the polymer underwent a transition from random coil to type II β-turn. We also observed that an aqueous solution of the triblock copolymer showed an increase in turbidity upon heating, indicating that there was some form of aggregation occurring, probably due to the transition of the block copolymer from a hydrophilic to an amphiphilic species as the VPGVG blocks became hydrophobic.

In this paper we describe our investigations of whether the stimulus responsive character of this class of elastin side chain block copolymers is affected by the same parameters as linear poly(VPGVG). For this purpose a series of polymers were made with varying degree of polymerization (DP) of the elastin fragments. The effect of DP, concentration, and pH on the phase transition temperature was examined. Furthermore, the nature of aggregation was studied in more detail with dynamic light scattering and electron microscopy. With this investigation we hope to further demonstrate that, by introducing a functional peptide into the side chain of a polymer, the functionality is transferred into the polymer itself and is still dependent on the physical parameters which affect the original peptide sequence.

Experimental Section

General Procedures. ^{1}H and ^{13}C NMR spectra were measured on a 400 MHz Bruker Inova400 machine with a Varian probe.

IR spectra were measured on an ATI Mattson Genesis Series FTIR.

Elemental analysis was performed on a Carlo-Erba Instruments EA1180 CHNO/S elemental analyzer

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Turbidity measurements were carried out on a Jasco J-810 spectropolarimeter with a temperature control unit. Samples were dissolved in a phosphate buffer of pH of 1, 2, or 3, composed of sodium chloride, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, and *o*-phosphoric acid. The samples were measured using a 1 mm quartz cuvette at different temperatures. The measurements were carried out at a fixed wavelength of 480 nm.

MALDI-TOF-MS spectra were measured on a Bruker Biflex III machine, with dihydroxybenzoic acid (DHB) as matrix. Samples were prepared by dissolving 2 mg of analyte in 1 mL of THF, after which this solution was mixed in a 1:1 ratio with a solution of 10 mg of DHB in 1 mL of H_2O containing 0.1% trifluoroacetic acid. This was then placed on a MALDI plate.

GPC measurements were performed using a Shimadzu GPC with Shimadzu RI and UV/vis detection, fitted with a Polymer Laboratories Plgel 5 μm mixed-D column, and a PL 5 μm guard column (separation range from 500 to 300 000 molecular weight) using THF or NMP as mobile phase at 35 and 70 °C, respectively. Polymer Laboratories polystyrene calibration kits were used.

Dynamic light scattering (DLS) measurements were carried out using an ALV/GmbH set up fitted with an ALV 125-laser light spectrometer, an ALV-5000 digital correlator, and a Lexel 500 mW Ar laser. The measurements were carried out at 514.5 nm, 200 mW, and an angle of incidence of 60°, 90°, and 120°.

Cryo-scanning electron microscopy (cryo-SEM) was performed on a JEOL JSM T300 operating at 30 kV. The sample solution was heated above the LCST and quenched in nitrogen slush. Afterward, the sample was freeze-fractured using standard procedures and transferred into the cryo-SEM. The sample was sublimed for 5 min before inserting into the sample chamber.

Reagents. CuCl (Aldrich, 97%) was purified by washing with glacial acetic acid three times and once with diethyl ether. Boc-L-valine (Fluka, ≥99%), Boc-L-proline (Fluka, ≥99%), glycine ethyl ester hydrochloride (HCl·H-Gly-OEt, Janssen, 99%), poly(ethylene glycol), M_n = 1000 g/mol (PEG 1000) (Fluka), ethyl 2-bromoisobutyrate (EBIB 98%, Aldrich), hydroxyl ethyl methacrylate (HEMA, Aldrich, 97%), 2-bromoisobutyric acid, (Aldrich, 98%), 2-isocyanatoethyl methacrylate (Aldrich, 98%), 2,2′-bipyridyl (Bipy) (Aldrich, 99%), *N,N*-dicyclohexylcarbodiimide (DCC) (Fluka, 99%), 4-(dimethylamino)pyridine (DMAP) (Across, 99%), DMSO-d_6 (Aldrich, 99.9%), *N,N*′-diisopropylethylamine (DIPEA) (Fluka, 99%), 1-hydroxybenzotriazole hydrate (HOBt) (Fluka, ≥98%), potassium hydrogen sulfate ($KHSO_4$) (Riedel-de Haën, 99%), sodium hydrogen carbonate ($NaHCO_3$) (Merck, 99.5%), and sodium sulfate anhydrous (Fluka, 99%) were all used as received.

