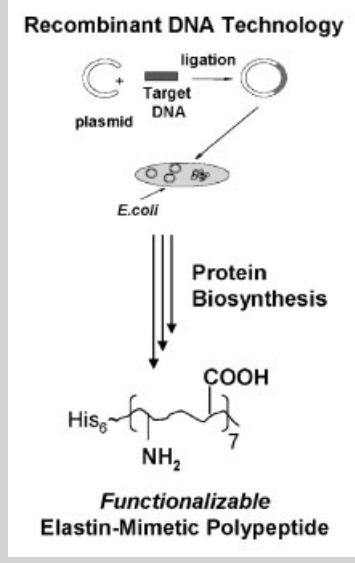
Summary: A new protein engineering strategy was utilized to synthesize an elastin-mimetic polypeptide. The primary structure represents an elastic motif composed of thirty amino acids with one lysine and one glutamic acid per repeat unit EMM = (VPGVG VPGKG VGPVG VPGVG VPGEG VPGIG). The gene was constructed using a Seamless Cloning method by generating three DNA cassettes which all encoded the EMM repeat unit, but with different flanking restriction recognition sites. The DNA cassettes were assembled to yield a gene that could be directly cloned into the multiple cloning site of pBluescript® II SK+. The resulting gene $(EMM)_7$ with approximately 650 base pairs in length was further cloned into the expression vector pET-28b. Protein biosynthesis in *E. coli* strain BLR(DE3) resulted in the 21.5 kDa repeating polypeptide His_6-$(EMM)_7$ yielding up to 50 mg · L^{-1} of cell culture. Secondary structure analysis by far UV circular dichroism revealed a minimum at 197 nm and a shoulder at 218 nm indicative for a random coil with some type II β-turn conformation content. Lower critical solution temperature (LCST) behavior strongly depends on salt and polypeptide concentration. Importantly, first cross-linking experiments indicate successful hydrogel formation with a surface structure reminiscent to natural elastin as visualized by SEM micrographs.



Biosynthesis of an Elastin-Mimetic Polypeptide with Two Different Chemical Functional Groups within the Repetitive Elastin Fragment

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Received: November 30, 2004; Revised: March 15, 2005; Accepted: March 24, 2005; DOI: 10.1002/mabi.200400213

Keywords: bioengineering; biopolymers; elastin; functional; polypeptide

Introduction

The development of new carrier materials with defined molecular structure and composition is becoming increasingly important not only from a fundamental point of view[1] but also for many applications in medical science, such as tissue engineering[2] and drug delivery[3] or for studying multivalent binding phenomena.[4] Polypeptides and proteins have been employed as a promising class of such materials due to their sequence specific structural properties in combination with biodegradability and biocompatibility.[5] Therein, elastin-mimetic proteins have been intensively investigated in the past due to the excellent correlation of sequence specificity and physico-chemical properties.[6] The majority of these elastin-mimetic polypeptides did not contain any chemical functional group, e.g., for post-analogous modification and were simply composed of the repetitive pentapeptide unit (Val-Pro-Gly-Val-Gly). Advances in recombinant DNA technology in the past decade, however, opened the possibility to engineer a variety of elastin-mimetic polypeptides with different sequences, e.g., containing cell-binding domains by employing different cloning strategies.[7] In addition, several research groups introduced lysine at the fourth position instead of valine.

 DOI: 10.1002/mabi.200400213

The resulting primary amino groups within the polypeptide opened many new opportunities for chemo-selective cross-linking and successive hydrogel formation for tissue engineering applications[8] or drug immobilization.[9]

To further expand the possibilities of chemical modification of elastin-mimetic polypeptides, the goal of this research was to introduce a second chemical functional group beside lysine residues. Therefore, we designed an elastin-mimetic polypeptide with two chemical functional amino acids per elastin repeat unit. Every 30 amino acids, which correspond to the length of one elastin repeat unit, we incorporated one lysine and one glutamic acid residue. The choice of the additional glutamic acid groups was guided by several considerations: (i) Many possibilities of different coupling reactions to primary amines or carboxylic acid groups are well documented in the literature,[10] (ii) if only amines or carboxylic acid groups are modified, the second chemical functional group can provide water solubility which is an important feature for protein-drug conjugates, and (iii) numerous effective protecting groups and deprotection methods for primary amines and carboxylic acid groups if needed are also well described in literature.[11]

Here we report on a new approach that takes advantage of the Seamless Cloning method using *Eam*1104 I sites during multimerization, but allows at the same time for direct cloning of the target gene in the expression system pET-28b using *Bam*H1 and *Hind*III sites without further modification of the expression plasmid. Cloning strategy, expression, and characterization of the target polypeptide by far ultraviolet (UV) circular dichroism (CD), turbidity measurements, NMR spectroscopy, and MALDI TOF are described. The new elastin-mimetic polypeptide possesses even carboxylic acid groups and seven primary amino groups within the repetitive elastin fragment, thus post-analogous modification with functional molecules such as probes, bioactive ligands, or drugs can be envisaged for various applications in medical science.

