

**MODIFICATION OF BEHAVIOR OF ELASTIN-LIKE POLYPEPTIDES BY
CHANGING MOLECULAR ARCHITECTURE**

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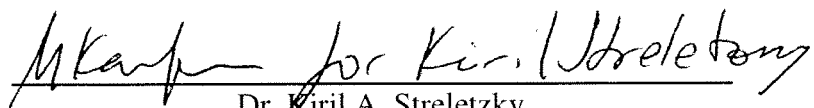
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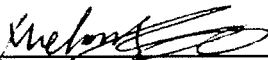
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ALI GHOORCHIAN

ABSTRACT

Elastin-like polypeptides (ELP) are environmentally responsive polymers that exhibit phase separation in response to external stimuli such as temperature, pH, light, and ionic strength. It has been shown that the sequence of the pentapeptide, its length, and the solution concentration are very important in the transition of the molecules from soluble to insoluble, but there has not been any detailed study of the effect of molecular architecture on the behavior of ELPs.

In this study we designed, synthesized and characterized ELPs with different architectures and chemical identities to probe the effect of molecular design on the microscopic and macroscopic behavior of ELP molecules and to compare them to the linear ELP molecules. These new architectures also helped us better understand the theory of folding and aggregation of ELPs. The design was based on constructing three-armed star molecules by tagging a trimer forming oligomerization domain to the ELP chains. ELPs were chosen to have different chemical identities by changing the pentapeptide sequence. The molecules were synthesized by molecular biology techniques and characterized by different methods.

Our results show that capping the three ELP chains forces the chains to fold into more extended rod-like constructs prior to aggregation. A mathematical model was developed

to predict the behavior of ELP chains at the transition temperature and it was shown that there is a difference between N- and C- terminal capping ELPs seem to fold at lower temperatures when their N-termini are held together. It was also shown that the constructs with both their ends capped can be designed such that they fold into a stable unit at much lower temperatures than the linear constructs without necessarily aggregation at higher temperatures.

The trimer constructs were also used to make micellar aggregates that were characterized by dynamic and static light scattering. It was shown that the size of the micelles can be controlled by adjusting salt concentration or by making mixtures of linear and trimer constructs. The micelles were also crosslinked into responsive stable nanoparticles.

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Chapter I

Background

1-1. Introduction

In nature there are many macromolecule-based systems with fundamental importance for living organisms that exhibit changes in structure and behavior in response to changes in their environment. Many biological systems function on the basis of some feedback communication with their surroundings which induce specific changes in the conformation of proteins, polypeptides, or nucleic acids. These changes in the conformation would then translate into changes in the function of the macromolecules and ultimately affect the living organisms. In essence many of the biologically related functions can be considered as responsive to environmental changes. The response can be a change in solubility, secondary structure, or even intermolecular association and can be triggered by a variety of natural stimuli including temperature and pH variations, among many other physiologically-induced changes. Understanding these biological responses and what induces them, their response to environmental stimuli, and the pathway of transition from one state to another has been the subject of countless studies and theories

throughout many decades. This understanding would help to provide us tools to utilize these environmentally-induced changes in designing new and improve biologically-inspired materials.

The most commonly studied responsive macromolecules are the ones that exhibit lower critical solution temperature (LCST) and transition from soluble to insoluble in response to environmental stimuli.^{1.1, 1.2} LCST is a temperature below which, a mixture components remain soluble and do not show any immiscibility for any composition. The best known representative of these materials is poly(N-isopropylacrylamide) (PNIPAA) which has an LCST of 32°C and has been studied for many years as a “smart” polymer for a variety of applications.^{1.1, 1.3-6}

The other common family of responsive macromolecules consists of peptide-based responsive molecules. The building blocks of these materials are usually naturally occurring amino acids and there is interest in them because of the advantages they offer in biologically-related applications. These soft, hydrated materials have many advantages over traditional chemically synthesized polymers. One major advantage of these materials is the possibility of precise control over the chemical identity and length together with their monodisperse distribution in the solution.^{1.7} Many of these materials have been shown to be biodegradable and generally biocompatible.^{1.8-10} Also the ability to add short peptide motifs and tags such as RGD, IKVAV, and GHK to the polypeptide makes these materials very attractive for bio-interface and cell attachment applications^{1.11-13}. These materials have also shown potential to be used as self-assembling units that can be used in designing 3D functional hydrogels.^{1.14, 1.15} Even their obvious shortcoming of being limited to naturally occurring amino acid residues is

becoming less of a restriction by the development of more artificial amino acids that can be used in making peptide-based materials.^{1.16-18}

Elastin-like polypeptides are a group of peptide-based materials which also have the LCST characteristics of responsive polymers and go through a transition in response to external stimuli.^{1.19} These polypeptides have similar characteristic of the elastin which makes them great candidates for many potential applications in which the flexibility and resilience of elastin is desired. The elastin-based materials are especially interesting since elastin and its unique characteristics have become the center of many material-related investigations and innovations in recent years because of its ability to repeatedly and reversibly go through stress cycles without failing.^{1.20, 1.21} Elastin-like polypeptides are designed based on observed repetitive sequences in mammalian elastin proteins^{1.22} with the most common being the pentapeptide Val-Pro-Gly-Xaa-Gly, in short VPGXG, in which Xaa can be any of the natural or synthetic amino acids except for proline.^{1.23} They are soluble below their lower critical transition temperature (LCST) and become insoluble and aggregate above their phase transition temperature.^{1.23} The protein-rich liquid phase from this phase separation is a coacervate and has been shown to have about 40% protein content.^{1.7} In addition to VPGXG, there are other sequences that have shown to have the characteristics of elastin-like polypeptides, including LGGVG^{1.24}, GXGGX^{1.25}, APGVGV^{1.26}, AVGVV^{1.27}, KGGVG^{1.28}, LGAGGAG^{1.29}, but most of the studies have focused on VPGXG as the representative of this family of peptide-based materials.

In this chapter we first look at different viewpoints on the theory of phase transition and aggregation of elastin-like polypeptides and the much debated origin of their

elasticity. In this aspect the role of water is especially of interest and the structural studies of these materials will be discussed. We will then present the synthesis and characterization methods of these materials and summarizes the work that has been done to utilize these biologically inspired materials in different medical and non-medical applications.

1-2. Theory of Transition

From macroscopic point of view the transition of elastin-like polypeptides from soluble to insoluble in response to environmental stimuli can be easily observed and quantified using UV-vis spectroscopy (Figure 1-1).

The transition temperature depends on the chemical identity, chain length, concentration, and architecture of the ELP in the solution^{1.7, 1.30, 1.31} and sometimes a combination of these factors can be utilized to design a specific ELP molecule with a known transition temperature for a particular application. The environmental stimuli to trigger this phase change are diverse and ELP molecules can be designed to respond to temperature, pH, ionic strength, light, and other stimuli.^{1.7, 1.32, 1.33}

a)



b)

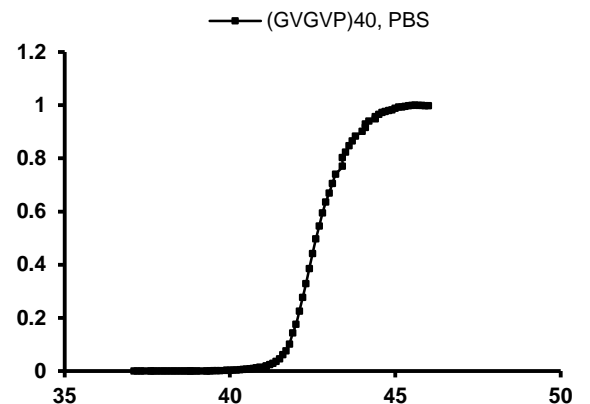


Figure 1-1. a) Transition of an ELP solution from soluble (left) to insoluble suspension of aggregates (middle), and finally phase separation (right). The phase separation happens after keeping the solution above the transition temperature for extended period of time. b) UV absorbance measurement of the (GVGVP)₄₀ solution from below to above the transition temperature.

The extensive studies by Urry showed that there is a predictable relationship between the hydrophobicity of the X residue in (VPGXG) and the temperature at which the ELP solution goes through the transition.^{1,7} This hydrophobicity scale has been shown to be a very accurate first estimate that can be used prior to designing and synthesizing new ELP molecules to predict their transition temperatures.

As much as it is a straight forward to observe and measure the transition temperature of elastin-like polypeptides, it is difficult and problematic to have a full picture of this transition at the molecular level. Early work by Urry^{1,34} showed that a cyclic polypentapeptide of (VPGVG)₃ in its crystalline state consists of three type II β -turns joined by Val-Gly-Val bridges. Based on this observation and using the (VPGVG) as the representative elastin-like polypeptide, they then employed circular dichroism (CD), NMR and dielectric relaxation studies to probe the transition of unordered polypeptides at low temperatures to more ordered structures at temperatures above the transition temperature.^{1,19, 1.35} They suggested that this transition is in agreement with their proposed phase diagram for ELP molecules^{1.36} and the change at molecular level is ultimately accompanied with the phase change in solution. But later studies showed that this molecular structure change is a gradual process that starts at temperatures well below the transition temperature.^{1.37} Urry and Cabello later suggested that the ordering of

polypeptides is actually the main contributor to the LCST behavior of ELPs.^{1,38} They studied the thermal behavior of ELP molecules using temperature modulated differential scanning calorimeter (TMDSC) in different salt concentrations and suggested that the process of inverse transition temperature (ITT) in ELPs is inherently different from LCST of the other macromolecules and the process of folding and aggregation leads to an increase in the order of the chains.

Based on Urry's model for ELP transition, the chains in the random conformation below their transition temperature start to form type II β -turn conformation as they approach their transition temperature and then at the transition temperature they fold in to a so called a β -spiral which is a helical arrangement of β turns.^{1,36} These folded constructs would then be stabilized by making trimers called twisted filament (Figure 1-2).

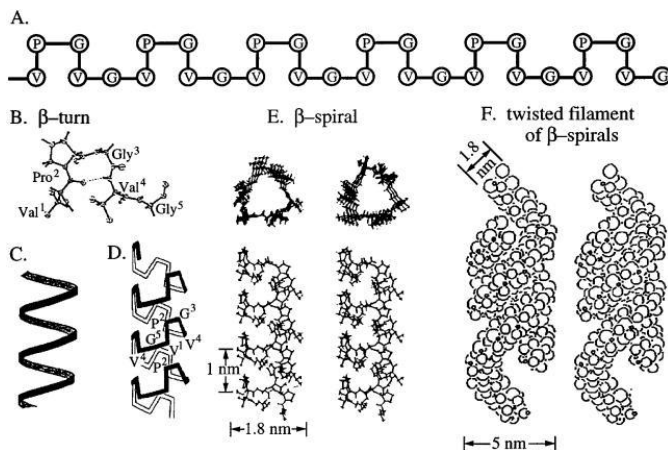


Figure 1-2. The formation of twisted filaments from folded ELP chains as suggested by Urry. The β -spiral formation from β -turns is believed to be precursor of ELP aggregation. (with permission from reference 1.39). Copyright 1997 American Chemical Society.

Numerous groups have studied different ELP sequences to probe this theory and an array of results has been reported. Many of the experimental studies, mostly using CD

spectroscopy and IR measurements, have suggested the existence of more ordered state of the polypeptides at higher temperatures that might be made of mainly β turns. In a study using infrared spectroscopy and computational methods on a 59 kDa ELP of 150 pentapeptide repeats of (VPGXG), Serrano et al. suggested that their results are consistent with β -spiral model of Urry.^{1.40} This is one of the few works which combines experimental data along with a mathematical model to study this transition. They also showed that increasing temperature to those of higher than the transition temperature affects the packing of the β -spiral strands to β -sheet structure. This is consistent with Urry's observation that heating up the ELP solution to temperatures well above their transition temperature causes more water to be expelled from the coacervate.^{1.36} Other studies by Arkin using (VPGXG) as the model molecule and employing multicanonical Monte Carlo simulations show good agreement to the Urry model.^{1.41, 1.42} Nicolini et al.^{1.43} characterized temperature and pressure induced inverse transition temperature of a single pentapeptide using DSC, CD, and FTIR showing the existence of type II β -turns and a change from unfolded to folded structure by increasing the temperature. They also showed that at high pressure the population of ordered structures decreases and more random coil is observed.

Despite the existence of the experimental and molecular simulation results pointing towards the existence of ordered structure for ELPs there are also many investigations showing less or no order for these constructs either below or above the transition temperature. Li et al.^{1.44} conducted one of the most detailed simulation studies of ELP molecules using (GVGVP)₁₈ as their template in a temperature range of 7°C to 42°C. Their starting point was the proposed β -spiral model and they showed that when the ELP

goes through the transition more turns can be observed above the transition temperature. But their simulations indicate that above T_i the ELP monomer can be described as a compact and amorphous structure which contains disordered β strands and fluctuating turns. It is noticeable that in this simulation a single chain was studied and the effect of other chains on the conformational behavior of the ELP chain was not taken into consideration. Yao et al. studied the structure of a (GVGVVP)₃ by using 1D NMR and their results suggested only a small fraction of the overall structure consists of type II β -strands.^{1.45} Later, Krukau et al. used a GVG(VPGVG)₃ as a molecular model to study the conformational transition of the ELPs by employing molecular simulations in water at temperatures between 7°C to 167°C and their results suggest that the breaking of hydrogen bonds of clustering water plays a very important role in the transition temperature but there is no folding of the ELP molecule upon reaching to the transition temperature.^{1.46} They suggested the existence of a random distribution of structured elements on the peptide chains even above the transition temperature. They also challenged the idea of having ELP chains in random coil below the transition temperature as they found a rigid conformation below the transition temperature. They suggest that the minimum of CD spectra at 195 nm which is used as an indication for the existence of random structure might be contributed to the presence of ordered polyproline II (PPII) structure. This idea has been challenged by the fact that increasing temperature causes the 198 nm minimum to disappear while another characteristic peak of PPII conformation at 212 nm remains almost the same in most of the CD spectra of ELP molecules at different temperatures. This is an indication that these two peaks represent two different species and so they most probably cannot be interpreted as PPII secondary structure.^{1.47}

Some researchers used elastin-like polypeptide sequences other than VPGVG to study the conformational change of the chains through the transition. One such molecule was (LGGVG)_n which was studied by some groups. Martino et al.^{1.48} first studied this ELP using CD spectroscopy and transmission electron microscopy. They suggested that the sequence XGGXG can better represent elastin molecules as it illustrates what they called “sliding β -turn”.^{1.24} They showed that a simultaneous presence of different conformations can be observed for this construct which includes type II β -turns, type I β -turns, and unordered structure and makes this ELP a structurally heterogeneous biopolymer. Using TEM they also observed twisted-rope aggregates which are in agreement to the Urry’s theory. Although it should be noted that their studies were done in organic solvents (TFE and HFIP) and it has been shown elsewhere that organic solvents and specifically TFE (trifluoroethanol) can promote the type II β -turn conformation for ELPs.^{1.49} Kumashiro group later studied this polypeptide using 1D and 2D NMR and concluded that the best scenario for describing the conformation of these polypeptides is what they call a “conformational ensemble”, in which a number of different conformations coexist.^{1.24, 1.50} They also confirmed the existence of type II β -turn structure in water and type I β -turn in TFE.^{1.51} This heterogeneity in conformation was also reported by Ahmed et al.^{1.52} who studied VPGXG as their model molecule and did a range of UV-resonance raman (UVRR) studies on linear and cyclic elastin. They suggested that there is an ensemble population of polypeptides especially for linear constructs which mostly includes type III β -turns and disordered β -strands and some type II β -turns. For the cyclic polypeptides at low temperatures they found that most of the population is unordered strands of type II and type III β -turns, but an increase in type II β -turns at temperatures above the transition

temperature was observed. However, their UVRR does not show any difference in population of type II β -turns for linear or cyclic constructs. The possible difference between the population of the turns is especially interesting considering the fact that most of the structural studies by Urry were done on cyclic short chains. Bochicchio et al. also used CD spectroscopy on elastin, abductin, and resilin proteins and showed the very close equilibrium between PPII, unordered, and β -turn conformations.^{1.49} These different results from different systems and the possibility of having a number of different conformations coexisting might explain the very different and sometimes contradictory results that have been reported, especially using molecular dynamic simulations, since these simulations are based on a series of initial boundary conditions which could be significantly different from one solution condition to another.