Dichloromethane (DCM) and ethyl acetate (EtOAc) were distilled from calcium hydride prior to use.

For the buffers sodium chloride (Merck, p.a), sodium dihydrogen monohydrate (Merck, p.a), disodium hydrogen phosphate dihydrate (Merck, p.a), and *o*-phosphoric acid (Merck, p.a., 85 wt % in water) were all used as received.

Monomer Synthesis. **Synthesis of Boc-Val-Gly-OEt**. Boc-Val-OH (5.64 g, 0.26 mmol) was dissolved in EtOAc (80 mL). To this mixture HCl·H-Gly-OEt (3.63 g, 0.26 mmol), DIPEA (8.9 mL, 0.52 mmol), and BOP (11.49 g, 0.26 mmol) were added. After stirring for 10 min another equivalent of DIPEA (4.45 mL, 0.26 mmol) was added dropwise to obtain a basic solution (pH > 9). The reaction was stirred at room temperature for 16 h. The reaction mixture was washed three times with 10 mL of a 1 M $NaHCO_3$ solution, once with 10 mL of water, once with 10 mL of brine solution, three times with 10 mL of a 1 M $KHSO_4$ solution, twice with 10 mL of water, and finally with 10 mL of brine. The ethyl acetate layer was dried with Na_2SO_4, and the solvent (ethyl acetate) was removed under reduced pressure, yielding the crude product. The crude product was dissolved in DCM and then added to diethyl ether. The impurities were filtered off and discarded, and the remaining diethyl ether was removed under reduced pressure. Pure Boc-Val-Gly-OEt (6.92 g) was obtained in 88% yield.

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.9 (CH(CH_3)$_2$, 6H, m), 1.2 (CH_3−CH$_2$, 3H, t), 1.35 (C(CH_3)$_3$, 9H, s), 2.1 (CH(CH$_3$)$_2$, 1H, m), 3.9 (HN−CH−C=O), 1H, m) 3.8 (NH−C$H_a$$H_b$−C=O, 1H, m), 4.0 (NH−C$H_a$$H_b$−C=O, 1H, m), 4.1 (CH$_3$−C$H_2$, 2H, q), 6.9 (−$H$N−CH−C=O, 1H, d), 8.1 (N$H$−CH$_2$, 1H, t).

Synthesis of the HCl Salt of H-Val-Gly-OEt. Boc-Val-Gly-OEt (6.92 g, 0.23 mmol) was dissolved in 2 M HCl/EtOAc (50 mL) and stirred for 90 min at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting product was extracted in DCM (50 mL), which was removed under reduced pressure. Pure HCl·H-Val-Gly-OEt was obtained in quantitative yield (5.49 g).

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.9 (CH−CH(CH_3)$_2$, 6H, m), 1.2 (CH_3−CH$_2$, 3H, t), 2.15 (CH−CH(CH$_3$)$_2$, 1H, m), 3.7 (CH−CH(CH$_3$)$_2$), 1H, m), 3.8 (NH−CH_aH$_b$−C=O, 1H, m), 4.0 (NH−CH$_a$$H_b$−C=O, 1H, m), 4.1 (CH$_3$−C$H_2$, 2H, q), 7.6 (N$H$−CH$_2$, 1H, s), 8.3 ($H_3N^+$−CH, 3H, s).

Synthesis of Boc-Pro-Gly-OEt. Boc-Pro-OH (10.02 g, 46.6 mmol), HCl·H-Gly-OEt (6.49 g, 46.6 mmol), HOBt (7.13 g, 46.6 mmol), and DIPEA (16.0 mL, 93 mmol) were dissolved in EtOAc (40 mL). To this solution, DCC (9.59 g, 46.6 mmol) was added. The reaction mixture was stirred for 16 h at room temperature, during which time a white precipitate (dicyclohexylurea (DCU)) was formed, which was filtered off. The reaction mixture was washed three times with 10 mL of 1 M $KHSO_4$, 10 mL of water, 10 mL of brine, three times with 10 mL of 1 M $NaHCO_3$, twice with 10 mL of water, and finally with 10 mL of brine. The mixture was then dried over Na_2SO_4, and solvent was removed under reduced pressure to obtain Boc-Pro-Gly-OEt (13.6 g) in 98% yield.

^{1}H NMR (400 MHz, DMSO-d_6): δ 1.2 (CH_3−CH$_2$, 3H, t), 1.4 (C(CH_3)$_3$, 9H, s), 1.7−2.0 (N−CH$_2$−CH_2−CH_2, 4H, m), 3.3 (N−CH_2−CH$_2$−CH$_2$, 2H, m), 3.8 (NH−CH_2−CO, 2H, m), 4.1 (CH$_3$−CH_2, 2H, q), 4.15 (N−CH−CO, 1H, m), 8.2 (NH−CH$_2$−CO, 1H, t).