Experimental Part

Elastin-Mimetic Monomer (EMM) Containing DNA Cassettes

Oligonucleotide (Figure 1) encoding for the (EMM) with the primary sequence (VPGVG VPGKG $(VPGVG)_2$ VPGEG VPGIG) was obtained from Novagen, Madison, USA, and assembled in pBluescript® II SK+ (Stratagene, La Jolla, CA). The complementary DNA strand was produced by using 50 ng of EMM-oligonucleotide and the primer pair 5′-AGTTAGGATCCCTCTTCAGTACC and 5′-AGTTAAAGCTTCTCTTCATACGCC in a PCR reaction using Pwo-Polymerase (Roche, Basel, CH) according to manufacturer's instructions. The resulting PCR-product was purified using High Pure PCR-Product Purification Kit (Roche, Basel, CH) and treated with the restriction enzymes *Bam*HI and *Hind*III for 2.5 h at 37 °C. The DNA fragment was purified using QIAquick Gel Extraction Kit (Qiagen, Venlo, NL) and ligated into an equally restricted pBluescript® II SK+ vector using T4-Ligase (Promega, Madison, USA) according to manufacturer's instructions. *E. coli* DH10B cells (Stratagene, La Jolla, USA) were transformed with the resulting construct. Plasmid DNA was isolated from selected clones using High Pure Plasmid Isolation Kit (Roche, Basel, CH). Successful cloning was verified by restriction mapping of purified plasmid DNA from several transformants. Resulting positive clones were further verified by DNA-sequencing and were denoted as pBluescript® II SK-EMM.[12]

Figure 1. Design of the oligonucleotide sequence and corresponding amino acid sequence for the elastin-mimetic polypeptide.

Construction of the Synthetic Elastin-Mimetic Gene

The plasmid pBluescript® II SK-EMM was digested with *Eam*1104 I (Stratagene, La Jolla, USA) by incubating 50 µg pBluescript® II SK-EMM for 16 h and 50 U *Eam*1104 I in a total volume of 500 µl and purified as mentioned above resulting in cassette **2** (Figure 2). Cassettes **1** and **3** were obtained by cleaving 5 µg pBluescript® II SK-EMM with either *Bam*HI or *Hind*III for 2 h at 37 °C and further treatment with *Eam*1104 I for 1 h in a total volume of 50 µl for each cassette. The purified cassettes **1**, **2**, and **3** were ligated in a 1:10:1 ratio for 1 h at 37 °C. *Bam*HI and *Hind*III digested pBluescript® II SK+ was added directly to this solution and ligated for 16 h at 16 °C. *E. coli* DH10B cells were transformed with the resulting constructs. Plasmid DNA was isolated from several colonies and analyzed for the presence and size of synthetic inserts by restriction mapping. Positive clones were further verified by DNA-sequencing. Ongoing work was performed by using plasmid pBluescript® II SK-$(EMM)_7$ containing seven repeat units of the monomeric elastin gene EMM. The target gene with the multimerized EMM unit was excised from its cloning vector by restriction digestion with *Bam*HI and *Hind*III, and ligated into an equally digested pET-28b expression

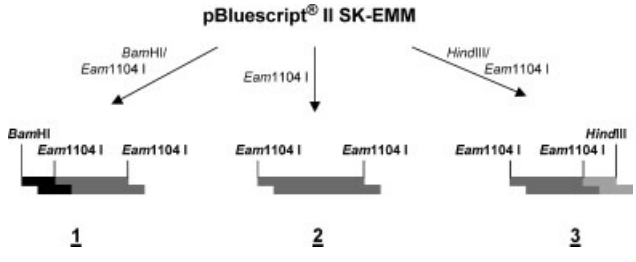

Figure 2. Construction of different DNA cassettes by sequential digestion of pBluescript SK II-EMM with *Bam*HI and *Eam*1104 I restriction sites for cassette **1** and with *Hind*III and *Eam*1104 I restriction sites for cassette **3**.

vector (Novagen, Madison, USA). The resulting plasmid pET28b-$(EMM)_7$ was transformed into the *E. coli* strain BLR(DE3) (Novagen, Madison, USA).

Bacterial Synthesis of His_6-$(EMM)_7$

(a) 5 mL Cultures

A single colony of the *E. coli* strain BLR(DE3) transformed with pET28b-$(EMM)_7$ was used to inoculate LB media (5 mL) containing kanamycin ($35\ g \cdot mL^{-1}$) and grown to stationary phase overnight. Three LB cultures (5 mL) were inoculated under the same antibiotic selection with the overnight culture to an optical density $OD_{600} = 0.1–0.2$ at temperatures of 25, 30, and 37 °C. The cultures were allowed to grow to an optical density of 0.8–1.0. Target protein expression was induced by addition of β-isopropylthiogalactoside (IPTG) to a final concentration of 1×10^{-3} M. Expression was allowed to proceed for 12 h and was analyzed by periodically taking samples by coomassie stained SDS-PAGE.

(b) 1 L Cultures

A single colony of the *E. coli* strain BLR(DE3) transformed with pET28b-$(EMM)_7$ was used to inoculate 8 L LB_{kana} media ($35\ \mu g \cdot mL^{-1}$ kanamycin). The 8 L culture was inoculated with cells from 200 mL of a starter culture, that was inoculated from frozen glycerine stocks (−80 °C) and grown overnight. The 8 L culture was grown without induction for 24 h at 30 °C with shaking at approximately 200 rpm. Due to leakiness of the T7 promoter, this process results in the efficient expression of pET28b-$(EMM)_7$ with typical yields of $50\ mg \cdot L^{-1}$ of culture media even in the absence of IPTG. As a comparison protein biosynthesis was also conducted with IPTG for protein induction under similar conditions as described for the 5 mL cultures at 30 °C. The cells were isolated by centrifugation at 6 000*g* at 4 °C for 20 min, resuspended in 50 mL lysis buffer (50×10^{-3} M Tris-HCl, 100×10^{-3} M NaCl, 8 M urea, 20×10^{-3} M imidazol), and lysed by a freeze-thaw cycle, sonicated three times for 30 s and centrifuged at 20 000*g* for 30 min RT. The supernatant was applied to affinity chromatography using chelating-sepharose charged with Ni^{2+} ions. Purification of His_6-$(EMM)_7$ was performed in the presence of 8 M urea on Äkta FPLC system (Amersham Biosciences, Uppsala, Sweden), using 5 column volumes (CV) for equilibration and loading of sample and 4–8 CV for washing. Bound polypeptide was eluted with 60×10^{-3} M imidazole. The His_6-$(EMM)_7$ containing fractions were pooled, dialyzed against H_2O_{dd}, and lyophilized.