1-3. Role of Water

Although there are numerous viewpoints on the molecular changes in ELP molecules when they go through their transition, there is very little doubt about the importance of water in this process. These mostly hydrophobic polypeptides are surrounded by water molecules which are called the water of hydrophobic hydration. Water of hydrophobic hydration was first studied by Frank^{1.53} who described these water molecules as “icebergs” surrounding the hydrophobic molecules. The idea of ice-like water molecules later evolved to more dynamic water molecules which have a relaxation frequency somewhat lower than that of the bulk water. In work done by Urry et al.^{1.39} they observed a temperature dependent relaxation of about 5 GHz, which is just below the frequency of bulk water, for two long ELP molecules. This first hydration shell covers most of the surface of the biomolecule at lower temperatures and increasing the temperature causes

this water network to break into small water clusters that cannot cover all of the area on the surface of the molecule and are less ordered.^{1.54} This results in more exposed hydrophobic residues to the bulk water which drives the aggregation of the molecules as they approach the transition temperature. The fact that the transition of biomolecules from soluble to insoluble correlates with the reordering of waters around the molecules was used to relate the transition temperature of ELPs to the water of hydrophobic hydration.^{1.7} It has been suggested that even the thermal characteristics of the water of hydrophobic hydration is different from that of the bulk water.^{1.55} Oleinikova et al. studied the heat capacity (C_p) of water molecules around hydrophilic and hydrophobic residues in both hydration shell and bulk water using molecular simulations.^{1.56} They showed that the C_p value of the water surrounding the biomolecule is actually higher than that of the bulk water but it starts to decrease when the temperature starts to elevate. It eventually approaches that of the bulk water at high temperatures and at these high temperatures the C_p is actually affected by the interaction of hydrophobic hydration water with the bulk water which means that the two species of the water molecules become more and more alike.

Since the change in the structure of water is accompanied with phase separation of these biomolecules and considering that the transition temperature is the line between the miscibility and immiscibility of the solution components, the Gibbs free energy should be zero at this point and so

$$T_t = \frac{\Delta H}{\Delta S} \quad (1-1)$$

Urry used this concept to develop the T_t -based hydrophobicity scale that can be used to predict the transition temperature of different ELP solutions based on their chemical

identity.^{1.7, 1.57} Also based on his theory, the folding of ELP molecules to more ordered constructs upon reaching to the transition temperature causes these chains to have more positive entropy. This entropy would be then compensated by the less ordered water molecules negative entropy at the transition.^{1.7, 1.39}

The water of hydrophobic hydration and its role in the transition of ELPs has been subject to many studies and experiments using different methods. Cabello et al. used scanning calorimetry to study poly(VPGVG) with the aim of better understanding the mechanism of protein folding and assembly.^{1.58} Their experiments were aimed at studying the clathrate-like structure of water molecules surrounding the hydrophobic moieties of the polypeptide chain from conditions of water deficiency to excess of water. This work was done based on using polypeptides with molecular weights of above 15 kDa and their results indicated that there are a maximum of 170 water molecules per pentapeptide. They also confirmed that these clusters of water are distributed in a non-homogeneous fashion with a broad range of energy distribution. The water molecules and their role in the structural stability of the polypeptide by making hydrogen bonds was investigated by changing the aqueous environment from water to D₂O by Cho et al.^{1.47} They measured the critical solution temperature of five different ELP molecules with different lengths and chemical identity. Using CD spectroscopy, ATR/FTIR, and DSC measurements they found β -turn structures in both water and D₂O solutions but the highest content of these β -turns were observed for less hydrophobic and shorter polypeptides (Figure 1-3). They also studied the difference between the LCST of the ELP molecules in water and heavy water and compared it to that of the polyisopropylacrylamide (PNIPAA). Base on their experiments there is a considerable

difference between Δ LCST and enthalpic cost of ELP and PNIPAA when the solvent is changed from water to D₂O. They suggested that this difference is mainly attributed to the different phase transition mechanisms in the two and the fact that hydrogen bonding plays a very important role in the ELP transition and restructuring above the transition temperature while there is minimal change in hydrogen bonding upon the hydrophobic collapse of PNIPAA molecules and unlike ELP molecules PNIPAA remains unstructured upon transition.

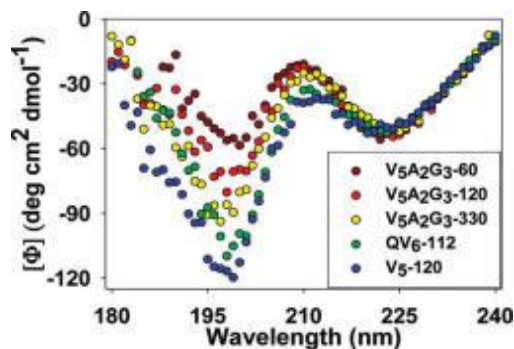


Figure 1-3. CD spectra of ELPs with different lengths and chemical identity. The minimum around 197 nm is less negative for longer hydrophilic constructs which is an indication of higher β structure at the same temperature. Reprinted with permission from reference ^{1.47}). Copyright 2009 American Chemical Society.

Since the water of hydrophobic hydration affects the transition of the elastin-like polypeptides through changes induced by the hydrogen bonding of water molecules with different amino acid residues, the effect of water on the structural transition of ELP molecules also depends on the chemical identity of the polypeptide. This was investigated for poly(GVGVP) and poly(AVGVP) using FTIR and raman spectroscopy in solution and in the solid state.^{1.27} It was suggested that below the transition temperature

for both polymers, water molecules make hydrogen bonds with a part of the amide groups and the rest of these groups interact with each other to form a β -sheet-like structure consisting of β -turns. But the difference between the two polypentapeptides is that the water is more structured around poly(AVGVP) molecules. Also, this polypeptide makes a more compact structure above the transition temperature and shows more resistance to rehydration which makes it more difficult to dissolve back into solution and, consequently, a hysteresis between heating and cooling UV spectra was observed. This mostly was attributed to the direct bonds between the amide groups and expulsion of water molecules from the spaces in between them, which makes it harder for the water to penetrate back in to the molecular structure below the transition temperature. On the other hand, poly(GVGVP) retains more water molecules on its amide groups above the transition temperature and consequently behaves reversibly when it goes through the transition temperature. This difference in the hydration shows how the chemical identity of a polypeptide can affect the structure of water around the molecule, which in turn changes the LCST behavior of the ELP molecule. Also from their studies, and in contradiction to the general belief that the transition of these biomolecules is mainly related to the restructuring of the water molecules around the hydrophobic moieties, they concluded that the restructuring of the biomolecule itself, for instance the decrease in β -sheet structure, is the more important factor in the transition. Their observation is in agreement with some other published data suggested that the interaction of water molecules with charged or polar moieties can be more influential in the transition than the interacting waters of hydrophobic hydration.^{1.59}

Not only the identity of the polypeptide affects the water structure around the molecule, but also the characteristics of the aqueous solvent can make a difference as to how the water molecules arrange around the biopolymer. This was studied specifically by having different salts and salt concentrations in the solution. Salts in general affect the solubility of proteins following the well-known Hofmeister series.^{1.60} In contradiction to traditional belief that these salts cause changes in hydrogen bonding among the bulk water molecules, more recent investigations suggest that the ions in fact interact with the biomolecules itself which results in an interfacial change between these molecules and their surrounding water molecules.^{1.61} This different interaction then causes the water molecule clusters around the biomolecule to arrange differently and so in the case of ELP molecules these interfacial waters affect the transition temperature of the polypeptides. Cho et al. investigated the effect of Hofmeister anions on the transition temperature of a (VPGVG)₁₂₀ and another construct with the same repeat number but more hydrophilic overall nature. They used 11 different sodium salts of the Hofmeister anions.^{1.62} Their results suggest that the hydrogen bonding of water molecules to the amide backbone becomes weak in the presence of the kosmotropic anions but in the case of chaotropic anions it is the hydrophobic hydration of the biomolecules which is being weakened and results in a lower transition temperature. In this case, they observed a strong correlation between the T_t of the ELP and the surface tension of the anions. Interestingly they found out a parallel but weaker salt-in effect even in the presence of chaotropic anions caused by direct binding of anions to amides. This latter effect actually works in the opposite direction with regard to the transition temperature of ELPs but has a smaller overall effect.

In short, water structure around the ELP molecules, is mostly believed to have a direct impact on the transition temperature, stability and structure of these molecules both below and above the transition temperatures and many factors including the chemical identity of the molecule, temperature, solvent, and salts affect the structure and stability of the water of hydrophobic hydration molecules.

1-4. Structural Studies

Structural studies of elastin-like polypeptides have proven to be a challenging task considering their non-crystalline structure and the fact that they go into large aggregates upon their transition. Consequently, there is no high-resolution NMR or X-ray crystallography of ELPs and most of the studies are based on interpretation of data from CD spectroscopy, IR, solid state NMR, or NMR studies of these molecules at temperatures below their transition temperatures. But the very nature of the transition and structural change has not been really resolved. Here we review some of the attempts to get a better understanding of the structure of elastin-like polypeptides below and above their transition temperatures.

The early work to probe the dynamic behavior and conformation transformation of these biomolecules come from Urry's research group over the course of three decades.^{1,7, 1.35, 1.36, 1.63-65} Using the model ELP sequence (VPGVG)_n they first showed the physical transformation of the ELP from low temperatures to temperatures above its transition and based on that he proposed that these molecules which are highly hydrated below their transition temperature, start to lose water above T_i and make a coacervate of about 37% polypeptide but then at even higher temperature there is a denaturation process that ends up losing more water to the point that the coacervate consists of about 68% protein.^{1.64}

They further studied this physical phenomena using UV and CD spectroscopy and came to the conclusion that these polypeptides consists of type II β -turn conformations which upon heating to the transition temperature form what they called a twisted filament (super coiled).^{1.34} They also showed that increasing temperature from below to above the transition temperature results in an increase in the ordered structure while keeping the solution at high temperature (around 80°C) leads to disruption of the structure.^{1.64} These observations were also accompanied by his observations of filament structure of cyclic (GVGVP)₂ and (GVGVP)₃ constructs.^{1.34} Much more recently, Karle and Urry successfully crystallized and studied another cyclic ELP sequence (APGVGV)₂. When it goes through its transition at 60°C, this ELP crystallizes out of the solution. They used x-ray crystallography to show the existence of type II β -turn at both ends of the construct.^{1.26} They showed that because of the hydrogen bonds among the residues of this molecule it forms an extended β -sheet.

Other researchers tried to probe the molecular structure of ELPs using IR or CD spectrometry^{1.27, 1.40, 1.52, 1.66} and also raman spectroscopy.^{1.67} Nicolini et al. used a combination of differential scanning calorimeter (DSC), CD and FTIR to study a short chemically synthesized polypeptide consisting of eight amino acids GVGVPGVG at high and low temperature and pressure.^{1.43} The temperature ranged from 2 to 120°C and the pressure up to about 10 bars. They showed that even this very short pentapeptide goes through the folding transition at about 36°C but then by heating up to very high temperature a denaturing was reported which they believe is a consequence of highly mobile chains that cause the hydrogen bonds to break. This is in agreement with Urry's observation for much longer polypentapeptides at temperatures around 80°C. The high

pressure study of the polypeptide revealed that the high pressure destabilizes the folded construct. They also suggest that folding is driven by the increase in the entropy of water at higher temperature.

Some groups also tried to probe the structural behavior of ELP constructs up to the transition temperature or in the solid state using NMR. Yao and Hong studied a model ELP consisting of three repeats of site-specific labeled VPGVG using solid state NMR in their attempts to better understand the structure of ELP and the source of its elasticity.^{1.45} They claimed that this peptide resembles the structure of longer VPGVG-based ELPs and therefore their results are valid even for long ELP constructs. They observed two types of compact and extended conformations. The compact conformation is reported to be about one third of all the conformational population. By conformational search for Pro-Gly pair they suggested that the compact structure is probably a type II β -turn structure although it is also possible for it to be a previously unclassified turn with Pro7 torsion angles of $(-70^\circ, 20 \pm 20^\circ)$ and Gly torsion angles of $(-100 \pm 20^\circ, -20 \pm 20^\circ)$. They proposed that the extended conformation was an unordered β -strand. For the origin of elasticity based on their NMR studies, they offered that entropic elasticity is the result of hydrophobic hydration molecules and not the conformational entropy of the polypeptides. Kurkova et al. studied the structure of two synthetic ELP molecules, poly(GVGVP) and poly(AVGVP) in water and D₂O at different temperatures using ¹H, ²H, ¹³C and ¹⁵N NMR spectroscopy.^{1.68} They assigned the signals by implementing COSY, NOESY, HXCORR, HSQC, HMBC and SSLR INEPT techniques and reported four different states for the molecular conformations of the two polypeptides based on their environmental temperature. In the first state they observed an extended and more random

and hydrated polypeptide chain conformation for both polypentapeptides. This state is reported to continue up to temperatures of about 300 K. In the second state they reported a more coiled and globular but still disordered state. This state persisted to temperatures close to the transition temperatures of the polypeptides. The difference between the two polypeptides was reported to be the broadness of the temperature intervals for each of them. For poly(AVGVP) they reported a rather narrow temperature region of about 3 degrees and it mostly behaves like a sudden change from the first state to the third one while for the other polypeptide this window was broader and overlapping with the next region. From temperatures of 303 to 313 K they observed a tightly coiled and compact state. The final state was an aggregated conformation for both constructs. Interestingly, they reported the simultaneous existence of second through fourth states in both polypeptides at all the temperatures above 299 K. This coexistence of different states of the polypeptides is very similar to previously discussed results and can explain the difficulties involving prediction or simulation of ELP behavior. They also suggested that only in the case of the poly(AVGVG) were β -turns observed in the second state and these turns were stabilized by hydrogen bonding between *Ala* carbonyl and *Val* group. The mixture of different conformations at a given temperature has also been observed in other NMR studies of ELP constructs^{1.24, 1.50, 1.51}. In another attempt to understand the structure of the ELP molecules, Gross et al. used a construct containing six repeats of GVGVP with a cysteine residue at the N-terminus.^{1.69} Based on DSC, CD, UV, IR and NMR spectroscopy data they suggest that this construct does not contain β -spiral conformations; rather an anti-parallel β -sheet is proposed to be the main component of the conformation of this ELP construct.

Other researchers tried to probe the role of individual residues, especially the Proline, in the structural conformation of ELP constructs. One of the first studies of this nature was done by Kim et al. in which they studied three ELP molecules based on the VPGVG sequence.^{1.70} they incorporated (2S)-proline in the first polypeptide, (2S,4S)-4-fluoroproline in the second, and (2S,4R)-4-fluoroproline in the third. They suggested that the substitutions of the proline in the polypeptide sequence have very noticeable effect on the transition and self-assembly of ELP molecules. The third construct showed a lower transition temperature and higher content of type II β -turns but the effect of the substitution in the second polypeptide showed an opposite trend. They concluded that the stereoelectronic effect can affect the self-assembly and the stability of the β -turn and it is directly related to the presence of the proline residue. They also confirmed their experimental results by employing density function theory and modeling the three different possible turns. The effect of proline on self-assembly of ELPs was also confirmed by more recent work^{1.71} where they studied the effect of proline number and spacing on the self-assembly and structure of elastin-like polypeptides. They observed that the spacing between the proline residues and the reversible self-assembly of the polypeptides are inversely proportional. They suggest the strong presence of β -sheet structures in proline-poor constructs which then lead to amyloid-like, irreversible aggregation of these molecules at higher temperatures. Glaves et al. studied the role of proline using point mutated ELP constructs.^{1.72} This work is a continuation of their previous work in which, by using molecular simulations and experimental data, they showed that there might actually be a folding process involved in the transition of the ELP molecules which cause the ordering of the polypeptides upon reaching their

transition temperature.^{1,73-75} Their results are very similar to the other reports on the role of the proline in the structural transition of ELP in that mutation of this proline completely altered the behavior of ELP. Their NMR studies and molecular dynamic simulations showed an unstructured conformation for these mutated ELPs while the wild type was observed to have the β -turn structure. In their work they also emphasized the importance of the proline *cis* or *trans* isomers for the stability of the polypeptides in the solution. Their results suggest a correlation between the increase in the population of the type II β -turn and the inverse temperature transition with *cis* to *trans* equilibrium in the solution at different temperatures. They suggested that high temperature lowers the Gibbs free energy barrier between the two isomers causes more prolines to go from their *cis* to *trans* conformation which then results in unstable polypeptides and ends up forming aggregates. This peptidyl-prolyl isomerization, they believe, can play an important role in aggregation and elastomeric behavior of ELPs. Interestingly their simulations showed very different conformations of polypeptides depending on which of the two isomerization forms of the prolines was used. This can add another critical parameter in performing molecular simulations for these systems and might explain some of the inconsistent results from different MD simulations. The proline isomerization was also studied by Valiaev et al. by force inducing the *cis* to *trans* isomerization of an ELP consisting of 180 repeats of pentapeptides.^{1,33} In this study they employed single molecular force spectroscopy method using AFM to characterize the molecular elongation and isomerization of ELP constructs as a result of external force. Their results confirmed the conformational change of the molecules due to the *cis* to *trans* isomerization of the proline residue.