Synthesis of the HCl Salt of H-Pro-Gly-OEt. Boc-Pro-Gly-OEt (13.6 g, 45.5 mmol) was dissolved in 2 M HCl/EtOAc (150 mL) and stirred for 90 min at room temperature. The excess HCl/EtOAc was removed under reduced pressure. The resulting product was extracted in DCM (50 mL), which was removed under reduced pressure, yielding 10.7 g of HCl·H-Pro-Gly-OEt (quantitative yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ 1.2 (CH_3−CH$_2$, 3H, t), 1.9−2.1 (N−CH$_2$−CH_2−CH_2, 4H, m), 3.3 (N−CH_2−CH$_2$−CH$_2$, 2H, m), 4.0 (NH−CH_2−CO, 2H, m), 4.2 (CH$_3$−CH_2, 2H, q), 4.4 (N−CH-CO, 1H, m), 8.2 (NH−CH$_2$−CO, 1H, t).

Synthesis of Boc-Val-Pro-Gly-OEt. HCl·H-Pro-Gly-OEt (10.7 g, 45.5 mmol) was dissolved in EtOAc (200 mL). To this solution Boc-Val-OH (9.89 g, 45.5 mmol), HOBt (6.99 g, 45.5 mmol), and 3 equiv of DIPEA (23.7 mL) were added. Finally, 9.39 g of DCC (45.5 mmol) was added. The reaction was stirred at room temperature for 22 h, during which time a white precipitate (DCU) was formed. This was filtered off, and the reaction mixture was washed three times with 10 mL of 1 M $KHSO_4$, 10 mL of water, 10 mL of brine, three times with 10 mL of 1 M $NaHCO_3$, twice with 10 mL of water, and finally with 10 mL of brine. The reaction mixture was then dried over Na_2SO_4 which was filtered off. The solvent was removed under reduced pressure, and the remaining solid was extracted with DCM (150 mL), which was removed under reduced pressure (15.27 g). The crude product was then purified by column chromatography using 5% MeOH in DCM as a mobile phase. Boc-Val-Pro-Gly-OEt was obtained in 61% yield (11.1 g).

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.8 (CH−(CH_3)$_2$, 6H, m), 1.2 (CH_3−CH$_2$, 3H, t), 1.4 (C(CH_3)$_3$, 9H, s), 1.9−2.1 (N−CH$_2$−CH_2−CH_2 and CH−(CH$_3$)$_2$, 5H, m), 3.3 (N−CH_2−CH$_2$−CH$_2$, 2H, m), 3.8 (NH−CH_2−CO and NH−CH(CH−(CH$_3$)$_2$)−CO, 3H, m), 4.2 (CH$_3$−CH_2, 2H, q), 4.4 (N−CH−CO, 1H, m), 6.6 (NH−CH(CH−(CH$_3$)$_2$)−CO, 1H, d), 8.2 (NH−CH$_2$−CO, 1H, t).

Synthesis of Boc-Val-Pro-Gly-OH. Boc-Val-Pro-Gly-OEt (11.1 g, 27.8 mmol) was dissolved in 153 mL of a mixture containing 70% dioxane, 10% water, and 20% 4 M NaOH. This was stirred for 1 h. The solution was then neutralized by adding 1 M $KHSO_4$ until a pH of 7 was obtained; the solvent was removed under reduced pressure. The crude product was redissolved in 100 mL of EtOAc and washed three times with 10 mL of 1 M $KHSO_4$ and twice with 10 mL of water. After drying over Na_2SO_4, EtOAc was removed, and the solid was extracted with 50 mL of DCM. The solvent was removed under reduced pressure, yielding 10.0 g of pure Boc-Val-Pro-Gly-OH (quantitative yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.8 (CH−(CH_3)$_2$, 6H, m), 1.4 (C(CH_3)$_3$, 9H, s), 1.9−2.1 (N−CH$_2$−CH_2−CH_2 and CH−(CH$_3$)$_2$ 5H, m), 3.3 (N−CH_2−CH$_2$−CH$_2$, 2H, m), 3.8 (NH−CH_2−CO and NH−CH(CH−(CH$_3$)$_2$)−CO, 3H, m), 4.4 (N−CH−CO, 1H, m), 6.6 (NH−CH(CH−(CH$_3$)$_2$)−CO, 1H, d), 8.2 (NH−CH$_2$−CO, 1H, t).

