Stimulus Responsive Behavior of Elastin-Based Side Chain Polymers

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Received October 8, 2004; Revised Manuscript Received November 29, 2004

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\(^5\) 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 \(\alpha\)-helix 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 \(\beta\)-turn, a stable structure 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 LCSTs. Pioneering work by Urry has shown 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 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 \(\beta\)-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 \(\beta\)-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. \(^1H\) and \(^13C\) 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 EA1100 CHNO/S elemental analyzer.
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 1, 2, or 3, composed of sodium chloride, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, and α-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 mass spectrometer, 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 H2O 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 Pigel 5 μm mixed-D column, and a PL 5 μm guard column (separation range from 500 to 3000 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 Laser 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 JEOI JSJ 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 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°.

**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), Mn = 1000 g/mol (PEG 1000) (Fluka), ethyl 2-bromoobutyrate (EIBB 98%, Aldrich), hydroxyethyl methacrylate (HEMA, Aldrich, 97%), 2-bromoisobutyric acid, (Aldrich, 98%), 2-isocyanatoethoxy methylacrylate (Aldrich, 98%), 2,2′-bipyridyl (Bipy) (Aldrich, 99%), N,N-dicyclohexylcarbodiimide (DCC) (Fluka, 99%), 4-(dimethylamino)pyridine (DAP) (Across, 99%), DSMO-d₆ (Aldrich, 99.9%), N,N'-dissopropylethylenimine (DIEPA) (Fluka, 99%), 1-hydroxybenzotriazole hydrate (HOBt) (Fluka, ≥98%), potassium hydrogen sulfate (KHSO₄) (Riedel-de Haën, 99%), sodium hydrogen carbonate (NaHCO₃) (Merck, 99.5%), and sodium sulfamic acid (Fluka, 99%) were 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 monoxide (Merck, p.a.), disodium hydrogen phosphate dihydrate (Merck, p.a.), and α-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 HCI-H-Gly-OEt (3.63 g, 0.026 mmol), DIEPA (8.9 mL, 0.052 mmol), and BOPT (11.49 g, 0.026 mmol) were added. After stirring for 10 min another equivalent of DIEPA (4.45 mL, 0.026 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 NaHCO₃ solution, once with 10 mL of water, once with 10 mL of hydrochloric acid, three times with 10 mL of a 1 M KH₂SO₄ solution, twice with 10 mL of water, and finally with 10 mL of brine. The reaction mixture was then dried over Na₂SO₄ which was filtered off. The solvent was removed under reduced pressure. The crude product was washed three times with 10 mL of 1 M KHSO₄, twice with 10 mL of water, and finally with 10 mL of brine. The mixture was then dried over Na₂SO₄, and solvent was removed under reduced pressure to obtain Boc-Pro-Gly-OEt (13.6 g, 45.5 mmol) in 88% yield.

**Synthesis of HCl Salt of H-Pro-Gly-OEt.**

H-Gly-OEt (3.63 g, 0.26 mmol), HOBt (7.13 g, 46.6 mmol), and DIEPA (16.0 mL, 93 mmol) were dissolved in EtOAc (40 mL). To this solution, DCC (9.58 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 KHSO₄, twice with 10 mL of water, and finally with 10 mL of 1 M NaHCO₃, twice with 10 mL of water, and finally with 10 mL of brine. The mixture was then dried over Na₂SO₄, and solvent was removed under reduced pressure to obtain Boc-Pro-Gly-OEt (13.6 g, 45.5 mmol) in 88% yield.

**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 DIEPA (16.0 mL, 93 mmol) were dissolved in EtOAc (40 mL). To this solution, DCC (9.58 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 KHSO₄, twice with 10 mL of water, and finally with 10 mL of brine. The mixture was then dried over Na₂SO₄, and solvent was removed under reduced pressure to obtain Boc-Pro-Gly-OEt (13.6 g, 45.5 mmol) in 88% yield.

**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).

**Synthesis of Boc-Pro-Gly-OEt.**

Boc-Pro-OH (10.02 g, 46.5 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 DIEPA (23.7 mL) were added. Finally, 9.39 g of DCC (45.5 mmol) was added. The reaction was stirred at room temperature for 24 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 KHSO₄, 10 mL of water, 10 mL of brine, three times with 10 mL of 1 M NaHCO₃, twice with 10 mL of water, and finally with 10 mL of brine. The reaction mixture was then dried over Na₂SO₄ 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-Pro-Gly-OEt was obtained in 61% yield (11.1 g).
re-dissolved in 100 mL of EtOAc and washed three times with 10 mL of 1 M KHSO₄ and twice with 10 mL of water. After drying over Na₂SO₄, 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).

1H NMR (400 MHz, DMSO-d₆): δ 0.8 (CH₃(=CH)₂, 12H, m), 1.7–2.1 (N–CH₂–CH₂–CH₂–CO–CH(CH₃)₂–CO–1H, m), 3.4–3.8 (N–CH₂–CH₂–CH₂–NO₂–CO–1H, m), 4.0 (O–CH₃–CH₂–O, 2H, s), 4.2 (NH–CH(CH₃)₂–CO, 2H, m), 4.3 (N–CH–CO, 1H, m), 5.6 and 6.0 (CH₂–CH₂–2H, s), 6.2 (NH–CH₂–COOH 1H, m), 7.6 (NH–CH(CH₃)₂–CO–1H, d), 8.2 (NH–CH₂–CO–1H, m), 8.3 (NH–NO₂–NH–2H, m).

Synthesis of Boc-Val-Pro-Gly-OEt. Boc-Val-Pro-Gly-OEt (5.00 mmol) was dissolved in 80 mL of EtOAc. To this mixture 6.9 mL of DIPEA (40.38 mmol) and 3.21 g of methacrylate (5.42 mmol) was added. The reaction mixture was stirred for 150 min. After neutralizing to pH 7 with 1 M HCl, the solvent was removed under reduced pressure. The product was redissolved in water, demineralized water (resulting solution pH 6.0). After acidifying the solution to pH 1 with 1 M HCl, the solution was heated at 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 Mₐ were determined by ¹H NMR spectroscopy in DMSO-d₆, using the resonances of the CH₃–O groups of poly(ethylene glycol) at 3.5 ppm 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 re-synthesize the VPGV-based monomer 1. This time a solution-based approach was used (see Scheme 1) instead of the previously published solid-phase approach, 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 that as the molecular weight of linear poly-VPGV is increased, the transition temperature is decreased.

To investigate whether our polymers were also affected in the same way, three polymers with different VPGV 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 ¹H 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 with the predictions of the theory described.
with the results described by Meyer and Chilkoti\textsuperscript{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.\textsuperscript{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 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{22} For our triblock copolymers there is already a free carboxylic acid group at the end of the peptide side
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. 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).

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 N2. 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.

Acknowledgment. The authors gratefully acknowledge the Netherlands Technology Foundation (STW) for financial support and R. Fokkink, Wageningen UR, for help with DLS measurements.

References and Notes


MA047923P