Nevertheless the actual structure of the ELP molecules and their pathway towards the aggregation is not yet clear but it is certain that both chemical nature of the ELP molecule and the environment can affect the process of phase transition in macroscopic and microscopic levels.

1-5. Synthesis.

In general two methods of synthesizing elastin-like polypeptides have been used, chemical synthesis and biosynthesis.

1-5-1. Chemical Synthesis.

This is the traditional method of synthesizing any polypeptide sequence in which solid state chemistry is employed to add the amino acids together in the desired sequence. Specifically Fmoc solid state chemistry^{1.76} or Boc protected solid state chemistry^{1.77} have been used for ELP construction. In his early works Urry used Boc-protected solid state synthesis to make different lengths of ELP molecules.^{1.36} But solid state chemistry is not limited only to earlier works in the field of ELPs. Recent work by Ohgo et al. employed solid state chemistry to synthesis short sequences of ELP molecules^{1.24}. The obvious shortcomings of this method are the difficulty of making long polypeptide sequences. In recent years, especially for making long ELP molecules, almost every group is using the more precise and predictable method of genetically engineered polypeptide synthesis.

1-5-2. Genetically Engineered Polypeptides.

Advances in molecular biology and gene manipulation in the past few decades have enabled researchers to design and synthesize almost any conceivable sequences in their laboratories with very high precision. For elastin-like polypeptides, the highly repetitive nature of these biopolymers made them even better candidates to be made by genetic

engineering. There are generally two approaches in genetically synthesizing polypeptides, concatemerization and directional ligation.

In concatemerization, a double stranded DNA sequence with two well-defined identical sticky ends is constructed using two single stranded forward and reversed sequence DNAs. The existence of the same sticky ends on both sides of the gene causes the ligation process to result in a random distribution of different lengths of the gene. The ligated DNA sequences with different lengths would then be used as the insert into a vector DNA digested at the same recognition cut sites as the two sticky ends of the annealed insert. The genes should then be separated based on their molecular weight. This is a random process and would result in a range of gene sequences with different molecular weights. This process can be specifically useful if different lengths of the gene is needed at once but there would be little to no control over what fraction of the end product would have a specific molecular weight.^{1.78}

The other gene engineering approach in synthesizing ELP molecules is recursive directional ligation (RDL) which became the most popular method of constructing these constructs soon after the method was shown to be applicable to silk elastin-like proteins by Cappello et al.^{1.79}. This method then was further developed by Meyer and Chilkoti to become a seamless and very flexible method of synthesizing ELP molecules.^{1.80} Based on this method, a double stranded DNA sequence with two different sticky ends is inserted into a linear vector which is doubly digested with the same two restriction enzymes of the insert DNA sequence. This plasmid is then amplified in vivo in a host bacterial cell. For the next step, two populations of the same plasmid would be used such that one population is cut at both sides of the insert and the other population is cut only at one side

such that it provides proper sticky ends for the insert DNA to be ligated to this linear plasmid. The result of the ligation of these two pieces of DNA would be a plasmid DNA of twice the size of the original DNA sequence. In theory this process can be repeated as many times as desired to result in long DNA sequences. We also used this approach in our lab to construct different lengths and sequences and architectures of ELP molecules (Figure 1-4).

There are many advantages of using this method over other synthesizing processes for ELP molecules and the most important one is the absolute control over the chemical identity and length of the genes and also the mono-dispersity of the final polypeptide from the expression of these genes. The main limitations of this method is that it is usually time consuming and expensive to scale up. Also the use of RDL method has shown to be problematic for very repetitive sequences like $(VPGVG)_n$ and usually longer sequences have been synthesized by interrupting the repetitive sequence by substituting the second Val residue in some of the pentapeptides. Recently, McDaniel et al. reported a modified RDL method that might overcome this limitation over a large range of molecular weights.^{1,81}

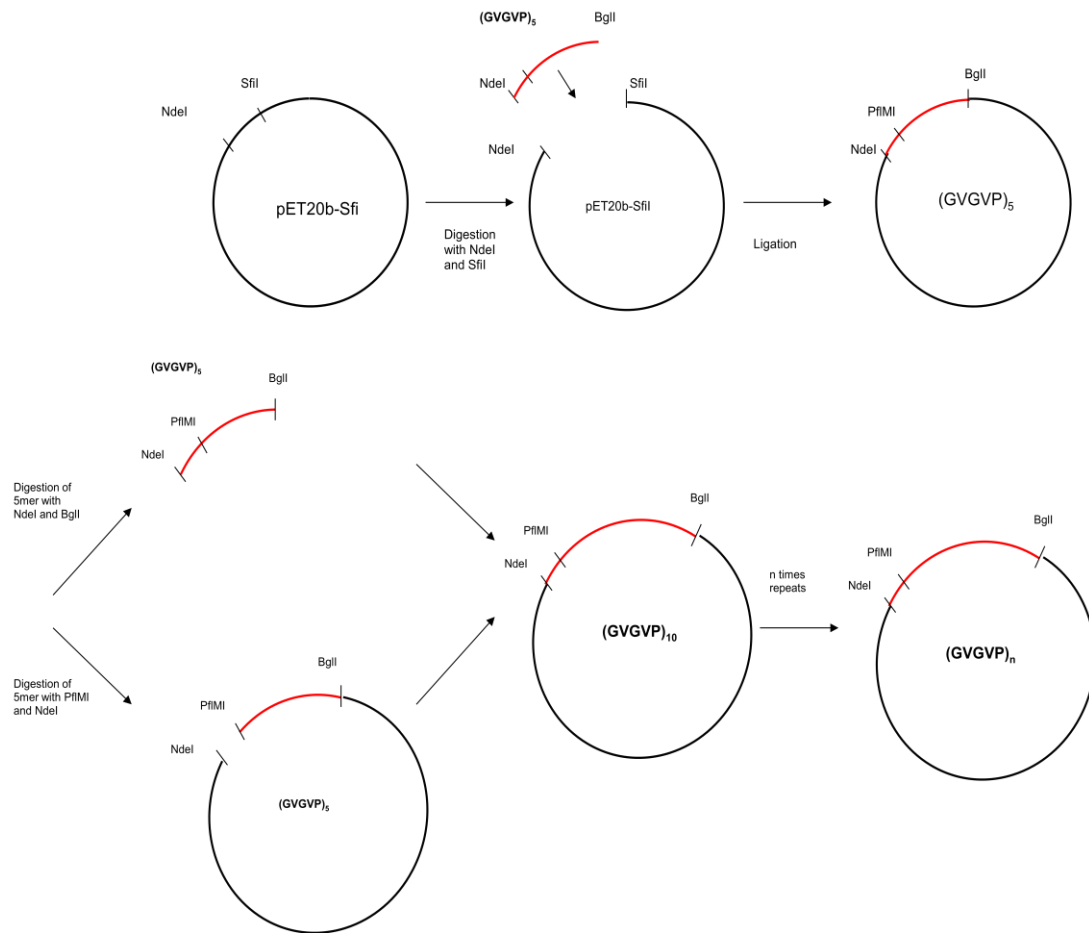


Figure 1-4. Schematic diagram of recursive directional ligation method used in our lab for ELP gene synthesis.

1-6. Characterization

Elastin-like polypeptides are characterized based on their molecular, physical, thermal, structural and rheological characteristics using many different techniques.^{1,82}

1-6-1. Biophysical properties. Most of the common techniques in characterizing proteins are also used in characterizing elastin-like polypeptides. Molecular weight of the constructs can be determined by mass spectroscopy and their concentration and purity is often characterized by electrophoresis-based methods like SDS-PAGE. The transition

temperature of the constructs are measured mostly by UV-vis spectroscopy although some analysts also have used differential scanning calorimetry (DSC) to measure the transition temperature and study the thermal behavior and of the molecules.^{1.57} Surface plasmon spectroscopy has been used for studying the surface behavior of ELPs.^{1.83}

1-6-2. Structural properties. The most well-known techniques in structural studies of proteins in general are nuclear magnetic resonance (NMR) and X-ray crystallography and although they have proved to be the most powerful techniques for this purpose their use for ELP molecules has been limited by the aggregation of the constructs above the transition temperature. Circular dichroism (CD) spectroscopy in which the absorption of left- and right-handed circularly polarized light is measured to determine unordered, alpha and beta conformations.

Hydrodynamic radius of ELP-based particles has been studied using dynamic light scattering (DLS) in which a beam of light passes through the aqueous sample. The light is scattered by the particles in different directions and part of it passes through a detector. The Brownian motions of the particles results in constantly changing measured intensity that fluctuates with time. The intensity-time correlation function would then be analyzed using Stokes-Einstein equation to calculate the hydrodynamic radius.

1-6-3. Rheological properties. The rheological properties of ELP solutions or ELP-based hydrogels can be estimated by measuring elastic, viscoelastic and dynamic modules together with dynamic viscosity as a function of stress strain, time and temperature.

1-7. ELP-based Materials

Because of the favorable characteristics of elastin-like polypeptides, including environmental responsiveness, general biocompatibility, biodegradability, and tunable behavior, they have been interesting candidates for developing new materials. Many of these materials are finding their ways into practical applications, but there are also many examples of materials based on these responsive biopolymers which are developed as proof of concept or towards the final goal of medical or biological applications. In this section, we present some of the work that has been done in the material world based on elastin-like polypeptides and then in the next section we present studies that have been done to use these materials in specific applications.

Most of the ELP-based materials can be categorized into three major groups: hydrogels, particles, and surface modifications. However, there are others forms in which these materials are being used including fibers or microfluidic devices that will be discussed later in this chapter.

1-7-1. Hydrogels

Hydrogels are three-dimensional networks of hydrophilic polymer chains which are solvated in water. Typically the mass fraction of water in this network is much higher than that of the polymer. The polymer chains in this network are held together by some kind of crosslinking between them. The crosslinking can be based on physical bonds such as hydrogen bonds, electrostatic forces, hydrophobic interactions, and/or chain entanglements or covalent bonds. Many of proposed applications of hydrogels are in the field of biomedical engineering and for that reason a great deal of effort has been made to

make biocompatible and biodegradable hydrogels that can be used in physiological conditions.

Hydrogels are also one of the most studied and most interesting classes of ELP-based materials. In fact they are the first set of materials that were made based on using elastin-like polypeptide when Urry applied γ -irradiation to an ELP coacervate and showed that the resulting gel has the responsive behavior of the ELP solutions as well.^{1.36} In his experiments he used 20 Mrad radiation at temperatures above the transition temperature of the ELP to crosslink the coacervate and showed that the resulting gel has considerable elastomeric characteristics. Use of γ -irradiation was a starting point in making hydrogels from ELP solutions and although it has also been used in more recent studies^{1.84, 1.85}, the random nature of this approach and practical problems of employing it, during the past three decades many other approaches have been introduced for ELP crosslinking.

Chemical crosslinking at specific sites along the backbone structure of the ELP has been shown to be a very effective way of making ELP-based hydrogels especially for those constructs containing lysine residue.^{1.86-92}

Another possible approach in crosslinking ELP constructs has shown to be enzymatic crosslinking. In this approach, transglutaminase (tTG) which is shown to be a more biocompatible substitute for the harsh chemical crosslinkers is used together with ELP molecules which contain both glutamine and lysine residues in their primary sequence.^{1.93-96} In this approach tTG facilitates the formation of a covalent bond between lysine and glutamine residues of biopolymer chains. The concept of using tTG as an enzymatic crosslinker has also been shown to work for other networks consisting of collagen or poly ethylene glycol (PEG).^{1.97, 1.98}

Although most of the hydrogels are formed based on covalent crosslinking of the chains^{1.85, 1.89, 1.92, 1.99, 1.100}, sometimes the polypeptide chains are designed such that a part of the chain can act as the crosslinker itself. This physical crosslinking has the advantage of eliminating the potentially toxic crosslinking agents.^{1.78} These constructs are usually based on a tri-block copolymer in which the end-blocks have lower transition temperature than the mid-block and increasing the temperature above the transition temperature of the end-blocks results in self-assembly of these blocks while the mid-blocks are still soluble.^{1.101-103} Recent studies have shown that some of these self-assembling tri-block copolymers can be extremely biostable *in vivo* and consequently very useful in biomedical applications.^{1.104} In an approach by Ma et al. aromatic-aromatic interactions of conjugated moieties to elastin-like polypeptides was used for self-assembly of polypeptide chains.^{1.105} In this study they showed the effectiveness of aromatic interactions between aromatic groups of fluorenyl, pyrenyl and naphthyl for physical crosslinking of different ELP molecules into gels with controllable characteristics.

Recently Sallach et al. reported a new approach in which they developed an ELP tri-block copolymer capable of both chemical and physical crosslinking.^{1.106} Their system included rigid domains followed by a covalent crosslinking site and the elastomeric domain.

Making these hydrogels by any of these different crosslinking techniques is only the first step in having a practically useful material. The next step is to tune the characteristics of these gels for specific applications. The advantage of chemical crosslinking of ELP hydrogels is the ability to control the mechanical properties and transition temperatures of the gel by designing the crosslinking density from the gene

level. Girotti et al. synthesized a fairly long ELP containing VPGIG and VPGKG as the main ELP sequences and REDV peptide sequence as a cell recognition sequence and finally VGVAPG for enzymatic hydrolysis of the scaffold.^{1.107} They chose this biopolymer sequence to tune the desired mechanical and bioactive properties and they showed the potential of such highly tunable design of ELP-based hydrogels. In more recent work, this group used the same polypeptide sequence and developed a salt leaching/gas-foaming technique to make highly porous hydrogels for which the porosity was controlled by the amount of sodium hydrogen carbonate salt incorporated into the gel during the crosslinking.^{1.108} This approach enabled them to fabricate three dimensional hydrogels with controllable pore size and functional moieties that can be used as a tissue engineering scaffold. The fabrication and control of porous hydrogels has also been studied by Annabi et al. using high pressure CO₂ and chemical crosslinking.^{1.109} Their results showed that the change of processing pressure from 30 bar to 150 bar can lead to a 60% increase in gel porosity and the resulting gel has highly interconnected pores and a good permeability.

Trabbik-Carlson et al. synthesized a number of ELP sequences using the generic sequence of VPGXG and chemically crosslinked lysine residues of the polypeptides using tris-succinimidyl aminotriacetate (TSAT).^{1.92} They also showed that the swelling characteristics and the material mechanical properties depend on the molecular weight of the ELP, solution concentration and the number and spacing of the lysine residues in the ELP sequence which all can be controlled very precisely. The crosslinking was done in a non-aqueous environment to prevent any conformational change of the chains while

crosslinking. This approach has been used by other researchers as well and the effect of solvent on the gel structure has been characterized^{1.87, 1.88}.