Characterization of His_6-$(EMM)_7$

Molecular weight, purity, and concentration of the target protein were studied using SDS-PAGE followed by coomassie blue staining (BioRad, Hercules, CA). MALDI-TOF mass spectrometry was further employed for precise molecular weight determination of expressed His_6-$(EMM)_7$ using a Bruker Biflex III instrument. The solution turbidity of His_6-$(EMM)_7$ was measured as a function of temperature to determine the effects of protein concentration and ionic strength on the inverse transition temperature. His_6-$(EMM)_7$ solutions were heated at a rate of $0.2\ °C \cdot min^{-1}$ with different protein concentrations as indicated, and the optical density was measured at 600 nm (OD_{600}) by a Cary 50 UV-visible spectrophotometer equipped with a water bath. The inverse transition temperature was defined as the temperature at which OD_{600} reached 50% of its maximum. Far UV CD spectra were obtained using a Jasco 715 spectropolarimeter equipped with a temperature control unit. All CD spectra were taken with a 0.1 cm path length quartz cuvette (Hellma) in H_2O_{dd} or in 100×10^{-3} M Na_2HPO_4 buffer for temperature-dependent measurements. To avoid evaporation, the cuvette was sealed with Teflon tape. The settings for wavelength scans were: bandwidth, 0.5 nm; response time, 2.0 s; speed, $20\ nm \cdot min^{-1}$; accumulations, 4. For NMR analysis a small fraction of the repetitive polypeptide His_6-$(EMM)_7$ was used to remove the His-Tag by BrCN cleavage according to a literature procedure.[13] Molar mass of the remaining polypeptide $(EMM)_7$ was confirmed by MALDI-TOF analysis. ^1H NMR and 2D TOCSY spectrum were recorded at 278 K on Bruker DMX 600 spectrometer in H_2O/D_2O (9/1, v/v) using presaturation for solvent suppression (Prof. Dr. H. Kessler, Department Chemie, TU München). Scanning electron microscopy (SEM) images were taken using a JEOL JSM-5900 LV instrument. Protein samples were prepared from hydrogels as described below, after removal of the solvent, vacuum coated with a gold layer and analyzed at 20 kV.

Crosslinking Experiments

A general procedure was as follows: His_6-$(EMM)_7$ (11.6 mg, 5.4×10^{-4} mmol) were dissolved in 40 µL DMF and added to a mixture of bis(sulfosuccinimidyl) suberate (4 eq, 1 mg, 2.2×10^{-6} mmol) in 10 µL DMF. After thorough mixing, the reaction mixture was left overnight at 4 °C. The stable gel that was formed was washed several times by adding 1 mL water for 10 min at 70 °C and 800 rpm. The final gel was stored in 1 mL water at 4 °C.

Results and Discussion

Gene Construction

Recently the seamless cloning procedure based on the usage of the Endonuclease *Eam*1104 I was introduced by several research groups for molecular cloning applications.[7b,14] Multimeric genes generated through the *Eam*1104 I sites, however, cannot directly be cloned into the pET expression system due to several other *Eam*1104 I sites within the pET plasmid family. Undesired cleavage of plasmid DNA can only be avoided by using 5′-methyldeoxycytosine (^{5m}dCTP) in the PCR amplification of the expression plasmid. Methylated dCTP is incorporated into the internal *Eam*1104 I recognition sites of the plasmid, thus preventing cleavage upon treatment with *Eam*1104 I. Subsequent screening of the amplified clones, however, indicated incomplete incorporation of ^{5m}dCTP and resulted in fragmentation of the expression vector.[7b]

We developed a new approach that takes advantage of the *Eam*1104 I site during multimerization, but allows at

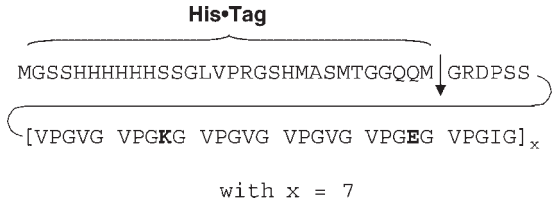

Figure 3. Amino acid sequence of the recombinant polypeptide His_6-$(EMM)_7$ indicating the cleavage site (arrow) after BrCN treatment with the remaining $(EMM)_7$ sequence.

the same time for simple cloning of the target gene into the recipient expression system pET-28b at the *Bam*HI and *Hind*III sites without further modification of the expression system. The DNA sequence shown in Figure 1 was designed with the following considerations.

The DNA cassette encoding the elastin-mimetic monomer (EMM) sequence was constructed from a chemically synthesized oligonucleotide and was designed to satisfy *E. coli's* codon bias. Oligonucleotide and the recipient plasmid, pBluescript® SKII+ were both treated with the restriction endonucleases *Bam*HI and *Hind*III, ligated and used to transform the *E. coli* strain DH10B. Recombinant pBluescript® II SK-EMM plasmids were identified by restriction mapping and the correct DNA monomer sequence was confirmed by sequencing.