Synthesis of Boc-Val-Pro-Gly-Val-Gly-OEt. Boc-Val-Pro-Gly-OH (5.00 g, 13.46 mmol) was dissolved in 80 mL of EtOAc. To this mixture 6.9 mL of DIPEA (40.38 mmol) and 3.21 g of HCl-Val-Gly-OEt (13.46 mmol) were added. Next, 2.78 g of DCC (13.46 mmol) was added, and the reaction was then stirred for 30 h during which time a white precipitate (DCU) was formed. This precipitate was filtered off, and the reaction mixture was washed three times with 10 mL of 1 M $KHSO_4$, 10 mL of water, 10 mL of brine, three times with 10 mL of 1 M $NaHCO_3$, twice with 10 mL of water, and finally with 10 mL of brine. After drying over Na_2SO_4, the solvent was removed, and the crude product was redissolved in 50 mL of DCM, which was subsequently removed under reduced pressure. After purifying by column chromatography using 60H silica and 5% MeOH/DCM as mobile phase, 4.02 g of pure Boc-Val-Pro-Gly-Val-Gly-OEt was obtained (yield 54%).

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.8 (CH−(CH_3)$_2$, 12H, m), 1.2 (CH_3−CH$_2$, 3H, t), 1.4 (C(CH_3)$_3$, 9H, s), 1.9−2.1 (N−CH$_2$−CH_2−CH$_2$, CH−(CH$_3$)$_2$, 6H, m), 3.4 (N−CH_2−CH$_2$−CH$_2$, 2H, m), 3.6−4.2 (NH−CH_2−CO, NH−CH(CH−(CH$_3$)$_2$)−CO and CH$_3$−CH_2, 8H, m), 4.3 (N−CH−CO, 1H, m), 6.6 (NH−CH-(CH−(CH$_3$)$_2$)−CO, 1H, d), 7.6 (NH−CH(CH−(CH$_3$)$_2$)−CO, 1H, d), 8.2 (NH−CH$_2$−CO, 1H, t), 8.35 (NH−CH$_2$−CO, 1H, t).

Synthesis of Boc-Val-Pro-Gly-Val-Gly-OH. Boc-Val-Pro-Gly-Val-Gly-OEt (3.01 g, 5.42 mmol) was dissolved in 29.8 mL of a mixture containing 70% dioxane, 10% water, and 20% 4 M NaOH. This was then stirred for 1 h at room temperature. After neutralizing to pH 7 with 1 M HCl, the solvent was removed under reduced pressure. 4.1 g (7.57 mmol) of Boc-Val-Pro-Gly-Val-Gly-OH was obtained (quantitative yield)

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.8 (CH−(CH_3)$_2$, 12H, m), 1.4 (C(CH_3)$_3$, 9H, s), 1.7−2.1 (N−CH$_2$−CH_2−CH_2 and CH-(CH$_3$)$_2$, 6H, m), 3.4 (N−CH_2−CH$_2$−CH$_2$, 2H, m), 3.6−4.2 (NH−CH_2−CO, NH−CH(CH−(CH$_3$)$_2$)−CO, 6H, m), 4.3 (N−CH-CO, 1H, m), 6.6 (NH−CH(CH−(CH$_3$)$_2$)−CO, 1H, d), 7.6 (NH−CH-(CH−(CH$_3$)$_2$)−CO, 1H, d), 8.35 (NH−CH$_2$−CO, 2H, t).

Synthesis of HCl·H-Val-Pro-Gly-Val-Gly-OH. Boc-Val-Pro-Gly-Val-Gly-OEt (4.1 g, 7.57 mmol) was dissolved in 50 mL of 2 M HCl/EtOAc and stirred for 1 h. The excess of HCl and solvent was removed under reduced pressure. The product was redissolved in 50 mL of DCM, which was subsequently removed under reduced pressure. 3.7 g (7.57 mmol) of HCl·H-Val-Pro-Gly-Val-Gly-OH was obtained (quantitative yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.8 (CH−(CH_3)$_2$, 12H, m), 1.7−2.1 (N−CH$_2$−CH_2−CH_2 and CH−(CH$_3$)$_2$, 6H, m), 3.4 (N−CH_2−CH$_2$−CH$_2$, 2H, m), 3.6−4.2 (NH−CH_2−CO, NH−CH(CH−(CH$_3$)$_2$)−CO, 6H, m), 4.3 (N−CH−CO, 1H, m), 7.6 (NH−CH(CH−(CH$_3$)$_2$)−CO, 1H, d), 8.1 (NH_3^+−CH(CH−(CH$_3$)$_2$)−CO, 3H, d), 8.2 (NH−CH$_2$−CO, 1H, m), 8.3 (NH−CH$_2$−CO, 1H, m).