Many of these crosslinked hydrogels cannot be used as injectable scaffolds and so Lim et al. developed a method for rapid and potentially *in vivo* crosslinking of ELP so that the ELP aqueous solution along with the viable cell can be directly injected into a defect site and immediately crosslinked in the presence of hydroxymethyl phosphines.^{1.110} Their approach has the advantage of producing water as the only byproduct which makes it ideal from biocompatibility point of view. Having the same concept of injectable liquid scaffold for *in vivo* gel formation in mind, Barbosa et al. developed a system based on incorporation of an osteoconductive-containing ELP sequence, in chitosan-based systems to improve bioactivity of the these systems in bone tissue engineering.^{1.111} In their system the gel was physically crosslinked by the interaction between chitosan and β -glycerophosphate and also chemically crosslinked using genipin. The transition temperature was tuned to 35°C. The system showed an apparent improvement of mechanical properties when studied *in vitro* in simulated body fluid and they showed that the gels have the ability of inducing calcium phosphate precipitation.

This last example shows that not only can ELP molecules be designed so that resulting hydrogels have suitable mechanical and biological characteristics for a specific application, but they also can be a part of hybrid systems in which a non-ELP system is modified by using ELP molecules to improve their characteristics.^{1.112-114} One good example of this combination is the use of ELP molecules in conjugation with collagen. In a work by Garcia et al., ELP chains are enzymatically crosslinked with collagen to give better mechanical strength to the collagen tissue-engineered implants.^{1.96} The ELP in this

study had the same sequence as the one used by Girroti earlier^{1.107} and the crosslinking was done using transglutaminase. The introduction of ELP into collagen scaffold was shown to be effective in improving collagen mechanical characteristics. The modulus and degradation temperature of the gel was shown to be affected by the ELP content of the gel and they were successful in growing certain line of cells on this new platform. A more detailed study of the characteristics of hydrogels containing ELP and collagen-derived peptides was done by Morihara et al.^{1.115} In this work they chemically copolymerized collagen model peptide (PPG)₁₀ and the pentapeptide (VPGVG) in different molar ratios using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) in an organic solvent at room temperature. Their results showed that high content of (VPGVG) in the hydrogel showed a sol-gel transition at temperatures above the transition temperature but at concentration between 58 to 82% of (VPGVG) in the hydrogel, a reversible transition from clear to cloudy suspension was observed instead. They suggested that in this hydrogel, ELP molecules act as the crosslinking nodes and their aggregation leads to the formation of the gel especially at high ELP concentration.

Although in most of the cases, the functionality of ELP-based hydrogels is incorporated into their gene sequence such that the final product has certain characteristics, there have been examples of modifying the ELP hydrogel after the gel formation.^{1.116, 1.117} This approach can sometimes be beneficial especially if a certain ligand cannot be produced in the bacterial systems or certain spacer needs to be added to the molecules after the bio-production of the polypeptides. Kaufmann et al. developed a system containing repeats of VPGXG in which X was chosen as V, K, E and I.^{1.14} They

then crosslinked the resulting coacervate above its transition temperature by bis(sulfosuccinimidyl)suberate in N,N-dimethylformamide (DMF). This hydrogel was modified by protecting lysine residues and then linear and cyclic RGD sequences were ligated to the gel. The conjugation efficiencies were shown to be between 25 to 65% and they also showed a clear difference in cell adhesion efficiency depending on the use of linear or cyclic RGD.

1-7-2. Particles and Micellar Systems

A very attractive area of research in ELP-based materials has been the use of them as building blocks for responsive micelles and nano- and micro-scale particles. The responsiveness and small size of these particles and their tunable properties together with their general biocompatibility and non-toxicity have been exploited especially in targeted drug delivery applications.

The first crosslinked particles based on elastin-like polypeptides were developed by Urry by γ -irradiation of small droplets of $(VPGVG)_n$.^{1.118} Although early Urry's experiment showed the possibility of formation of responsive particles and drug loading and unloading by using ELP molecules, it was not until more recently that particles of more biologically appropriate and controlled size were developed. A recent study of nano-particles formed by γ -irradiation of $(VPGVG)_{251}$ aggregates, showed the effect of heating rate of the ELP solution on the size distribution of the final particles.^{1.119} Based on this study, heat shocking the solution before irradiation can lead to the formation of particles as small as 150 nm in diameter. Osborn et al. showed the possibility of making use of coacervate droplets of ELP to make crosslinked particles with micron-size diameters.^{1.120} Ge et al. even showed the possibility of making these droplets *in vivo* in

E.coli and tobacco cells.^{1.121} Herrero-Vanrell et al. developed a system in which the sizes of the particles were controlled to be about 300 to 400 nm and the drug was encapsulated in them by a novel electrospray technique.^{1.122} The advantage of their system was their ability to control the size of these particles by changing the chemical identity and length of the polypeptides. But even these particles are too big for some drug delivery applications for which particles below between the sizes of 10 to 200 nm in diameter are expected to be the most effective ones because of long-term circulation.^{1.123} For smaller sizes, other researchers have developed systems of amphiphilic block copolymers to make micellar particles with smaller sizes.^{1.124-127}

Micelles are self-assembled aggregates of surfactant-like molecules with a hydrophilic head group and hydrophobic tails. Micelles are formed when the concentration of monomers exceed a certain limit, called critical micelle concentration at a constant temperature. The driving force behind the self-assembly is the hydrophobic effect. At low concentrations the hydrophobic tails are solvated by water molecules and as the monomer concentration increases, the entropy penalty of keeping these molecules soluble in the more ordered network of water molecules gets larger to the point that aggregation of the hydrophobic tails becomes energetically favorable. In other word, the monomer free energy comprises a hydrophobic term (g) from the hydrophobic tail and electro-static energy (g') from the hydrophilic (charged) head group. As the difference between the two energies ($g' - g$) approaches zero the components in the system become immiscible and start to aggregate into micelles with different geometries.^{1.128}

The geometry of the micelles is affected mainly by the size of the head group and the length of the hydrophobic tail and the way in which the molecules pack into the micelle.

The packing of the monomers into the micelle is characterized by what is known as packing factor. Whatever the shape of the micelles, the hydrophobic core of the micelle should satisfy two criterion, first $V/v=A/a_0=N$ in which V is the total volume of the micelle, v is the volume of a single hydrophobic chain, A is the total surface area of the micelle, a_0 is head group area, and N is the aggregation number and then, the farthest distance from the interior to the hydrocarbon-water interface cannot exceed the maximum length of the hydrocarbon chain (l_c).^{1.128} For these conditions to be true for a spherical micelles with radius of R ,

$$\frac{3v}{a} = R \quad (1-2)$$

Considering the fact that the radius of this sphere cannot be larger than l_c , the spherical micelles can only exist if

$$\frac{v}{a_0 l_c} \leq \frac{1}{3} \quad (1-3)$$

With the same concept it can easily be shown that for packing factor of 1/2 the micelles take the geometry of a cylinder while bilayer structure is expected for packing factor of about 1. For values in between, a transition from one state to another is expected. For instance, for packing factor of about 0.38 and 0.44, globule shape and toroid shape micelles are expected respectively.^{1.128, 1.129}

Write et al. developed an ABA block copolymer consisting of hydrophobic and hydrophilic ELP molecules and successfully encapsulated small drug molecules in the particles ranging from 50 to 90 nm in diameter.^{1.130} In another study^{1.131} six different ELP block copolymers with different lengths and hydrophilic to hydrophobic ratios were studied and a two-step aggregation process was observed in which the block copolymers go into micelles when hydrophilic to hydrophobic ratios were between 1:2 to 2:1. The

micelle sizes were controlled by the ELP length and the hydrophobic content of the chains. In addition to temperature triggered micelle formation, it has been shown that block copolymers can be designed such that formation of nano-particles can be a result of both pH and temperature and in fact same construct can produce different size of particles depending on the environmental stimuli that is applied.^{1.132, 1.133}

In addition to using hydrophilic and hydrophobic ELP blocks to make micellar particles, other researchers used a hydrophilic head group fused to the hydrophobic ELP chain to induce the self-assembly of micelles. In a recent study particles with less than 100 nm diameter were obtained by adding different lengths of polyaspartic acid to the C-terminus of an ELP chain^{1.134} and the size of the particles were controlled by changing the length of the polyaspartic acid. More recently, Ghoorchian et al. successfully made size-controlled micellar particles as small as 20 nm in diameter using the fusion of a negatively charged trimer forming oligomerization domain called foldon to the C-terminal of (GVGVP)₄₀ (Figure 1-5).^{1.135} The size of the particles is controlled just by adjusting the salt concentration of the solution.

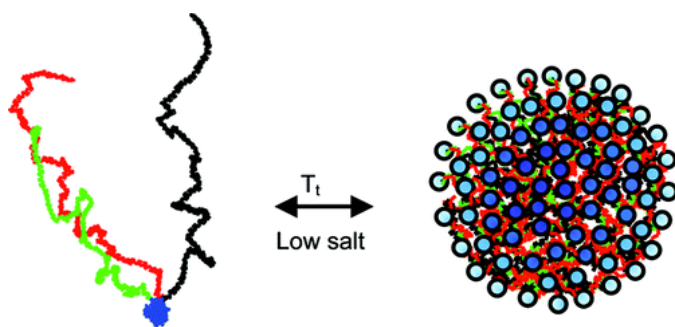


Figure 1-5. Micelle formation of three-armed star elastin-like polypeptide molecules. Below the transition temperature the molecules are as random coil in the solution. Above the transition temperature at low salt concentration the charged head groups decorate the exterior of micelles consisting of aggregated

ELP molecules. Reprinted with permission from reference^{1.135}. Copyright 2010 American Chemical Society.

In a different approach and instead of using fused blocks of ELP molecules, Kolbe et al. used separate chains of positively and negatively charged ELPs and deposited them on CaCO₃ micro particles by employing a layer-by-layer technique.^{1.136} The core was then dissolved and a hollow capsule with layered shell was obtained.

The concept of using inorganic nano-particles as the basis for ELP assembly was also studied by Huang et al. by developing optically responsive assemblies of gold nanorods and ELP molecules.^{1.32} In this approach, optically responsive gold nanorods generate heat when exposed to near IR light. This heat then induces the aggregation of ELP molecules which are covalently bonded to the surface of these nanorods, which results in a detectable optical response.

1-7-3. Surface modifications

Biological surface modification has been an interesting multi-disciplinary area of research for many years and involves the study and modification of surfaces which act as interface between biological environments and synthetic materials. There are many applications of these modified surfaces including medical implants, biosensing, tissue engineering, and biomimetic materials.^{1.78, 1.137} The surface modifications can be either static, in which the biological receptors are fixed permanently on the surface or in more recent approaches, dynamic, in which the surface modifiers can be switched between two different modes.

Elastin-like polypeptides have been shown to be interesting candidates to be used as surface modifiers considering their controllable responsive nature and the possibility of

attaching different functional domains and recognition sequences to them. Other stimuli-responsive polymers like poly(N-isopropylacrylamide) have also been used in surface modification and they are proven to be useful in certain applications^{1.138, 1.139}, but the advantages of ELP-based materials make them more attractive for biological applications in which more flexibility in design and application is needed. The change of hydrophobicity in ELP molecules can be used as a way to change the characteristics of the surface while the possibility of adding almost any peptide sequence to them or even chemical conjugation of non-peptide molecules to these constructs can be a way of functionalizing the surfaces. Early work using ELP molecules for biological surface modification was done by the Chilkoti group.^{1.140} In their work polypentapeptides based on VPGXG sequence were covalently attached to a glass surface and the possibility of enhancing or preventing the attachment of proteins to the surface was observed. They later used ELP-thioredoxin fusion protein and selectively immobilized it on a hydrophobically treated surface just by increasing the temperature.^{1.141}

The fact that a surface can be patterned using ELP-tagged proteins and can then be activated to interact with other proteins or cells near the surface has practical applications.^{1.142} Chilkoti group showed that it is possible to use ELP inverse transition behavior to immobilize ELP-fused proteins directly from cell lysate without any processing of the lysate.^{1.143} The surface patterning is not limited only to 2D surfaces but even 3D microstructures have been shown to have the potential of being patterned using ELP molecules.^{1.144} Martin et al. showed that changing the temperature changes the dimensions of the micropatterned scaffold but the topography stays the same. This of course can be useful in cell culturing.

Although the characteristics of ELP molecules make them very interesting base material for developing biologically active surfaces, this aspect of ELP-based materials has not been explored as much as hydrogels or nano-particles but even from the existing experimental results it is evident that ELPs can provide a platform that can be easily tuned to capture very different target molecules.^{1.145} This especially can be useful in developing ELP-based sensors or capturing systems.^{1.146} As an example Valiaev et al. developed a sensing system by modifying the cantilever tip of an AFM that can detect biomolecules based on the changes that they induce on the local environment,^{1.147} which shows the flexibility and possibilities of developing novel systems for biomolecule detection.

1-8. Applications

1-8-1. Drug Delivery

The idea of delivering drug to specific organs without exposing healthy tissues to the adverse effects of it is not new and goes back to more than a century ago when in 1906 Paul Ehrlich suggested the use of a drug carrier to “*the organ in question*”.^{1.148} The drug carrier can attach or encapsulate the drug and guide it towards the point of interest. Attaching the drug to a high molecular weight carrier is especially useful to prolong the circulation time of the drug in the body before drug clearance and mostly relies on the enhanced permeability and retention effect (EPR).^{1.149-153} EPR effect is the result of rapid growth of tumor vasculature which makes the tumors much more porous than healthy tissues and enables macromolecules to penetrate them.^{1.154} In addition to the EPR effect, attaching macromolecules to small drugs can increase the circulation time just based on the higher apparent molecular weight.^{1.155} An example of this approach is the conjugation

of polyethylene glycol (PEG) to adenosine deaminase (ADA) which leads to longer biological half-life of the compound.^{1.156, 1.157} Although this is one of the easier approaches in targeting cancerous tissues it is not always the best one especially considering the fact that the drug is still circulating all through the body and there is always some penetration to the healthy tissues along with the possibility of the clearance of the drug from the body. Even when the drug reaches the cancerous tissues the heterogeneity of the tumors makes the effective and complete delivery of the drug much more complex since the cancer cells only occupy up to one tenth of the total tumor volume.^{1.158} As a result, other approaches to facilitate the delivery of the carrier to the target, like ligand-mediated targeting^{1.159, 1.160}, temperature mediated targeting^{1.161, 1.162}, and pH-mediated targeting^{1.163} have been widely investigated.