DNA cassette **2** (Figure 2) was obtained from the pBluescript® II SK-EMM vector using *Eam*1104 I, which generates seamless junctions for directional cloning as reported previously.[6a] Gene fragments with flanking *Bam*HI and *Eam*1104 I sites or *Eam*1104 I/*Hind*III sites can be generated by sequential digestion of pBluescript® II SK-EMM and optimizing the time of digestion with the enzyme *Eam*1104 I since this enzyme will not efficiently recognize the *Eam*1104 I site close to the generated *Bam*HI or *Hind*III site. Based on this phenomena DNA cassettes **1** and **3** were produced by digestion of the plasmid pBluescript® II SK-EMM first with *Bam*HI or *Hind*III, respectively, for 2 h followed by digestion with *Eam*1104 I for another hour. For the cloning of the DNA cassettes, a molar mixture of the DNA cassettes **1**, **2**, and **3** of 1:10:1 was employed in the ligation reaction together with the *Bam*HI and *Hind*III digested pBluescript® II SK+ vector prior transformation into DH10B cells (Figure 2). Main consideration for the molar ratio was to be able to generate a gene composed of multiple DNA cassettes encoding for the elastin-mimetic monomer to allow efficient cross-linking of the resulting protein later on.

Transformed colonies were screened for positive clones. Presence of a multimer with 650 bp corresponding to an elastin-mimetic polypeptide with seven repeat units was confirmed by restriction mapping with *Bam*HI and *Hind*III. The complete amino acid sequence of the polypeptide is shown in Figure 3.

Polypeptide Production and Purification

The *E. coli* strain BLR(DE3) was selected for recombinant protein production. In this strain, a gene encoding T7 RNA polymerase is incorporated into the bacterial chromosome under lacU*V5* control and protein expression is induced by IPTG addition. To detect protein expression levels, protein biosynthesis was first performed in 5 mL cultures using a final concentration of 1×10^{-3} M IPTG. Aliquots were taken periodically after induction of protein synthesis ($t = 1$, 2, 3, and 12 h), analyzed by SDS-PAGE, and visualized by coomassie blue staining. In the first set of experiments, protein expression was performed at three different temperatures of 25, 30, and 37 °C. Figure 4 shows the results which suggest the expression level to increase with induction time up to 12 h. Moreover, it was found that highest protein levels were produced at temperatures of 25 and 30 °C. Therefore, a temperature of 30 °C for further upscaling was chosen.

Large scale protein expression of the elastin-like polypeptide in the pET system was performed by a methodology described by D. Urry and A. Cilkoti, which is based on the leakiness of the T7 promoter, that allows polypeptide production without usage of IPTG but utilizing gentle shaking for 24 h at 30 °C.[15] Protein biosynthesis affords a translational fusion protein with an amino-terminal His_6-Tag, that provides a convenient method of protein purification using immobilized metal-affinity chromatography. Isolation of target protein was accomplished under denaturing conditions to avoid protein losses due to inclusion bodies. Fractions of purified His_6-$(EMM)_7$ eluted with 60×10^{-3} M imidazole displayed a high degree of purity as visualized by SDS-PAGE and coomassie staining. Quantitative analysis of protein yield gave 50 $mg \cdot L^{-1}$ bacterial culture without IPTG induction supporting the results reported by Urry and Chilkoti et al.[15] In comparison when IPTG was used to induce protein production as described for the 5 mL cultures protein yield as low as 11 $mg \cdot L^{-1}$ was obtained. The polypeptide displayed excellent solubility in water, DMF, and DMSO.

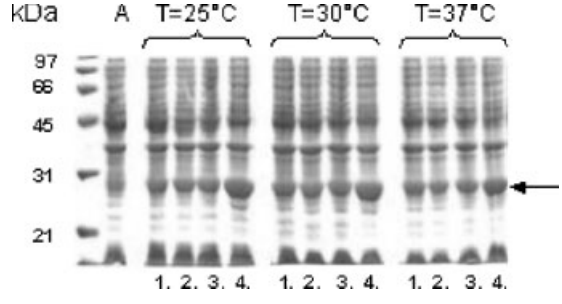

Figure 4. Coomassie stained SDS-PAGE for analysing the biosynthesis of His_6-$(EMM)_7$ at various temperatures of $T = 25$, 30, and 37 °C after (1) $t = 1$ h, (2) $t = 2$ h, (3) $t = 3$ h, and (4) $t =$ 12 h of induction (A = negative control without IPTG induction; arrow indicates additional band for protein production).

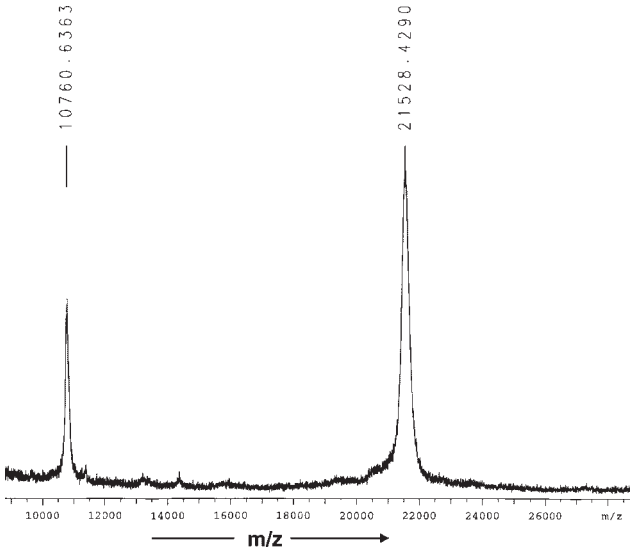

Figure 5. MALDI TOF/MS spectrum of His_6-$(EMM)_7$ with the signals for m/z and $(m/z)/2$.

Characterization of His_6-$(EMM)_7$

The molecular weight of polypeptide as determined by MALDI-TOF mass spectrometry confirmed the correct molar mass of 21 528 Da which is in excellent agreement with the theoretical expected value of 21 514 Da (Figure 5).