Synthesis of Methacrylate-Functionalized Val-Pro-Gly-Val-Gly (1). HCl·H-Val-Pro-Gly-Val-Gly-OH (1.3 g, 2.71 mmol) was dissolved in 30 mL of Milli-Q water. To this solution 0.57 g of $NaHCO_3$ (6.8 mmol) was added to obtain a basic reaction mixture of pH = 8. Next, 767 μL of 2-isocyanatoethyl methacrylate (5.42 mmol) was added dropwise while stirring vigorously. The reaction mixture was stirred for 150 min. After washing twice with 5 mL of DCM, the pH was lowered to 1 with 1 M HCl. Finally, the product was extracted from the water layer with butanol, which was subsequently removed under reduced pressure. The product was redissolved in water, and after freeze-drying, 1.4 g (yield 89%) of pure product was obtained.

Table 1. Molecular Weight Data, Determined by ^{1}H NMR, and Transition Temperatures, Measured at pH 1, for Polymers A, B, and C

sample	DP of VPGVG block	M_n (kg/mol)	transition temp (°C)
A	$\tilde{n}$ = 5.3	7.0	47
B	$\tilde{n}$ = 7.2	9.4	44
C	$\tilde{n}$ = 11.1	15.2	33

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.8 (CH−(CH_3)$_2$, 12H, m), 1.7−2.1 (N−CH$_2$−CH_2−CH$_2$, CH−(CH$_3$)$_2$ and CO−CH(CH_3)═CH$_2$, 9H, m), 3.4−3.8 (N−CH_2−CH$_2$−CH$_2$, and NH−CH_2−CO, 6H, m), 4.0 (O−CH$_2$−CH$_2$−O, 2H, s), 4.2 (NH−CH(CH−(CH$_3$)$_2$)−CO, 2H, m), 4.3 (N−CH−CO, 1H, m), 5.6 and 6.0 (C═CH_2, 2H, s), 6.2 (NH−CH$_2$−COOH 1H, m), 7.6 (NH−CH-(CH−(CH$_3$)$_2$)−CO, 1H, d), 8.2 (NH−CH$_2$−CO, 1H, m), 8.3 (NH−CO−NH, 2H, m).

^{13}C NMR (SO(CD$_3$)$_2$): δ 17.94, 18.30, 19.12, 24.52, 29.15, 30.77, 31.33, 55.92, 58.52, 59.67, 64.12, 125.81, 135.80, 157.83, 166.50, 168.46, 170.52, 171.27, 171.63, 171.91.

MALDI-TOF-MS: *m/e* 605 (M^+ − H + Na); 621 (M^+ − H + K); 627 (M^+ − 2H + 2Na).

Synthesis of Bifunctional PEG ATRP Macroinitiator (Di-α,ω-bromoisobutyrate-PEG). This was performed according to the previously published procedure.[24]

Polymerization of Methacrylate-Functionalized VPGVG, 1, to an ABA Block Copolymer. The polymerization of methacrylate-functionalized Val-Pro-Gly-Val-Pro (**1**) was carried out according to the previously published procedure.[24] This time the polymerizations were carried out 60 °C until the desired conversion was achieved. After polymerization, the mixture was poured in diethyl ether. After decantation of the ether layer, the brown polymer precipitate was redissolved in demineralized water (resulting solution pH 6.0). After acidifying the solution to pH 1 with 1 M HCl, the solution was heated to 70−80 °C and centrifuged. The water layer was decanted, and the resulting crude product was redissolved in water. After freeze-drying the pure product was obtained.

The number of units of monomer **1** added to the PEG bifunctional initiator and subsequently the number-average molecular weight M_n were determined by ^{1}H NMR spectroscopy in DMSO-d_6, using the resonances of the CH$_2$−O groups of poly(ethylene glycol) at 3.5 and the signal of the urea group at 6.2 (see Table 1).

Results and Discussion

To begin our investigation into the effects of molecular weight, concentration, and pH on our elastin-based block copolymers, it was necessary to resynthesize the VPGVG-based monomer **1**. This time a solution-based approach was used (see Scheme 1) instead of the previously published solid-phase approach,[24] as this allowed us to more easily produce a larger quantity of this short peptide.