One way to overcome some of the difficulties related to delivering polymer-drug conjugates to the tumor site is the encapsulation of the drug in a carrier and then release of it at the specific destination.^{1.164} For any carrier to be used for drug delivery, it should be stable in the physiological conditions and be able to keep the drug encapsulated or attached to it for as long as needed for the delivery. There should also be a way of releasing the drug at the site and most importantly it should be biocompatible and selective towards the target cells^{1.165} and be able to overcome different transport barriers.^{1.166, 1.167} Of course different applications need different release rates and mechanisms. In general the drug can be released from the particle either by slow, constant diffusion, by bolus initiated by external stimuli, or a combination of them.^{1.164, 1.168, 1.169} A good example of using polymeric particles for slow release of drug is the well-known Norplant which consists of small silicone capsules that release contraceptive

drug during a five-year period. Liposomes and micelles have shown to have good potentials for drug loading and targeted delivery using external stimuli.^{1.168, 1.170} The stimulation by which the drug is released might also be different from one case to another. Early studies used magnetic beads in the particles to control the release of the drug by applying a magnetic field.^{1.171} Ultrasound and electric current have also been used as external stimuli for controlled release of the drugs.^{1.172-175} However, many of the recent works are leaning towards designing responsive particles which can be stimulated by more physiologically controllable stimuli such as temperature and pH.^{1.176-179} Even when the carrier is delivered to the specific site, the problem of it diffusing into the cell to deliver the drug can reduce the efficiency of the system. For that matter, in some studies a cell penetrating domain is added to the carrier to enhance the targeting and overcome the poor pharmacokinetic parameters related to most of the particle-based delivery systems.^{1.180, 1.181}

A different approach to deliver drugs, instead of systemic delivery, is to use a drug depot and locally administrate the drug in form of a gel to the specific site without exposing the other organs to the drug.^{1.182, 1.183} The biggest limitations of this approach are that the exact site of the tumor should be known and the implantation might be very invasive.^{1.82}

The tunable responsive behavior of elastin-like polypeptides, their general biocompatibility and biodegradability and the possibility of designing them to respond to many different environmental stimuli have attracted great attention to develop drug delivery systems from early studies by Urry^{1.184} to much more recent advancements in this field.^{1.117, 1.122, 1.127, 1.161, 1.185-189} The fact that hydrophobicity along with the size and

molecular weight of the carriers have shown to be effective in the success of drug delivery system and these parameters are well under control in ELP-based materials, is also a good reason to use them in targeted drug delivery.^{1.123, 1.190, 1.191}

Just like any other macromolecule with high molecular weight, an ELP with transition temperature above the body temperature which is conjugated to a hydrophobic drug can be used to deliver the drug solely based on EPR effect^{1.192} and the molecular weight of ELP has shown to have direct effect on the drug accumulation in the tumor.^{1.191} In this approach the ELP can be designed to be very hydrophilic and thus stay soluble while the hydrophobic drug molecules might come together and make an even higher molecular weight soluble micelle-like drug carrier. This simple attachment of ELP molecules to certain therapeutics or antibodies has been shown to be useful in attacking some inflammatory diseases.^{1.193, 1.194} Although this ELP-drug conjugation can enhance the drug delivery yield and has all the characteristics of polymeric delivery systems, it does not utilize most of the ELP advantages including its tunable transition from soluble molecules to insoluble aggregates. Noticeably it has been shown that the conjugation might affect where the drug will end up to be in the cell and while the free drug mostly accumulate in the nucleus the conjugated drug might disperse in cytoplasm of the cell.^{1.195} In a controlled study using ¹⁴C-labeled ELP conjugated to doxorubicin, Liu et al. showed that the use of an ELP with a very high transition temperature can slightly increase the uptake of the drug in the cells, but it is still much less effective than using an ELP construct which also has the capability of responding to local hyperthermia.^{1.196} For that reason many researchers have tried to exploit the temperature responsiveness of the ELPs in designing drug delivery vehicles.^{1.186, 1.197-199} In fact when the ELP is designed

such that its transition temperature is just slightly above the body temperature, mild hyperthermia can be applied to the site of the tumor and that causes the aggregation of ELP-drug conjugate molecules at the site. This accumulation of high molecular weight particles facilitates the penetration of the drug to the cancerous cells.

In addition to increasing the drug uptake, the delivery systems based on mild thermal treatment of ELP-drug conjugates have been shown to be effective in inhibition of proliferation of cancer cells^{1.187} and was helpful in overcoming the drug resistance to some extent.^{1.185} This might be a result of different cytotoxicity mechanism of the conjugated drugs. Another advantage of using mild hyperthermia for ELP-conjugated drugs has been shown in the systems in which targeting ligands (RGD, NGR) are used to increase the uptake of the drug in the cells. In these cases the existence of binding ligand can sometimes increase both specific and non-specific binding but use of external stimulus to trigger tumor-specific multivalency has shown to be effective in reducing the non-specific binding.^{1.131, 1.200, 1.201}

Another approach for delivering drugs to the site of interest using ELP-based materials is by designing systems based on hydrophobic-hydrophilic ELP block copolymers that assemble into micellar particles and encapsulate the drugs and release it at the target site in response to external stimuli.^{1.131, 1.202} These micelles usually have a very narrow size distribution and thus a better control over their retention in the blood stream can be achieved.^{1.78} The micelles can also be designed such that they respond to different environmental stimuli. It has been shown that the drug can be released either by the dissociation of drug carriers at the target cells or by the aggregation of monomers or micelles at higher temperatures at the site of interest.^{1.203, 1.204}

The possibility of loading drugs in the ELP-based micelles has been verified in a number of studies.^{1.122, 1.130, 1.205, 1.206} Kim et al. studied a series of different block copolymers that have the capability of self-assembling into micelles and can also be crosslinked to responsive nano-particles.^{1.207} It has been shown that ELP-based nano-particles can be used in gene delivery applications.^{1.208, 1.209}

Although the use of an environmentally responsive system improves the drug delivery yield especially when it is accompanied with targeting ligands, similar to any other systemic drug delivery systems, the penetration of the drug into the cell which is the last transportation barrier can still be challenging.^{1.210, 1.211} This is where cell penetrating peptides (CPP) can be added to the ELP-based systems.^{1.181} In general, CPPs are short peptides that can effectively penetrate into the cell membranes and enter the cytoplasm.^{1.212} They are either amphipathic helical peptides with high lysine content or arginine-rich peptides, such as Antp or TAT.^{1.213} Meanwhile, it has been shown that attaching these peptides to macromolecules or nano-particles facilitates their transport through the cell membranes as well.^{1.214} In a study by Bidwell et al. a therapeutic peptide was attached to a 59.1 kDa ELP carrier and then four different CPPs were added to the N terminal of the ELP to study the possible improvement of the cellular uptake of the drug.^{1.180} Their results showed that the existence of the CPP clearly increases the drug accumulation in the cells and there is also a difference between different CPPs in their ability to penetrate into the cells.

A third approach in using ELP-based materials for drug delivery is by intratumoral injection of ELP solutions that are designed to form a gel in situ at the body temperature. This approach can be used for both delivering drug to cancerous tumors which are

accessible to the surgeon and their position is well known and also for delivering antibiotics to the sites which are susceptible to infection especially in orthopedic applications.^{1.215} In this approach the transition temperature of the ELP-drug conjugate is usually lower than the body temperature. Early work on localized drug delivery were done using charged ELP constructs coupled with ionic drugs.^{1.216} Later hybrid materials consisting of both elastin-like polypeptides and silk-like polypeptides were used to make use of the physical properties of the silk along with solubility and tunable properties of ELPs.^{1.79, 1.217} It has also been shown that triblock ELP copolymers with the ability to make physical crosslinking can go through gelation and might be used as injectable gels.^{1.217}

Liu et al. developed a system in which two different ELP constructs with molecular weight of about 50kDa but different transition temperatures were labeled with a fluorescent dye and then injected to a tumor.^{1.218} One ELP construct was designed to undergo a sol-gel transition at the body temperature and the other one was designed to stay soluble before and after the injection. Their results show a clear difference between the diffusion of the drug in these two cases such that the drug had a much longer retention time when entrapped in the gel.

The increase in retention time was quantified earlier in another work and was shown to be 25-fold longer than a soluble protein with the same molecular weight.^{1.219} In addition to longer retention time and half-life of the drug in the tumor, this method was more effective in slowing the growth of the tumor in mice.^{1.197, 1.218}

1-8-2. Tissue engineering

Elastin-derived materials have long been sought for their potential to be used in tissue engineering applications primarily because of their elastic characteristics and general biocompatibility. Elastin in tissue engineering can originate from naturally occurring matrices like the elastin in autografts, allografts, xenografts, and decellularized extracellular matrices or from synthetic sources like elastin-like polypeptides, silk elastin-like polypeptides and their hybrid molecules^{1.220} and they have been used in a range of applications like skin substitutes and wound healing^{1.221, 1.222}, vascular grafts^{1.223-225}, bladder reconstruction^{1.226}, intervertebral restoration^{1.227}, liver tissue repair^{1.228, 1.229} and artificial extra cellular matrices.^{1.230} Recent advances in gene manipulation and protein engineering provided strong tools for engineering elastin-like polypeptides with higher control over their functionality and mechanical characteristics for tissue engineering.^{1.188, 1.231}

Early works by Urry group showed the possibility of adding cell recognition sequence (RGD) to elastin-like polypeptide and the functionality of these sequences *in vitro*.^{1.232, 1.233} The resulting polypeptide was shown to be non-toxic to bovine aortic endothelial cells. Later, Betre et al. showed the possibility of using un-crosslinked ELPs as injectable tissue engineering scaffolds and the ability of ELP to promote chondrogenesis *in vitro*.^{1.223} Below 35°C the soluble ELPs allow the incorporation of cells within the solution but upon injection, a gel-like matrix forms that would serve as the scaffold for the growth of the cells. They later showed that the ELP aggregates can promote the cartilage tissue growth and accumulation.^{1.223, 1.234}

Although the use of un-crosslinked ELP has shown to be feasible for tissue engineering, in many cases it is necessary to use a prefabricated scaffold that can provide a suitable growth environment for the cells or to prevent the platelet coagulation.^{1.235} For that reason crosslinked materials in the form of gels^{1.236}, films^{1.89}, and fibers^{1.237} have been produced using different crosslinking methods.

Vascular grafts and blood vessels have been synthesized for decades and are one of the commonly used biomaterials,^{1.224, 1.238, 1.239} but most of the current commercially available synthetic biomaterials including polyethylene terephthalate and expanded polytetrafluoroethylene show thrombogenicity as a result of protein adsorption and platelet activation^{1.240} and immunogenic response leading to chronic inflammation^{1.241} in addition to the general lack of elasticity for arterial applications.^{1.242} In this case, ELPs are good candidates to be used in making small diameter vascular grafts considering their tunable characteristics^{1.100, 1.243-246} and their relative biocompatibility.^{1.184} To exploit some of these characteristics and to overcome the shortcomings of other synthetic materials the Tirrell group developed a system based on ELP molecules containing RGD and CS5 to be used as artificial extra cellular matrix and studied endothelial cell adhesion for vascular graft applications.^{1.91, 1.247} Their results showed that RGD is a better choice to enhance the cell adhesion and in later work, they showed that the density of RGD also affects the cell adhesion and higher density of this peptide sequence results in more robust cell adhesion and spreading.^{1.230} Joen et al. showed the possibility of stimulating fibroblasts and neuroblasts on RGD-containing ELP matrix.^{1.248} Caves et al developed a system of artificial extra cellular matrix composed of ELP protein matrix reinforced with synthetic collagen fibers.^{1.249} In their experiments ELP sequence was chosen to be a block

copolymer consisting of two hydrophobic ends and a hydrophilic mid-block and additional crosslinking domains. The collagen microfibers were produced using wet spinning technique and then embedded within a thin film of crosslinked ELP.^{1.250} The reinforced thin film was then rolled into small diameter vascular grafts. The combination of collagen and ELP was shown to be effective and they were successful in controlling the collagen fiber orientations while the structural resilience of ELP made the material stronger.

More recently, the same group showed that the collagen reinforced ELP matrix which has a tailored mechanical behavior can also be used in soft tissue repair *in vivo*.^{1.251} To combine the strength and elasticity in a single material, Wise et al. used a different method to synthesize vascular grafts with tailored mechanical properties.^{1.252} They synthesized an elastin/polycaprolactone hybrid material by electrospinning the recombinant human tropoelastin in combination with polycaprolactate and showed that the resulting material has enhanced endothelial cell interactions along with low thrombogenicity while keeping up with the mechanical properties of internal mammary artery. In some applications, instead of using ELP to synthesize the material for arterial vessels, elastin-like polypeptides have been used to produce a biocompatible, thromboresistant coating that can be used on top of conventional materials to make small diameter grafts.^{1.225, 1.235, 1.253} A study by Srokowski et al. showed the effectiveness of using three different ELP sequences in coating small tubular surfaces and their positive effect on reducing fibrinogen adsorption and platelet adhesion in contact with flowing blood.^{1.142}

The other important area in which elastin-like polypeptides have been explored in tissue engineering applications is cartilage or soft tissue repair.^{1.223} Early studies used uncrosslinked ELP solutions with transition temperatures just below the body temperature for injection to the defect site.^{1.254} The aggregated ELP has shear modulus about three orders of magnitude higher than that of the soluble ones,^{1.223} but this shear modulus is still at least four orders of magnitude lower than the natural cartilage.^{1.255} and for that reason in most of the tissue repair applications, some kind of crosslinking has been implemented and hydrogels have been widely used in tissue engineering applications.^{1.256} One logical approach to the crosslinking of ELP in cartilage tissue repair would be to modify the ELP such that it goes through crosslinking in the body without adding any extra component. This was done by enzymatic crosslinking of ELP solution containing lysine and glutamine by making use of the tissue transglutaminase.^{1.257} McHale et al. showed that this hydrogel has at least two orders of magnitude larger force moduli in comparison to non crosslinked gel.^{1.95} An improved system with much faster reaction time was later developed by Lim et al.^{1.110} and *in vivo* experiments showed the possibility of using this technique to repair cartilage tissue in big animals.^{1.258} The same group also used physical crosslinking of block copolymers as another approach for developing cartilage repairing systems.^{1.259} In fact physical crosslinking or self-assembling of modularly designed biomaterials for tissue engineering is an effective way of developing materials for tissue engineering platforms.^{1.260} The modular design of ELP construct has also been used by Heilshorn group to develop 3D artificial extra cellular matrices that has the potential to be used in regenerative therapeutics.^{1.261-263} In all of these cases, the choice

of the crosslinking method has shown to be effective on the cell viability and growth and for that reason the crosslinking should be tailored to the specific application.^{1.264}

Other than synthetic ELPs, naturally occurring elastin-like polypeptides obtained from human tropoelastin gene has also been used in regenerative applications.^{1.265} Some studies have shown the biocompatibility of ELP matrix based on exons 20-21-24 in reconstruction of rabbit osteochondral defects.^{1.183}

In addition to cartilage tissue regeneration, ELPs have been used to treat chronic wounds. These are the wounds that do not go through the healing process in the normal healing time of up to 12 weeks^{1.266} and are mainly associated with poor dermal or epidermal remodeling. These include wounds from autoimmune disease, arterial and diabetic foot ulcers, and decubitus wounds.^{1.267} In many of these cases, natural elastin, which has been shown to be effective in dermal wound healing,^{1.220} or a combination of elastin and collagen is used for dressing the chronic wounds.^{1.267-269} These can be in the forms of collagen scaffolds coated with elastin^{1.270}, collagen/elastin membranes^{1.271} and also some commercially available collagen/elastin dressings like Matriderm.^{1.272} Koria et al. developed a system based on ELP fused to the keratinocyte growth factor (KGF).^{1.222} KGF has also been shown to be effective in reepithelialization and wound healing.^{1.273} The fusion protein was shown to go in to submicron-size particles above the transition temperature of ELP and enhance the healing of wounds in diabetic mice by improving dermal and epidermal regeneration. Interestingly the use of ELP resulted in significant granule formation *in vivo*.