The structural identity of the repetitive polypeptide has been characterized by ^{1}H NMR spectroscopy. 2D TOCSY was used for correct peak assignment and to characterize overlapping signals [Figure 6(b)]. In order to simplify NMR analysis, the His_6-Tag of a small fraction of the His_6-$(EMM)_7$ polypeptide according to a literature procedure (Figure 3) was cleaved off and this sample was used for the NMR experiments.[13] ^{1}H NMR spectrum of $(EMM)_7$ was performed in H_2O/D_2O. The spectra were calibrated against D_2O as reference and all signals were attributed to different amino acid groups indicating excellent agreement with data in the literature.[13,16–18] Beside the signals that can be assigned to the most abundant amino acids valine, glycine, and proline several peaks can be clearly assigned to the lysine and glutamic acid groups, which supports the presence of these two amino acids in the repetitive polypeptide. According to these data, the γCH_2 and δCH_2 groups of the lysine amino acids can be clearly assigned to the peaks around 1.40 and 1.70 ppm, respectively, and also the Glu-αCH and Lys-αCH can be clearly seen at 4.45 ppm. Moreover, the side chain β-CH_2 and γ-CH_2 of glutamic acid have been nicely resolved in the 2D TOCSY spectra as indicated in Figure 6(b).

The secondary structure of His_6-$(EMM)_7$ was measured by far UV CD spectroscopy (Figure 7) at 0.15 mg $\cdot$ mL^{-1} in water at 20 °C. Inspection of Figure 6 shows a typical CD spectrum for a non-coacervated solution of soluble elastin displaying a minimum at 197 nm and a shoulder at 218 nm indicative of type II β-turn conformation. Similar spectra have been reported for human κ-elastin[19] and artificial elastin.[20]

Temperature-dependent CD spectra show an increase in the ordered conformation on increasing temperature from 5 to 40 °C in PBS buffer at pH = 7.4 (Figure 8). Although the overall shape of the curve remains the same, some important differences can be observed. The minimum around 200 nm is shifted compared to water from 197 to 204 nm in PBS buffer at 5 °C whereas the plateau region is still centered around 220 nm. With increasing temperature from 5 to 20 °C and finally to 40 °C, a slight shift of the minimum from 203 to 206 nm is observed. At the same time, the plateau region at 220 nm changes also to a discrete minimum and the relative intensity of the signals at 203 nm and 220 change from 1.39 at $T = 5$ °C to 1.23 and finally 1.11 at $T = 40$ °C. Such a behavior can be attributed to an increase of type II β-turn conformation with increasing temperature, which is an important requirement for the inverse temperature conformational change that has also been found for peptides based on the sequence (VPGVG) or domain 26 of tropoelastin.[21] Interestingly the temperature-dependent CD spectra reveal already significant changes in the secondary structure of the protein at very low protein concentration but no macroscopic coacervation is observed during CD measurements indicating again that protein concentration is a crucial parameter to visualize macroscopic coacervation and particle formation in the range of several hundred nanometer in size.

Lower Critical Solution Temperature (LCST) Behavior

The temperature-dependent phase behavior of His_6-$(EMM)_7$ was studied in 100×10^{-3} M PBS buffer, pH = 7.5 by temperature-dependent turbidity measurements showing no coacervation of the solution at concentrations of 5 mg $\cdot$ mL^{-1} over a wide range of temperature (20–95 °C). Due to the ampholytic character of the protein, it was anticipated that the inverse transition temperature T_c would be very sensitive towards ionic strength. Figure 8 summarizes the turbidity profiles of His_6-$(EMM)_7$ in 1 M NaCl solution at different protein concentrations of 1, 10, and 50 mg $\cdot$ mL^{-1}. Not surprisingly the inverse transition temperature of His_6-$(EMM)_7$ indicated strong dependency on polymer concentration ranging from 35 °C for 50 mg $\cdot$ mL^{-1} protein to 63 °C for very low protein concentrations of 1 mg $\cdot$ mL^{-1} (Figure 9). A similar pronounced effect of the salt concentration on the LCST behavior of elastin-mimetic proteins has been recently reported by Chilkoti et al. for a lysine-containing elastin-mimetic protein.[13b] Moreover, the critical coacervation temperature T_c decreases with increasing protein concentration of the His-$(EMM)_7$ from 46×10^{-6} M to 2.325×10^{-3} M and the data