Block Length. It has been shown by Meyer and Chilkoti[21] that as the molecular weight of linear poly-VPGVG is increased, the transition temperature is decreased.

To investigate whether our polymers were also affected in the same way, three polymers with different VPGVG block lengths were synthesized using ATRP (see Figure 1). The polymerizations all had first-order rate kinetics, and the degrees of polymerization of each polymer could be determined using ^{1}H NMR spectroscopy, by comparing the peaks due to poly(ethylene glycol) at 3.5 ppm and the peaks due to urea at 6.2 ppm (see Table 1).

The three polymers were dissolved in a phosphate buffer of pH 1, and the transition temperature was determined (see Table 1). The turbidity measurements clearly showed a decrease in the transition temperature as the chain length was increased. This is in agreement

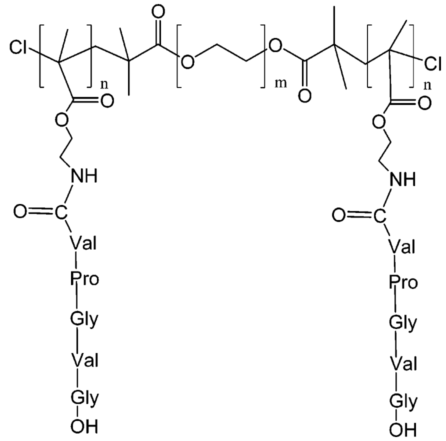
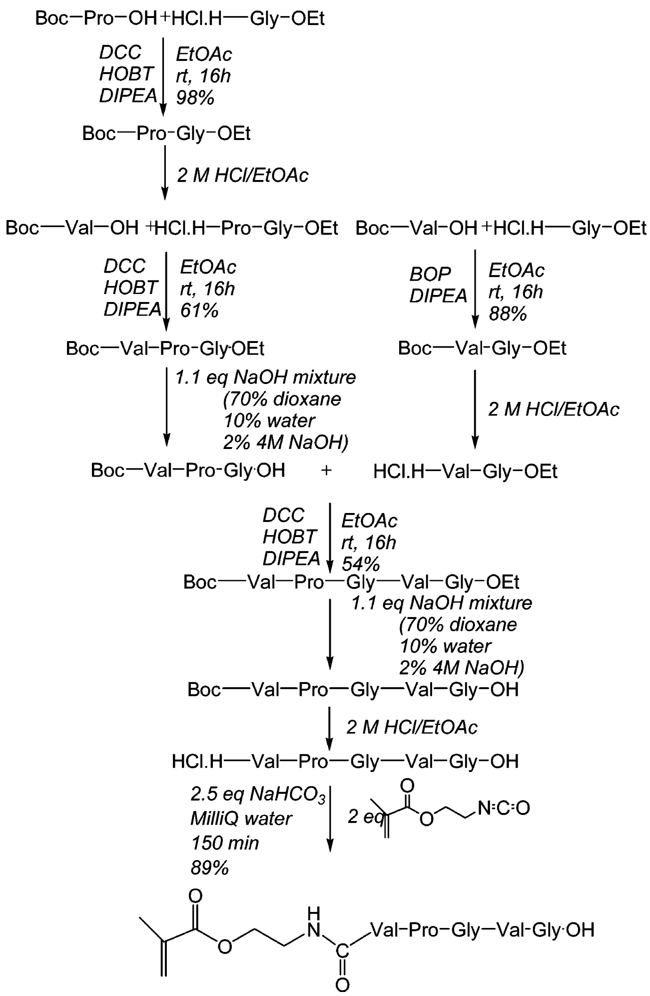


Figure 1. Chemical structure of elastin-based side chain block copolymers.

Scheme 1. Solution Phase Synthesis of VPGVG-Based Monomer 1



with the results described by Meyer and Chilkoti[21] for linear elastin. It was suggested that as the linear VPGVG polymers become shorter, they become more ordered, increasing the energy required to change from one structure to the other.[20] The changes in transition temperature which are observed with our block copolymers, however, are more pronounced than those observed for linear poly(VPGVG). When the number of VPGVG units on each side of the triblock copolymer is increased from 7 to 11, we see a change in LCST from 44 to 33 °C. According to Meyer, to obtain a similar