Apart from soft tissue engineering, ELPs have been used in bone reconstruction and mechanical characteristic enhancement of biomaterialized composites.^{1.274} Wang et al.

developed an ELP system including well-defined charged distribution and used it to construct ELP-hydroxyapatite (HAP) composites. The HAP-ELPs were then incorporated into calcium phosphate cements (CPC) which resulted in a mechanically robust material.^{1.10}

1-8-3. Purification

The need for recombinant proteins for medical and industrial applications and the difficulties involved in most of the purification processes like cellulose binding domains^{1.275}, thioredoxin^{1.276}, and affinity-based chromatography, including high cost, the need for specialized equipment, and hurdles towards scale-up of the bench-top procedures^{1.277} have made a good case for scientists to look into other means of purifications that can be customized to the system of interest. Elastin-like polypeptides have proven to maintain their thermal transition characteristics even after fusing to other protein domains including their environmental responsiveness^{1.278} and was first used by Meyer and Chilkoti as a fusion tag for protein purification.^{1.279} In their work, different lengths of ELP genes were fused to the C-terminus of *E. coli* thioredoxin and they purified the protein by both metal affinity chromatography and inverse transition cycling. In the case of ELP-fused protein, the ELP was then cleaved by using thrombin. Their results proved the possibility of purifying thioredoxin with very high yield by exploiting the inverse transition cycling of the tagged ELP. More recently they showed that lower molecular weight anionic ELP tags as small as 4.3 kDa, dramatically increase the recovery of the protein.^{1.277} It has also been shown that in principle this purification technique is a general procedure and can be modified to fit many systems including green fluorescent protein, blue fluorescent protein, and chloroamphenicol acetyltransferase,^{1.280}

or even to purify plasmid DNA^{1.281} or antibodies^{1.282, 1.283}. The process can be used to recover very dilute protein samples as well,^{1.284, 1.285} although the fusion order can make a considerable difference in the expression and activity of the target protein such that short ELP tags fused to the C-terminus of the desired protein lead to the highest level of expression, purification, and activity of the protein.^{1.286}

One important step in using ELP tags to purify a target protein is the final cleavage of the ELP from the protein. This is usually done by designing a cleavage site between the two polypeptides and applying protease digestion after the purification by inverse temperature cycling. Protein splicing elements have long been studied in systems dealing with fusion proteins and have been used along with chromatographic columns to purify recombinant proteins. Maybe the most well-known example among them are inteins.^{1.287, 1.288} In addition to inteins, many other self-cleaving tags including Sortase A (SrtA)^{1.289}, N-Terminal protease^{1.290}, FrpC module^{1.291}, and cysteine protease domain (CPD)^{1.292} have been used in developing protein purification systems, but inteins still remain the main self-cleaving tags. A recent review of self-cleaving fusion tags is published by Li.^{1.293}

In the case of ELPs, inteins have also been engineered to construct self-cleaving purification systems.^{1.294-296} In a study by Banki et al. the intein-mediated self-cleavage of ELP from the target protein was achieved by lowering the pH to 6 during the cold centrifugations.^{1.297} Self-cleavage has also been shown to be possible by small temperature shifts during the purification^{1.279, 1.294} or even addition of reducing agents like DTT.^{1.295} Although these self-cleaving ELP-tagged systems are not yet commercialized, they have shown great potential towards industrial use especially that they can be used without the need for additional steps such as chromatography^{1.298} and it has been shown

that the whole process of purification and cleavage can be done at temperatures as low as the room temperature by salt substitution which makes it even more economically attractive for industrial processes.^{1.299}

One issue with many of these self-cleaving systems is the need for purification of the protease after being used to make it reusable again and make the process cost-effective. This process should be done usually by conventional affinity column processes which make it less attractive for commercial-scale production. Very recently, Lan et al. developed a system in which an ELP-tagged protease was used along with a cleavable ELP-tagged target protein (Figure 1-6).^{1.300} This procedure allows for a single-step purification of the protein and removal of the tag while the protease can be reused immediately for another purification.

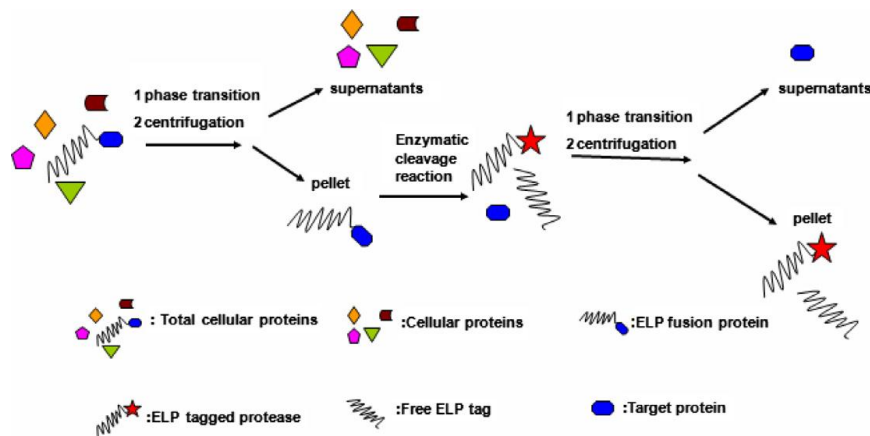


Figure 1-6. Use of ELP tags for protein purification. ELP molecules were tagged to the target protein and mixed with another ELP tagged protease. The enzyme cut the target protein and then pellet out of the solution together with the ELP molecules detached from the target protein. Reprinted with permission from Reference ^{1.300}. Copyright 2011 Elsevier.

In some cases, the addition of ELP to the target protein not only creates an improved pathway to purify the protein, it can also help increasing the expression of the protein as

well. Hu et al. showed the effect of ELP fusion on the expression yield of antimicrobial peptides and used the same tag for purification of the peptides.^{1.301} The effectiveness of ELP tags in expression of proteins has also been shown in plant-derived proteins.^{1.193, 1.302, 1.303} Plants are especially interesting in scaling up the processes to industry with low cost and high versatility.^{1.304} Floss et al. showed the possibility of using transgenic plants as bioreactors for ELP fusion proteins.^{1.305} They also used transgenic tobacco plants and expressed and purified (HIV)-neutralizing antibodies with and without tagged ELP in the plant seeds.^{1.306} Their results showed the enhancement of accumulation of protein in the presence of ELP while making the purification much easier.

The ELP fused proteins have also been shown to have the capability of being used as heavy metal removal and purification. In a study done by Lao et al. Cadmium contaminants were successfully removed and washed from the soil with a very high yield using a phytochelation molecule genetically fused to an ELP.^{1.307} In a different study, MerR which is a bacterial metalloregulatory protein and binds to mercury, was fused to (VPGVG)_n of various lengths and was shown to be extremely effective in reducing the mercury level of water to as low as drinking water.^{1.308}

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Chapter II

Molecular Architecture Influences the Thermally Induced Aggregation Behavior of Elastin-Like Polypeptides

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2-1. Abstract

Elastin-like polypeptides are thermally responsive polymers that exhibit phase separation above a transition temperature. The effect of molecular architecture on the temperature responsive behavior of elastin-like polypeptide solutions was investigated by characterization of solutions of two families of three-armed star polypeptides and linear polypeptides. These biosynthesized polypeptides have precise lengths and amino acid sequences. Transition temperatures were measured as a function of molecular weight and solution concentration and compared to their linear counterparts. Like their linear counterparts, the transition temperature is linearly related to log concentration. A mathematical relationship was used to fit the transition temperature data of different polypeptide lengths to a volume-based concentration using the polymer coil volume. The results of this model suggest that the linear ELP is in a random coil conformation at the

transition temperature while the three-armed ELP is in a compact extended coil conformation, consistent with different pathways for aggregation.

2-2. Introduction

Elastin-like polypeptides are environmentally responsive biopolymers which are based on peptide sequences originally found in nature^{2.1} consisting of repeats of the pentapeptide (G β G α P) in which α can be any of the 20 naturally occurring amino acids while β can be any of those amino acids except for proline.^{2.2} An important characteristic of these polypeptides is their LCST (lower critical solution temperature) behavior, resulting in phase separation of the soluble polypeptides into a protein rich coacervate and a protein lean phase above what has been referred to as an inverse transition temperature.^{2.3} This phase separation is reversible and the transition temperature can be modified by changing the chemical identity and length of ELP molecules as well as their concentration in solution.^{2.2, 2.4} Phase separation can also be triggered by other stimuli, including pH, ionic strength, and light.^{2.3, 2.5-7} The reversible phase separation has been the subject of many studies in the past three decades and numerous applications have been proposed for these materials based on these characteristics, such as drug delivery^{2.8}, tissue engineering^{2.9, 2.10}, surface engineering^{2.11}, and microfluidic devices.^{2.12}

One interesting aspect of these materials is the dependency of their transition temperature on the sequence, length, and concentration in the solution.^{2.4, 2.13} These characteristics have been exploited to modify the transition temperature of ELP solutions to make them suitable for different potential applications. Although length and

concentration dependency of the transition temperature in linear ELPs have been thoroughly described, the role of molecular architecture has not yet been investigated.

To study the effect of molecular architecture on ELP behavior, in conjunction with length and concentration, we present here the characterization of polypeptides designed by adding a trimer forming oligomerization domain to the C-terminal of the linear ELP molecules.

The trimer construct was chosen in an attempt to induce polypeptide association based on the most widely accepted theory of ELP folding and aggregation. According to this theory^{2,3, 2.14}, heating up the ELP sample causes the unordered polypeptide chains to go through a gradual transition to more ordered, and more hydrophobic folded structure mainly consisting of β turns. At the transition temperature these “ordered” hydrophobic constructs are stabilized by other chains, presumably as twisted filaments of three chains, which then aggregate and phase separate. The association of three chains as a twisted filament has been supported by a recent study in which combining ELP with a trimer forming α -helical coiled coil motif resulted in the ends of the ELP chains coming into close proximity above the transition temperature.^{2.15} It has also been shown that ELP molecules fused to a self-assembling domain go through similar conformational transition as they approach their transition temperature.^{2.16} Based on this mechanism, the aggregation of the ELP exhibits significant concentration dependence since it proceeds only after associating as a twisted filament.^{2.14} Consequently, alterations in the geometry of the ELP that encourage the formation of twisted filaments could potentially affect the overall folding and aggregation of the chains. It should be noted that even in the absence of specific stoichiometric interactions, a high local concentration caused by close

proximity of the chains might help the chain-chain interactions and consequently the aggregation of the molecules.

As described previously,^{2,17} the trimer forming foldon domain, which is a part of the bacteriophage T4 fibrin protein, can be added as a fusion protein to either termini of ELP chains to form a three-armed ELP. Here we report on the characterization of different lengths of such three-armed star-like elastin-like polypeptides and the dependency of their aggregation temperatures on length and concentration, as compared to linear counterparts and we also study the effect of ELP/foldon fusion order in the molecular behavior of these constructs. The study of these different architectures provides new data used to improve the model for predicting the transition characteristics of ELP solutions.

2-3 Materials and Methods:

2-3-1. Gene Design and Preparation

The design and synthesis of elastin like-polypeptides with and without the foldon trimerization domain were done using the recursive directional ligation technique as explained in detail elsewhere.^{2,4, 2.17} The final sequences of the linear ELP, ELP-foldon and foldon-ELP genes are MGH(GVGVP)_nGWP, MGH(GVGVP)_nGWP-GYIPEAPRDGQAYVRKDGEWVLLSTFL and MGHGVGYIPEAPRDGQAYVRKDGEWVLLSTFLGP-(GVGVP)_nGWP respectively in which *n* is the number of the pentapeptide repeats and was chosen to be 20, 40, or 60. For longer linear ELP constructs, a cysteine residue was introduced at the C terminal end of the ELP sequence resulting in a sequence of MGH(GVGVP)_nGWPC where *n* was 20, 40, or 60. Disulfide bond formation between two chains makes a linear ELP chain with

twice the length of the expressed polypeptide. The transition temperature of ELP-cys dimers (in non-reducing conditions) is in agreement with that of a linear ELP of twice the length and so can be used interchangeably (Figure 2-1). The polypeptides are referred to as $(\text{GVGVP})_n$, $(\text{GVGVP})_n$ -foldon and foldon- $(\text{GVGVP})_n$ where n is the total number of pentapeptide repeats. The genes were prepared in pET20b vectors and transformed into BL21(DE3) expression strain of *E. coli*.

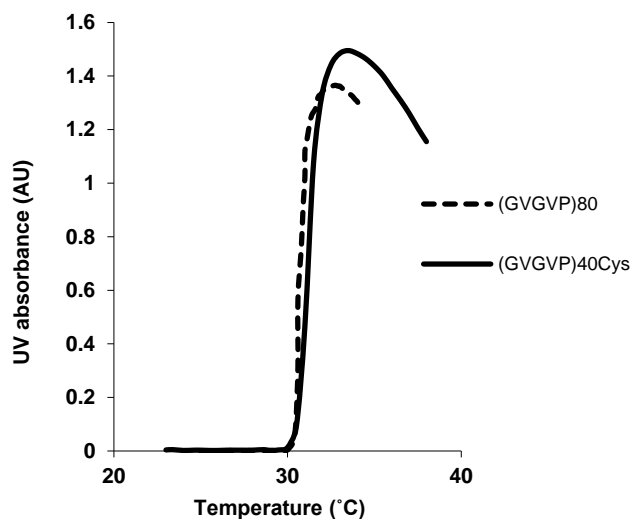


Figure 2-1. UV absorbance curves of $(\text{GVGVP})_{80}$ and $(\text{GVGVP})_{40}\text{Cys}$ in non reducing conditions both at 25 μM

2-3-2. Protein Expression and Purification.

Expression of polypeptides were started using 20 ml of overnight starter culture from frozen stock which was then added to 1 L of LB medium in a 2 L Erlenmeyer flask supplemented with 0.1 mg/ml of ampicillin and shaken at 37°C to OD_{600} above 0.9. Then the culture was induced by adding 0.1 mM IPTG. The cells were harvested at least 5 hours after the induction by centrifugation for 20 min at 3000 \times g. The cells were then frozen overnight and later lysed by sonication in phosphate buffer saline, pH 7.4 (PBS)

supplemented with 2 mM EDTA. The lysed cells were then centrifuged at 4°C for 20 min at 20,000×g to separate the soluble cell compounds, including ELP, from the insoluble cell lysate. The soluble fraction was then heated up to at least 40°C for the ELP to precipitate out of solution. Centrifugation at about 40°C and 15000×g was then used to separate the soluble impurities from the insoluble ELP. The final pellet was then resuspended in PBS and the cold and hot cycles were repeated at least two more times to purify the ELP or ELP-foldon proteins. Protein samples were diluted using PBS to their final concentrations.

2-3-3. Protein Characterization.

Gel electrophoresis using a 4-20% gradient Tris-HEPES-SDS gel (Thermo Scientific) confirmed the purity and molecular weight of all the samples, and also confirmed the formation of the ELP-foldon trimers. The samples were prepared in loading buffer containing 0.1% SDS and heated to boiling temperature for 5 min and then cooled to ambient temperature prior to loading on the gel. The molecular weights of the polypeptides were additionally confirmed by ion spray quadrupole/time-of-flight mass spectrometry (AB/Sciex). The concentrations of the purified proteins were determined based on UV absorbance at 280 nm measured on a Biomate3 (Thermo Scientific) using calculated extinction coefficients.^{2,18} Folding of the foldon domains were additionally confirmed by a 228 nm peak in circular dichroism spectra obtained on an Aviv 215 CD spectropolarimeter.

Transition temperatures of ELP and ELP-foldon constructs were obtained using UV absorbance of solutions at 350 nm measured on a Shimadzu 1800 UV-vis

spectrophotometer equipped with a temperature controlled sample holder unit. The spectra were obtained at 0.1°C steps with a temperature ramp of 1°C/min.

2-4. Results and Discussion

2-4-1. Protein Expression

ELP constructs with and without foldon were expressed and purified with yields between 100 and 200 mg/L of culture. As has been shown previously for (GVGVP)₄₀-foldon,^{2,17} each of the ELP molecules containing foldon, fold as a trimer, as illustrated by SDS-PAGE (Figure 2-2). The trimers in this study include (GVGVP)₂₀-foldon, (GVGVP)₄₀-foldon, (GVGVP)₆₀-foldon, foldon-(GVGVP)₂₀, and foldon-(GVGVP)₄₀ while the linear constructs are (GVGVP)₄₀, (GVGVP)₈₀, and (GVGVP)₁₆₀.

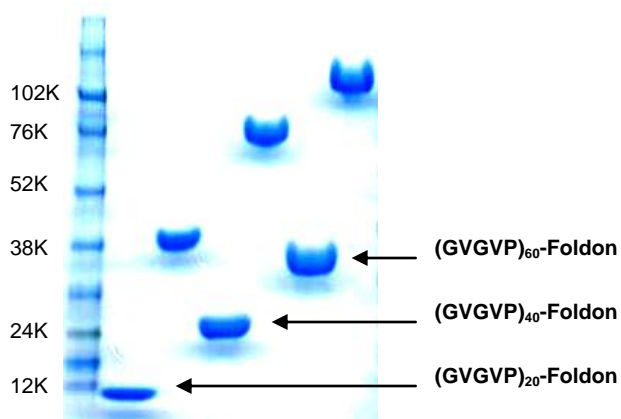


Figure 2-2. SDS-PAGE of (GVGVP)₂₀-foldon, (GVGVP)₄₀-foldon and (GVGVP)₆₀-foldon. When samples were loaded after about 5 minutes of boiling, the foldon domain was disrupted and the polypeptides appeared as monomers on the gel (lanes 2, 4, 6 from left), theoretical molecular weights of 11.9 kD, 20.1 kD, and 28.3 kD, respectively. But when the samples were loaded with no prior heating, the foldon domain remained as a trimer and bands with three times the molecular weight of the monomeric ELP-foldon constructs were observed on the gel (lanes 3, 5, 7 from left).