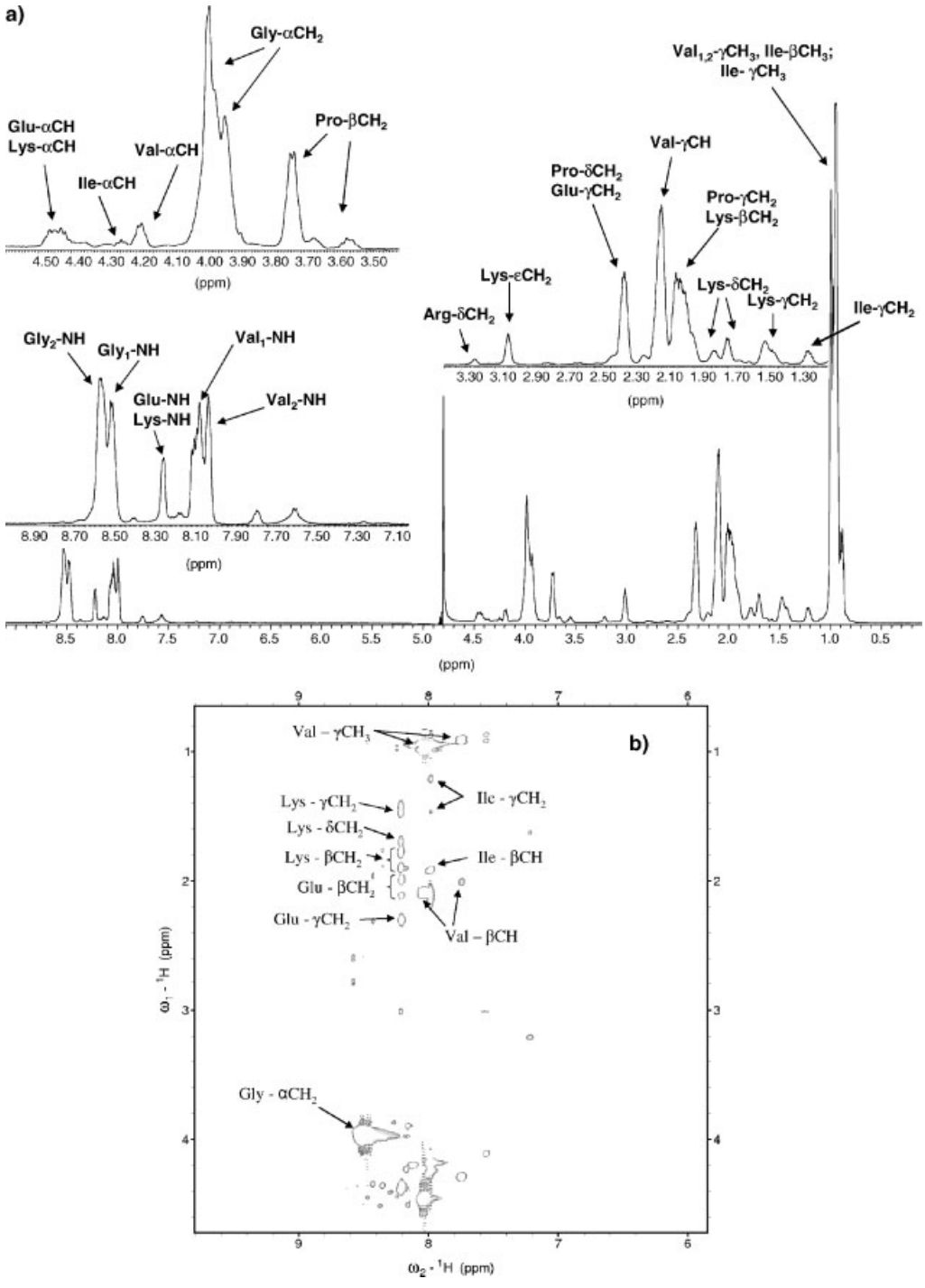

Figure 6. (a) ^{1}H NMR spectrum of $(EMM)_7$ in D_2O/H_2O (1:9, v/v), 600 MHz, $T = 20\,^{\circ}C$) and (b) part of a 2D TOCSY NMR (D_2O/H_2O (1:9, v/v), 600 MHz, $T = 20\,^{\circ}C$) with selected group assignment.

could be well fitted using a logarithmic function of protein concentration with an r^2 of 0.996 ± 0.005 which is in excellent agreement with other elastin-mimetic proteins as reported recently.[22]

Cross-Linking and Hydrogel Formation

One possible application of the elastin-mimetic repeating polypeptide His_6-$(EMM)_7$ is in the field of hydrogels for

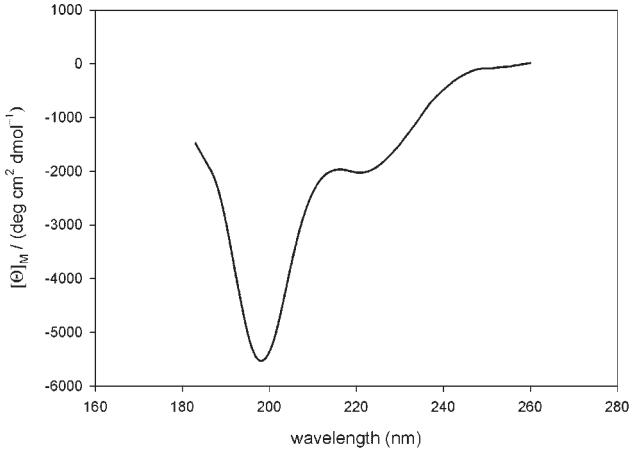

Figure 7. Far UV CD spectroscopy of His_6-$(EMM)_7$ at 0.15 mg · mL^{-1}, $T = 20$ °C.

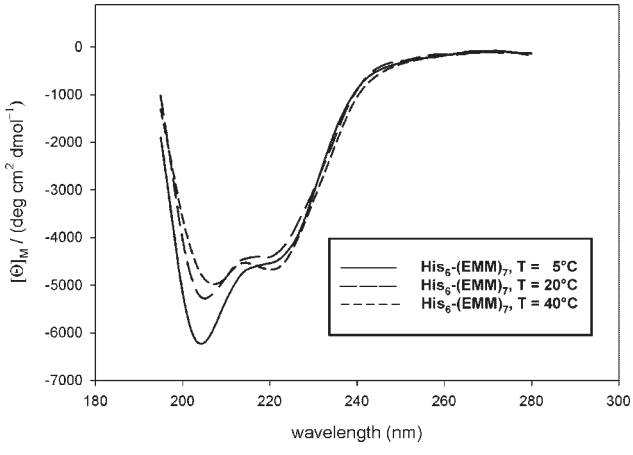

Figure 8. Far UV CD spectroscopy of His_6-$(EMM)_7$ at 0.13 mg · mL^{-1} in 100×10^{-3} M Na_2HPO_4 buffer, pH 8.0 at $T = 5$, 20, and 40 °C.

biomedical applications such as self-regulating delivery vehicles for bioactive molecules due to their expected pH and ionic strength responsive features and as three-dimensional matrices that organize cells or direct the formation of