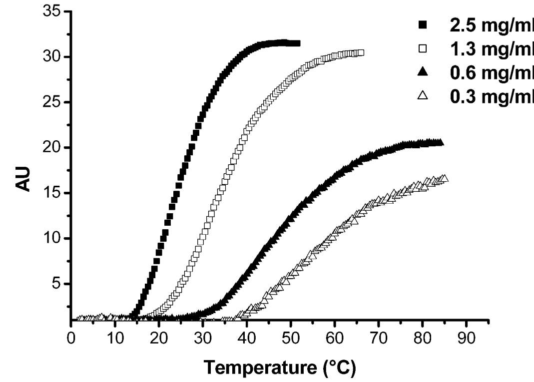


Figure 2. Turbidity measurements of different concentrations of triblock copolymer C taken at pH 1.

change in transition temperature from 50 to 35 °C with linear VPGVG, the chain length had to be increased from 30 to 60 units. This suggests that there is an additional reason for the relatively large change in transition temperature observed with our block copolymers. One difference between our side chain block copolymers and linear VPGVG is that as the molecular weight of our polymers is increased, the polymethacrylate backbone is also extended. As the backbone becomes longer, its influence on the triblock copolymers properties becomes more pronounced, as the hydrophobicity of the end block is increased. The VPGVG side chains of the polymers with a higher degree of polymerization are in a more hydrophobic environment and can therefore more readily undergo the inverted phase transition, lowering the transition temperature more than is observed with a similar molecular weight change in linear elastin.

Concentration. The second physical parameter of interest is concentration. It has been shown that as the concentration was increased, the transition temperature decreased.[19,21]

To investigate the effect of concentration on our block copolymers, four different concentrations of the triblock copolymer C, in a buffer of pH 1, were made, and their transition temperatures measured.

From the turbidity measurements (see Figure 2) we can clearly see that as the concentration increases, the transition temperature decreases; the same trend as is observed for linear VPGVG. For linear VPGVG Urry et al.[19] proposed that this change in transition temperature is caused by the fact that the transition from random coil to β-spiral is a cooperative process. This means that as the concentration is increased, this cooperative effect plays a larger role in the transition process, decreasing the transition temperature.

This explanation could also apply to our block copolymers. As the concentration of our block copolymer increases, the cooperative effect becomes more pronounced, reducing the transition temperature in a similar manner as for linear polyVPGVG.

pH Dependence. It has been shown that by replacing the second valine in linear poly(VPGVG) with an acidic or basic amino acid, the properties of the polypeptide can be changed, allowing the transition temperature to be manipulated by varying the pH. For example, if valine is replaced by glutamic acid, the transition temperature can be increased by increasing the pH.[22] For our triblock copolymers there is already a free carboxylic acid group at the end of the peptide side

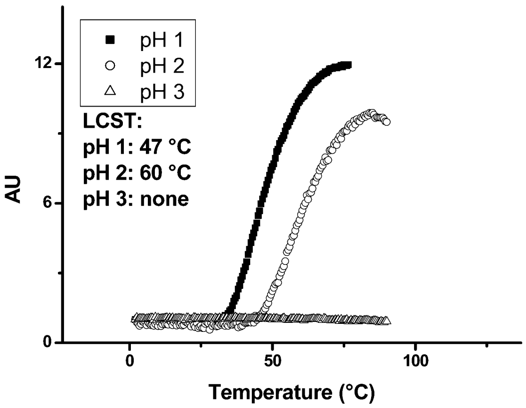


Figure 3. Turbidity measurements of triblock copolymer A at pH = 1, 2, and 3.

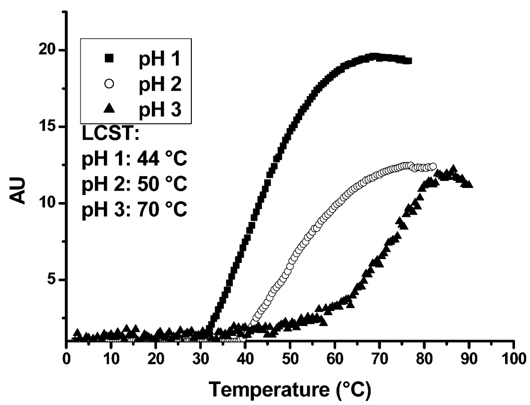


Figure 4. Turbidity measurements of triblock copolymer B at pH = 1, 2, and 3.