2-4-2. Transition Temperature Determination

The transition temperature at which the ELP molecules aggregate, producing a turbid solution is commonly measured using UV-vis spectroscopy. This transition temperature can be measured as the cloud point (T_c), i.e. the onset of turbidity determined from intersection of the tangent lines of zero absorbance and the highest slope of the curve on a UV absorbance spectrum.^{2,19} Since this transition is rather sharp, many groups working with ELPs have defined the transition temperature (T_t) for a constantly warmed sample as the temperature at the mid-point between the baseline and maximum in the absorbance versus temperature curve, which is comparable to the point of the highest slope of the curve.^{2,3} For high molecular weight ELP molecules or for high concentration solutions, there is a rapid change in turbidity, i.e. nearly infinite slope, which results in nearly identical values to a cloud point measurement. However, for short chains and/or low concentration solutions there can be a considerable difference between the two values due to a smaller slope in the turbidity curve. A comparison between (GVGVP)₆₀-foldon and (GVGVP)₂₀-foldon at 25 μ M shows a clear difference between the two spectra (Figure 2-3a). For the shorter ELP chains using the midpoint as the transition temperature results in a significantly higher T_t , particularly at lower concentrations. This method of measurement is not adequate for identifying the transition temperature of these samples since the resulting temperature does not represent the point at which the ELP molecules start to phase separate and is dependent on the heating rate of the sample. Since some of our samples are relatively low molecular weight, we use the cloud point (T_c) to most accurately reflect the transition temperature of the ELP solutions (Figure 2-3b).

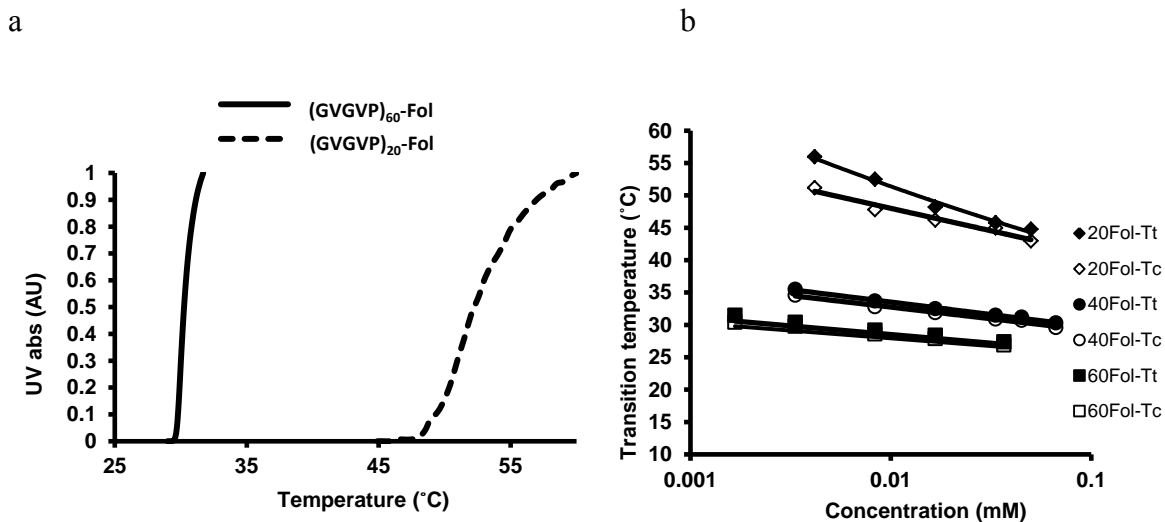


Figure 2-3. a) UV absorbance of (GVGVP)₆₀-foldon and (GVGVP)₂₀-foldon at 25 μ M concentration in PBS as a function of temperature. In order to determine the transition temperature for ELP solutions, the samples were warmed at a rate of 1°C/min and the UV absorbance at 350 nm was recorded. As is observed for the (GVGVP)₆₀, the curve is quite sharp, and the mid-point of the curve (T_t) is nearly equivalent to the onset of turbidity (T_c). For the lower molecular weight (GVGVP)₂₀-foldon, the slope of the curve is smaller, resulting in different values for T_t and T_c . b) The concentration dependency of the transition temperatures of ELP-foldon constructs using both T_c and T_t . For high molecular weight constructs the values are essentially identical but for shorter chains a considerable deviation in the values is observed.

This was not a concern for many previously published studies since the measurements were made using higher molecular weight constructs and medium to high solution concentrations for which the values from the mid-point and the onset of turbidity are essentially equivalent (Figure 2-3b). For these studies, we are able to directly compare our data to those of previous studies. Meanwhile, the use of cloud point as the transition temperature gives us the flexibility to include a much wider range of data in the study of the length and configuration of ELP constructs.

2-4-3. Modeling the Transition Temperatures

It has been shown for linear ELP constructs that the transition temperature decreases with increasing concentration of the ELP solution and increasing molecular weight. However, at high concentration the transition temperature reaches a minimum for ELP constructs containing same pentapeptide sequence regardless of their length.^{2,2, 2.4} Based on the phase diagram originally presented by Urry^{2,3}, Meyer and Chilkoti developed a model to predict the transition temperatures of ELP solutions as a function of concentration and length for different linear elastin-like polypeptides.^{2,20} This model predicts a so-called critical point where the transition temperatures for all lengths converge, i.e. the temperature and concentration at which the transition temperature reaches a minimum. Our experimental data for linear and trimer ELPs are first analyzed using this model in order to compare the results to those of previously reported linear ELP constructs.

The model was originally developed using measurements of ELP solutions with concentrations mostly below 2 mg/ml. Since the critical point occurs at much higher concentrations, it was determined by extrapolation. In this model the transition temperature (T_t), is a linear function of the logarithm of concentration (C) following the equation

$$T_t = T_{cr} - K_c \ln(C/C_{cr}), \quad (2-1)$$

in which

$$K_c = k_t/L, \quad (2-2)$$

where C_{cr} is the critical concentration, k_t is a constant with the unit of °C, and L is the number of pentapeptide repeats.

We have previously reported that transition temperatures of (GVGVP)₄₀-foldon also exhibits linear dependency on the natural logarithm of the concentration at low concentration values,^{2,17} but here with a broader range of sizes of ELP-foldon, we find that the data can be fit to the model. The molar concentration of the trimers, i.e. 1/3 the concentration of the ELP-foldon monomers, was used, and the total number of pentapeptides in the trimers were used for L . To fit the experimental data, the transition temperatures (T_c) were measured at different concentrations (C) and the first estimate of the critical concentration (C_{cr}) and critical temperature (T_{cr}) were obtained from the intersection of plots of transition temperature vs. natural logarithm of concentration. Subsequently, the transition temperature for all different concentrations of either linear or trimer constructs were then fit to the equation using least square analysis to determine the critical parameters along with k_t . The model is able to fit our experimental data for both linear and trimer constructs (Figures 2-4a and 2-4b, respectively). The calculated parameters from the fit for linear constructs are in good agreement with previously reported values (Table 2-1). Based on this analysis, the critical temperature is about 2°C lower and the critical concentration is one order of magnitude larger for the ELP-foldon constructs compared to the linear critical values. It is also interesting to note that the k_t value is observed to be about 1.5 times larger for the trimers than the linear ELP.

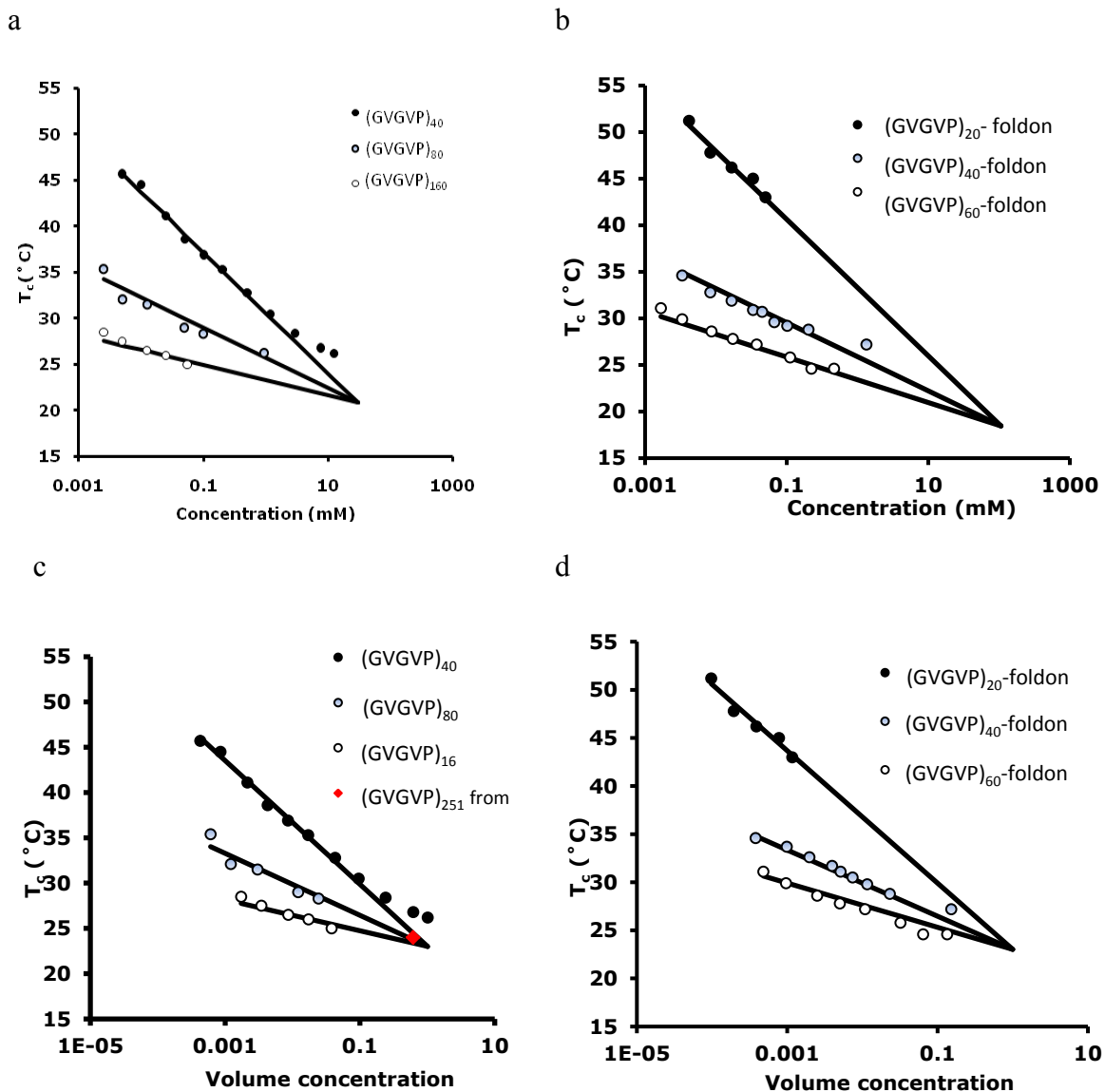


Figure 2-4. Transition temperatures versus log concentration for linear and trimer ELPs. The fit of our experimental data for linear ELP constructs to the Meyer and Chilkoti model^{2,4} (a) results in good agreement with the previously reported data. The ELP-foldon data (b) can also be fit well with this model, but results in a lower critical transition temperature than found in the linear fit. The critical concentration is about one order of magnitude larger than that of the linear fit. The same data is fit using modified model based on volumetric concentrations (c, d). The common critical transition temperature calculated by this approach is 23.0°C, which is close to the reported experimental critical transition temperature for high molecular weight, high concentration (GVGVP)_n solutions.^{2,3}

	Linear	Trimer	Linear from Meyer and Chilkoti ^{2,4}
k_t (°C)	114.5	190.8	129
C_{cr} (mM)	30.0	106.4	25
T_{cr} (°C)	20.8	18.5	20.8

Table 2-1: Fit parameters for linear and trimer constructs from molar concentration data.

An observation from our data is that at high concentrations shorter ELP molecules, particularly obvious for (GVGVP)₄₀, show a deviation from linearity in the T_t versus log concentration plots (Figure 2-4a). It appears that for these shorter constructs, no matter how high the concentration, the T_c will not decrease to T_{cr} . Although the cause of this deviation is not clear, this higher temperature plateau might be the result of chain end effects, which reduce the stabilizing effect of chain association and consequently increase the transition temperature above what is predicted by T_{cr} .

2-4-4. Limitations of the Model

As mentioned above, the critical temperature is the minimum temperature at which ELPs containing the same amino acid repeats will aggregate out of the solution.^{2,3,2,4} This temperature is thought to be independent of the length of the ELPs and is based on the idea that all of the ELP constructs at high enough concentration act like high molecular weight ELP. Considering the results from the fits of this model, there are reasons to believe that the use of molar concentration in the model does not result in critical parameters that merit attributing this physical meaning to.

First, the critical transition temperature for linear (GVGVP)_n using molar concentration in this model is 20.8°C, consistent the original reported value,^{2,4} and 18.5°C for the

trimer. However, there has not been any report of transition temperatures of the (GVGVP)_n system below 24°C, which is the value at high concentration and molecular weight.^{2,3}

Secondly, the critical point is at a high concentration where there are significant chain-chain interactions such that the transition temperature is no longer dependent on chain length, but solely the interactions of the ELP chains with each other and the solvent. One might expect that this temperature should also be independent of molecular configuration. However, using molar concentration results in a difference between the critical transition temperatures of linear and trimer constructs. It should be noted that it is reasonable that the molecular configuration may influence the length and concentration dependence of the transition temperature at concentrations below the critical point, where the differences in conformations between the branched and linear systems can affect chain-chain interactions.

Finally, in the model, the critical concentration value is dependent on the choice of the type of concentration producing significantly different results. In fact, the critical parameters were originally defined by Urry on a mass concentration phase diagram of ELP solutions.^{2,21} So for this model to be directly comparable to Urry's phase diagram the model should utilize mass concentration instead of molar concentration. Using our experimental data, we have found that the model indeed can be used to fit mass concentration data for both linear and trimer constructs resulting in different values of T_{cr} and C_{cr} , compared to the fit with molar concentration (Figure 2-5).

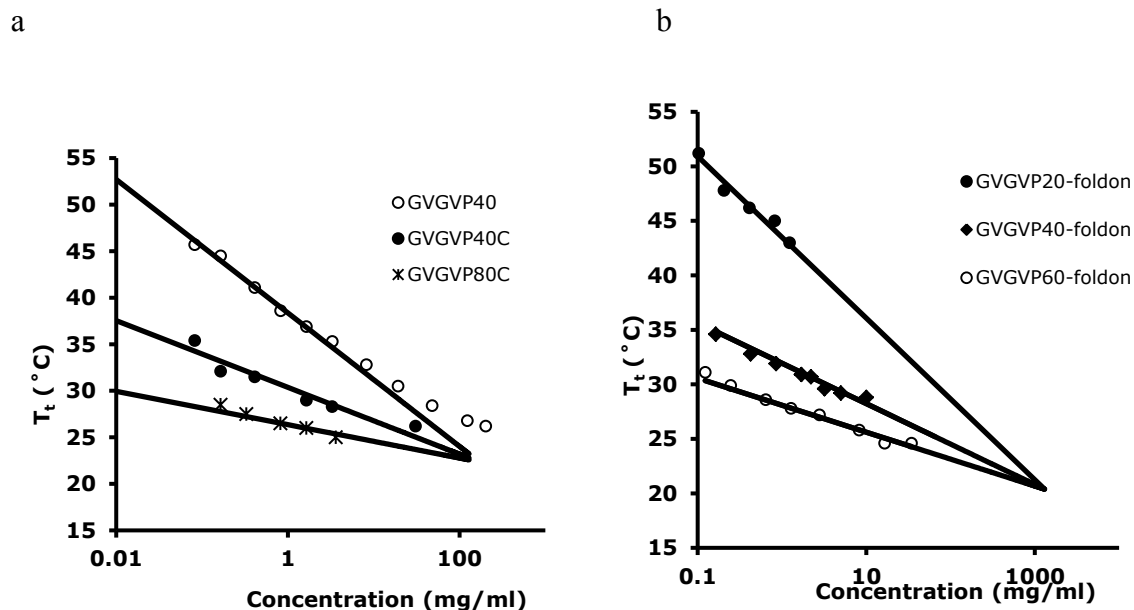


Figure 2-5. Fit of experimental data to Meyer and Chilkoti model using mass-based concentration for both linear (a) and trimer (b). The results show different values of critical transition temperature and concentration in comparison to the molar-based fit for both constructs. And they both result in lower values of T_{cr} than the experimental values.