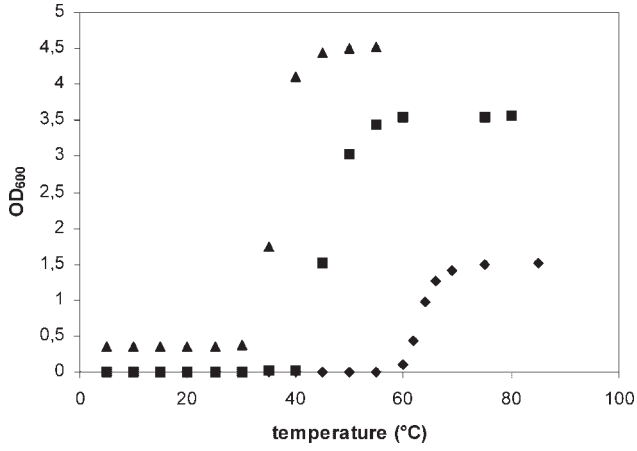

Figure 9. LCST behavior of His_6-$(EMM)_7$ in 1 M NaCl aqueous solution. (◆) 1 mg · mL^{-1}, $T_c = 63$ °C; (■) 10 mg · mL^{-1}, $T_c = 48$ °C; (▲) 50 mg · mL^{-1}, $T_c = 35$ °C.

a desired tissue.[23] Therefore, some preliminary chemical cross-linking of the His_6-$(EMM)_7$ using bis(sulfosuccinimidyl) suberate as cross-linker was performed in order to reveal, if the additional glutamic acid residues would have any effect on hydrogel formation of the elastin-mimetic polypeptide. Gel formation occurred within 5–15 min reaction time and was left for completion overnight at 4 °C. SEM micrographs (Figure 10) of the gels show quite different surface morphologies depending on polypeptide concentration, a rough surface with structural features on the μm scale and a filamentous surface structure that resembles more natural elastin indicating that the additional glutamic acid groups do not disturb chemical cross-linking of the elastin-like polypeptide. However, many more experiments have to be made to fully understand and to control the surface structure of the elastin-based hydrogels which is known to depend on many parameters such as solvent used during cross-linking, protein concentration, and gel processing. Ongoing studies focus on the physical characterization of the gels that should have interesting pH and ionic strength responsive swelling properties and the chemical modification of the gels with various bioactive ligands.

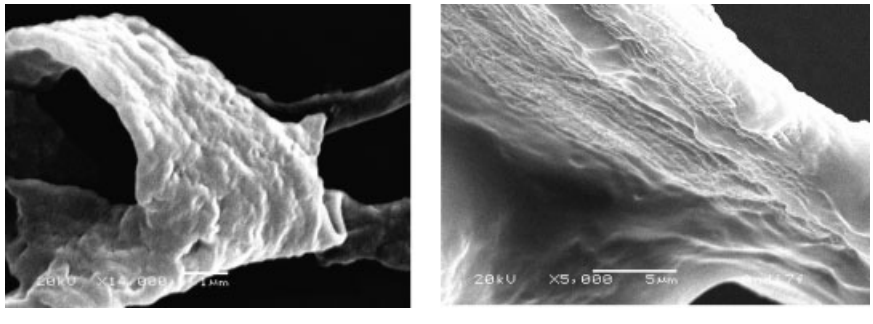

Figure 10. SEM micrographs of His-$(EMM)_7$ cross-linked with bis(sulfosuccinimidyl) suberate in DMF at (a) 10 wt.-% and (b) 20 wt.-% of the elastin-mimetic polypeptide His_6-$(EMM)_7$.

Conclusion

In summary, we have presented the biosynthesis of a new elastin-mimetic polypeptide by a procedure that enables the usage of the Seamless cloning method based on *Eam*1104 I restriction sites, but eliminates at the same time the necessity for multiple PCR reactions on the cloning or expression plasmid. This cloning method should be applicable to the production of any repeating polypeptide and combines the advantages of the Seamless cloning method with an easy and rapid assembly of a target gene. The resulting repeating polypeptide His_6-$(EMM)_7$ has a molecular weight of 21 500 Da and temperature-dependent CD spectra displayed a predominant type II β-turn conformation between 5 to 20 °C and 40 °C. The LCST behavior strongly depends on polypeptide and salt concentration. Most importantly the repeating polypeptide possesses several carboxylic acid groups and primary amino groups at defined positions within the backbone and, therefore, presents a versatile starting material for many applications in biomedicine where bioactive ligands, drugs, or probes have to be covalently attached to a molecular defined polymeric carrier.

Acknowledgements: The authors thank Prof. Dr. *J. Buchner* (Lehrstuhl für Biotechnologie, TU München) for his generous support of this work. D. Kaufmann would like to thank the *SFB 486* for financial support.

[1] [1a] S. J. M. Yu, V. P. Conticello, G. H. Zhang, C. Kayser, M. J. Fournier, T. L. Mason, D. A. Tirrell, *Nature* **1997**, *389*, 167; [1b] A. E. Barron, R. N. Zukcermann, *Curr. Opin. Chem. Biol.* **1999**, *3*, 681; [1c] K. Kirshenbaum, R. N. Kuckermann, K. A. Dill, *Curr. Opin. Struct. Biol.* **1999**, *9*, 530; [1d] J. C. Nelson, J. G. Saven, J. S. Moore, P. G. Wolynes, *Science* **1997**, *277*, 1793.

[2] [2a] D. W. Urry, A. Pattanaik, J. Xu, T. C. Woods, D. T. McPherson, T. M. Parker, *J. Biomat. Sci. Polym. Ed.* **1998**, *9*, 1015; [2b] A. Panitch, T. Yamaoka, M. J. Fournier, T. L. Mason, D. A. Tirrell, *Macromolecules* **1999**, *32*, 1701.