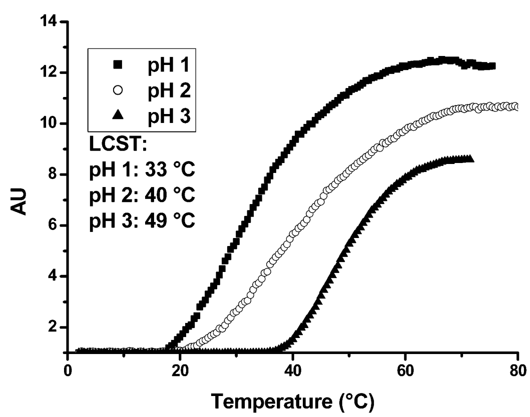


Figure 5. Turbidity measurements of triblock copolymer C at pH = 1, 2, and 3.

chain; therefore, it was not necessary to introduce a pH-sensitive amino acid.

The triblock copolymers A−C respectively were dissolved in three phosphate buffers with pH's of 1, 2, and 3. The turbidity measurements clearly showed that as the pH increased for each polymer, the transition temperature also increased (see Figures 3, 4, and 5) until there was no longer a transition point for triblock copolymer A at pH = 3.

It is clear that the same trend is observed for our triblock copolymers as for linear poly(VPGXG), in which X is an acidic residue. For substituted linear poly(VPGVG) the change in transition temperature is thought to be due to a change in the hydrophobicity of the VPGXG sequence.[22,25] As the pH is increased, the acidic residue becomes deprotonated, making the peptide sequence more hydrophilic, thus increasing the transition temperature. This explanation is also applicable to our triblock copolymer systems. As the acidic end groups become deprotonated, the end blocks become more hydrophilic, increasing the temperature at which hydrophobic dehydration occurs. This results in our triblock copolymer systems having the same behavior as linear poly(VPGXG).

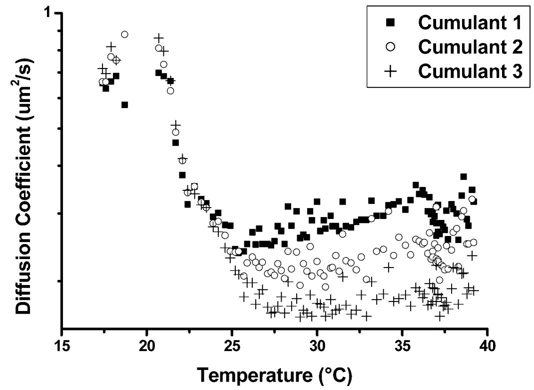


Figure 6. Temperature dependence of the diffusion coefficient as determined by dynamic light scattering, for triblock copolymer C at pH = 1, taken at a concentration of 1 mg/mL.

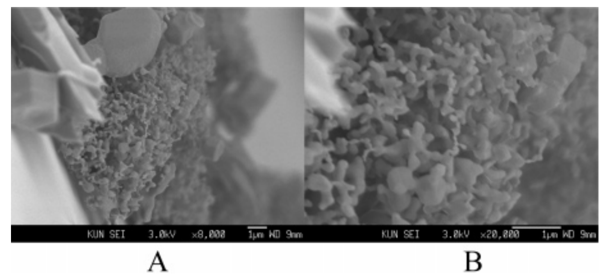


Figure 7. Cryo-SEM images of a 1 mg/mL solution of triblock copolymer C, at pH = 1, frozen in above its LCST: (A) 8000 times enlargement; (B) 20 000 times enlargement.

Aggregation. In our previous article we suggested that the mechanism of aggregation is due to a change in the amphiphilicity of the block copolymers as they are heated. To investigate what sorts of aggregates are formed upon heating, we analyzed block copolymer C with dynamic light scattering measurements in a buffer solution of pH 1. Figure 6 shows the changes in diffusion coefficient with temperature. The large deviation between the first, second, and third cumulants at higher temperatures indicates that the measured particles are not spherical. This type of change is indicative of network formation.

The idea of network formation was furthermore supported by cryo-SEM of the same solution (Figure 7). The solution was heated above the block copolymer transition temperature and then quenched in liquid N_2. After freeze-fracturing, the morphology of the block copolymer above its LCST could be determined by cryo-SEM. The cryo-SEM pictures clearly indicated the presence of a network.

Conclusion

In this paper we have shown that the LCST behavior of a series of side chain elastin-based block copolymers are influenced by the same parameters as linear poly(VPGVG). By increasing polymer concentration or molecular weight the transition temperature is lowered, by increasing pH the transition temperature increases.

This proves that it is possible to incorporate a structural peptide into a polymer and have it retain its functionality, resulting in functional synthetic polymers which behave in a similar way as the original peptide sequence or protein on which they are based.

We have also found more evidence that the mechanism of aggregation for our peptides is due to a change in aggregation in our block copolymers. From cryo-SEM and DLS measurements we can see that upon heating no defined structures are present, but instead a network is clearly formed.

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