[3] [3a] A. Chilkoti, M. R. Dreher, D. E. Meyer, D. Raucher, *Adv. Drug Delivery Rev.* **2002**, *54*, 613; [3b] M. Haider, Z. Megeed, H. Ghandehari, *J. Control. Rel.* **2004**, *95*, 1.

[4] J. E. Gestwicki, C. W. Cairo, L. E. Strong, K. A. Oetjen, L. L. Kiessling, *J. Am. Chem. Soc.* **2002**, *124*, 14922.

[5] [5a] J. C. M. van Hest, D. A. Tirrell, *Chem. Commun.* **2001**, *19*, 1897; [5b] H. A. Lashuel, S. R. LaBrenz, L. Woo, L. C. Serpell, J. W. Kelly, *J. Am. Chem. Soc.* **2000**, *122*, 5262; [5c] D. Cunliffe, S. Pennadam, C. Alexander, *Eur. Polym. J.* **2004**, *40*, 5; [5d] G. W. M. Vandermeulen, H. A. Klok, *Macromol. Biosci.* **2004**, *4*, 383.

[6] [6a] D. W. Urry, *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 819; [6b] L. Debelle, A. J. P. Alix, *Biochimie* **1999**, *81*, 981; [6c] L. Debelle, A. M. Tamburro, *Int. J. Biochem. Cell Biol.* **1999**, *31*, 261; [6d] M. Martino, T. Perri, A. M. Tamburro, *Macromol. Biosci.* **2002**, *2*, 319.

[7] [7a] D. T. McPherson, C. Morrow, D. S. Mineham, J. Wu, E. Hunter, D. W. Urry, *Biotechnol. Prog.* **1992**, *8*, 347; [7b] R. A. McMillan, T. A. T. Lee, V. P. Conticello, *Macromolecules* **1999**, *32*, 3643; [7c] J. Lee, C. Macrosko, D. W. Urry, *Biomacromolecules* **2001**, *2*, 170; [7d] D. E. Meyer, A. Chilkoti, *Biomacromolecules* **2002**, *3*, 357; [7e] J.-I. Won, A. E. Barron, *Macromolecules* **2002**, *35*, 8281.

[8] [8a] E. R. Welsh, D. A. Tirrell, *Biomacromolecules* **2000**, *1*, 23; [8b] K. Di Zio, D. A. Tirrell, *Macromolecules* **2003**, *36*, 1553; [8c] A. Girotti, J. Reguera, J. C. Rodríguez-Cabello, F. J. Arias, M. Alonso, A. M. Testera, *J. Mater. Sci.: Mater. Med.* **2004**, *15*, 479.

[9] M. R. Dreher, D. Raucher, N. Balu, O. M. Colvin, S. M. Ludeman, A. Chilkoti, *J. Contr. Release* **2003**, *91*, 31.

[10] [10a] G. T. Hermanson, "*Bioconjugate Techniques*", Academic Press, New York 1996; [10b] S. V. Han, Y. A. Kim, *Tetrahedron* **2004**, *60*, 2447; [10c] J. P. Tam, Q. T. Yu, Z. W. Miao, *Biopolymers* **1999**, *51*, 311.

[11] P. Kocienski, "*Protecting Groups*", 3rd edition, Thieme, Stuttgart 2004.

[12] J. Sambook, E. F. Fritsch, T. Maniatis, "*Molecular Cloning: A Laboratory Manual*", 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989.

[13] R. A. Mc Millan, V. P. Conticello, *Macromolecules* **2000**, *33*, 4809.

[14] K. A. Padgett, J. A. Sorge, *Gene* **1996**, *168*, 31.

[15] [15a] C. Guda, X. Zhang, D. T. McPherson, J. Xu, J. H. Cherry, D. W. Urry, H. Daniell, *Biotechnol. Lett.* **1995**, *17*, 745; [15b] D. E. Meyer, A. Chilkoti, *Biomacromolecules* **2002**, *3*, 357.

[16] D. W. Urry, K. Okamoto, R. D. Harris, C. F. Hendrix, M. M. Long, *Biochemistry* **1976**, *15*, 4083.

[17] D. Kurková, J. Kříž, P. Schmidt, J. Dubal, J. C. Rodríguez-Cabello, M. Alonso, *Biomacromolecules* **2003**, *4*, 589.

[18] A. Bundi, K. Wüthrich, *Biopolymers* **1979**, *18*, 285.

[19] L. Debelle, A. J. P. Alix, S. M. Wei, M.-P. Jacob, J.-P. Huvenne, M. Berjot, P. Legrand, *Eur. J. Biochem.* **1998**, *258*, 533.

[20] D. W. Urry, B. Starcher, S. M. Partridge, *Nature* **1969**, *222*, 795.

[21] [21a] C. Spezzacatena, T. Perri, V. Guantieri, L. B. Sandberg, T. F. Mitts, A. M. Tamburo, *Eur. J. Org. Chem.* **2002**, *1*, 95; [21b] H. Reiersen, A. R. Clarke, A. R. Rees, *J. Mol. Biol.* **1998**, *283*, 255; [21c] S. A. Jensen, B. Vrhoovski, A. S. Weiss, *J. Biol. Chem.* **2000**, *275*, 28449.

[22] D. E. Mayer, A. Chilkoti, *Biomacromolecules* **2004**, *5*, 846.

[23] [23a] J. Kost, R. Langer, *Adv. Drug Del. Rev.* **2001**, *46*, 125; [23b] J. L. Druy, D. J. Mooney, *Biomaterials* **2003**, *24*, 4337.