2-4-5. Modification of the Model

Because of the inconsistencies in T_{cr} for different molecular architectures and the ability to redefine the concentration unit, we sought a concentration definition that could provide physical meaning to the critical point. To do this, we propose to use a concentration based on the volume of the polypeptide coils at the transition temperature, specifically the ratio of the total polymer coil volume to the solution volume. We consider the polymer coil volume to be the equivalent hydrodynamic volume, V_e , which is related to the average polymer conformation, indicating the geometry of the coil and the degree of swelling. For a given chain conformation there is a scaling relationship

between the molecular weight (M) and hydrodynamic volume. Consequently for a polymeric chain in the solution^{2,22}:

$$V_e \propto M^n, \quad (2-3)$$

where, the exponent n describes the scaling relationship. For theta conditions, where the radius of gyration squared (R_g^2) is proportional to M , the value of n is $3/2$.

The volume concentration, based on equation (2-3) becomes

$$C_v = C_m L^n k', \quad (2-4)$$

where C_m is molar concentration and k' is a constant. At the critical point we have arbitrarily defined the critical volume concentration, C_v , to be equal to 1, where the total volume of the polymer coils is equal to the solution volume, allowing k' to be a fit parameter. Experimental data for both the linear and trimer constructs were fit together by coupling the equations 2-1 and 2-4 using a single T_{cr} and individual n , k' , and k_t values for the linear and trimer architectures (Figure 2-4c,d and Table 2-2).

	Linear	ELP-foldon trimer
k_t (°C)	119	179
k' (L.mol ⁿ⁻¹ .gr ⁻ⁿ)	3.3×10^{-4}	2.0×10^{-6}
T_{cr} (°C)	23.0	23.0
n	1.51	2.30

Table 2-2. Fit parameters for linear and ELP-foldon trimer constructs using volumetric concentration

The resulting critical transition temperature is 23.0°C, which is higher than the fit using molar or mass concentration and is closer to the transition temperature observed for high molecular weight linear constructs.^{2,21} It is notable that a single temperature could be successfully fit to the data for both the linear and trimeric constructs, which was not

possible using either mass or molar concentrations. This suggests that using volumetric concentration may result in parameters that have physical meaning, where the critical point indicates the volumetric concentration at which significant chain-chain interactions occur. One interpretation could be that this is the point where ELP chains start to overlap.

From the fit, k_t values were again found to be related by a factor of 1.5 (as was observed with fits for all other types of concentration). The constant k_t relates the concentration dependence of the T_c to the number of pentapeptide repeats in the ELP (Equations 2-1 and 2-2). The factor of 1.5 may indicate that the concentration dependence of T_c is more accurately related to the maximum contour length of the ELP (for our system the total ELP repeats is 1.5 times the ELP repeats in the contour length) rather than the total amount of ELP, but data on other polymer configurations would be needed to confirm this.

2-4-6. Interpretation of Model Parameters

The results of fitting using volumetric concentration provide additional information that can be used to elucidate the effect of architecture on the behavior of ELP molecules. The exponent n indicates how the polymer coil size scales with molecular weight and is similar to the exponent, a , in the Mark-Houwink-Sakurada relationship between intrinsic viscosity ($[\eta]$) and molecular weight (M) with the constant K ,

$$[\eta] = K M^a, \quad (2-5)$$

except since mass concentration is used for intrinsic viscosity, $n = 1 + a$. The determined value of 1.51 for the linear construct is consistent with the polymers in a theta coil conformation at their transition temperature.^{2,22}

Previous findings show that ELPs begin to exhibit β -secondary structure which were interpreted as β turns, becoming more ordered and less flexible, as they approach their transition temperature.^{2,23} This structuring is a gradual process which starts well below and increases up to the transition temperature.^{2,14} The equilibrium between the β and unordered structure at the transition temperature is affected by the length and hydrophobicity of the chains and with unordered regions being particularly prominent for more hydrophobic chains including poly(GVGVP)^{2,24, 2,25}. The finding that the (GVGVP)_n is in a random coil conformation at the transition temperature is consistent with this process.

The trimer constructs on the other hand have a higher n value, about 2.3, which is consistent with an extended coil/rod like state. This difference in conformation can be explained within the accepted theory of folding and aggregation of the ELP molecules.^{2,3, 2,14} The chains go through the transition from random coil to more ordered state when they approach their transition temperature. However, neighboring chains can potentially stabilize these ordered chains by forming twisted filaments of three chains leading to aggregation and formation of a coacervate. In our trimeric system, there are three chains attached at one end, which provides the proper stoichiometry for twisted filaments at higher local concentration than the bulk solution. This could lead to an earlier and more stable formation of a twisted filament resulting in a non-spherical conformation of the trimer prior to aggregation, leading to the higher n value.

The formation of a twisted filament conformation could also explain the larger critical concentrations observed for the trimer conformation. The twisted filament is a more compact conformation having a smaller hydrodynamic radius than comparable linear

constructs. The critical concentration being defined as the point of significant interactions of the chains is higher for the more compact conformation. As an example, the critical molar concentrations can be calculated from equation 2-4 using k' , the length of the polypeptide, and the critical volume concentration. For (GVGVP)₁₂₀ this results in critical concentration of 2.2 mM, while the comparable molecular weight trimer, (GVGVP)₄₀-foldon, has a critical concentration of 8.3 mM, which is nearly a factor of four larger than that of the linear constructs.

We have noted that, although any form of concentration in the model can be used to predict the concentration and length dependence of ELPs, except for the volumetric concentration, they fail to correctly determine the critical transition temperature. Despite the fact that most of the ELP solutions are used well below their critical point, the prediction of the critical transition temperature is still of importance especially when dealing with cross-linked networks. In these networks, no matter what the initial length of polypeptide, cross-linking can potentially result in an essentially infinite molecular weight gel and the transition temperature theoretically approaches T_{cr} . The precise prediction of this temperature can be important if such gels are considered to be used in sensitive application such as drug delivery in which the functioning of the system is dependent on the response of the gel at the designed temperature.

2-4-7. Foldon-ELP constructs

To further expand our results and to investigate the effect of foldon positioning in the construct, we made and used trimer constructs by adding foldon to the N-terminus of the ELP molecules. The two studied constructs were foldon-(GVGVP)₂₀ and foldon-(GVGVP)₄₀. Their molecular weight and trimer formation were confirmed by SDS-

PAGE electrophoresis and CD spectroscopy the same way that were done for other constructs. The transition temperatures at different protein concentrations were fit to equation 4 the same way as the ELP-foldon data. The critical concentration for this construct was also kept constant at 1 and all other parameters were fit simultaneously with the linear data (Figure 2-6 and Table 2-3).

	Linear	Foldon-ELP trimer	ELP-foldon trimer
k_t (°C)	119	224.5	179
k' (L.mol ⁿ⁻¹ .gr ⁻ⁿ)	3.6×10^{-4}	3.5×10^{-6}	2.0×10^{-6}
T_{cr} (°C)	23	23	23.0
n	1.48	2.6	2.30

Table 2-3. Fit parameters for linear and foldon-ELP and ELP-foldon trimer constructs using volumetric concentration

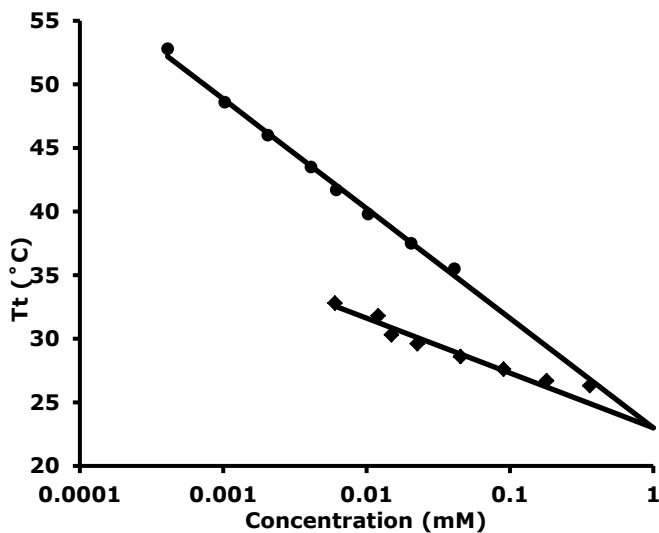


Figure 2-6. Transition temperatures versus log concentration trimer foldon-ELPs.

Comparing the fit parameters of foldon-ELP to that of the linear and ELP-foldon constructs shows an increase in n and k_t . The higher n value compared to linear constructs is expected and can be understood based on the previous discussions. But interestingly the n value is even higher than that of the ELP-foldon trimers. This can be an indication of certain differences between the folding and aggregation of these constructs based on the position of the trimer forming domain. The higher n value for foldon-ELP can be an indication of even more extended and stiffer folded constructs.

To better understand the conformational differences between these constructs circular dichroism (CD) spectroscopy was done using samples of ELP-foldon and foldon-ELP at the same solution concentrations and temperatures below the transition temperatures (Figure 2-7). ELP-foldon construct at this temperature shows the behavior fairly similar to that of a random coil polypeptide with a minimum around 196 nm. This minimum is considered to be the characteristic feature of random coil polypeptides.^{2,26} On the other hand, the CD spectrum for foldon-ELP shows a minimum around 217. This minimum is an indication of much higher population of ordered ELP construct and shows a clear difference between the secondary structures of these two constructs. This is in agreement with previous studies using coiled-coil self-assembling polypeptides at two ends of the ELP constructs.^{2,27} The CD spectra of different constructs will be discussed in more details in Chapter 6.

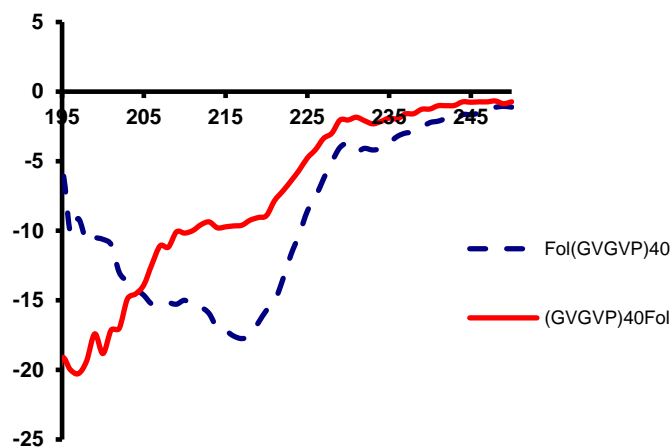


Figure 2-7. Circular dichroism spectroscopy of Foldon-(GVGVP)₄₀ and (GVGVP)₄₀-foldon at 10 μ M protein concentration and 25°C.

The difference between the two constructs can be contributed to the folding process of the ELP chains. We suggest that the folding of ELP molecules might be a directional process that starts from the N-terminus of the ELPs. Adding the foldon to this end of the molecules and bringing the chains in a very close proximity induces the folding of the constructs. In case of ELP-foldon, adding the foldon domain was shown to induce the folding process, but as observed with foldon-ELP, it seems that the foldon at the N-terminus has a greater effect on the folding of ELPs.

2-5. Conclusion

In this study the role of molecular architecture on the thermal transition behavior and the polypeptide coil structure of elastin-like polypeptides was investigated by developing a three-armed star ELP system using a trimer forming oligomerization domain. The domain was added to either N- or C-terminus of the ELP. The transition temperatures of

linear and trimer constructs at different concentrations were fit to a mathematical model using a volume-based concentration that scales with molecular weight, providing a physical interpretation of the critical transition temperatures for ELP constructs. This new fit resulted in critical transition temperatures in good agreement with experimentally observed critical parameters for high molecular weight constructs. This approach also enabled us to study the different behavior of linear and branched ELP molecules at their transition temperature. It was shown that the trimer goes into a more extended conformation at its transition temperature while the linear construct keeps a more random coil conformation. Between the two trimer construct, it was shown that when foldon was added to the N-terminus of the ELP, it had a greater effect on their folding. The results are consistent with the theory of twisted filament formation of ELPs above their transition temperature.

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Chapter III

Thermoreversible Micelle Formation Using a Three-Armed Star Elastin-like Polypeptide

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3-1. Abstract

We have designed, synthesized, and purified a novel three-armed star polymer consisting of an elastin-like polypeptide (ELP) followed by a negatively charged trimer-forming oligomerization domain at the C-terminus. In low salt conditions, the water-soluble trimers assemble into micellar particles as small as 25 nm in diameter when heated to temperatures above their transition temperature. The size of these micelles can be controlled by adjusting salt and cosurfactant concentrations. They are stable at elevated temperatures but will dissociate into the individual trimers when the temperature is again decreased below the transition temperature. Their behavior at high temperatures is quite different than typical ELP constructs, which initially form large aggregates followed by phase separation into a coacervate and soluble fraction. The polypeptide consists of 40 pentapeptide repeats of glycine-valine-glycine-valine-proline (GVGVP) followed by the

27 amino acid foldon domain. It is expressed in *E. coli* and purified by thermal transition cycling.

3-2. Introduction

Elastin-like polypeptides (ELP) are a part of the family of responsive polymers that have been studied extensively for the past two decades.^{3.1-3} These polymers consist of repeats of the sequence G α G β P, where α can be any of the 20 naturally occurring amino acids and β can be any except for proline.^{3.1} These polypeptides are similar to responsive synthetic polymers like PNIPAA in many ways. This includes the fact that both exhibit LCST (lower critical solution temperature) behavior by phase separating above a specific transition temperature.^{3.1, 3.4} When these materials are prepared as bulk hydrogels, this behavior results in significant volume changes when passing through the transition temperature. These so called responsive materials have been used for many different applications including drug delivery^{3.5-6}, tissue engineering^{3.7-8}, surface engineering^{3.9-11}, nano sensors^{3.12}, and hydrogels.^{3.1, 3.13-14} The environmental stimuli for both synthetic polymers and elastin-like polypeptides can be chosen based on the intended application and can include temperature^{3.1-2}, pH^{3.15-16}, ionic strength^{3.16}, or light^{3.17-18}.

An area that elastin-like polypeptides have attracted much attention is the molecular self-assembly of ELP constructs into nano- and micro-scale constructs.^{3.19-23} Several different block copolymers (either AB or ABA) consisting of ELP chains with different hydrophobicities have been designed.^{3.5, 3.24} these constructs assemble into particles when triggered by external stimuli that cause one of the ELP chains of the copolymer to become hydrophobic, leaving the second soluble ELP block to act as a hydrophilic head group that stabilizes the formation of micellar or vesicular particles.

Drug delivery using self assembled particles^{3.25-26} is an attractive area for ELP because of the ability to precisely control the structure of these polypeptides^{3.27} and their natural biocompatibility.^{3.28} ELP constructs have demonstrated efficacy in properties desirable for drug delivery carriers including targeted delivery^{3.29}, good loading capacity^{3.30}, and low clearance in animals^{3.31}. The most important environmental stimuli for a drug delivery carrier are the ones which have physiological relevance, i.e. pH difference between the healthy and diseased cells, temperature, concentration difference, or a combination of these^{3.32-33} and ELP molecules have the capacity to be designed and constructed to respond to any of these environmental stimuli. Another advantage of ELP self-assembled particles is their small size. These particles can be 100 nm or less, which is much smaller than typical ELP aggregates. The smaller size can be an advantage *in vivo* by decreasing antigenicity and reducing clearance.^{3.24}

Although the specifics of ELP aggregation at the molecular level are still debated, an early description of the process suggested that the ELP chains transition from a random coil conformation to a folded β -spiral which is hydrophobic.^{3.1, 3.34} The folded polypeptides are partially stabilized by forming a twisted filament of three chains and phase separating in the form of a coacervate.^{3.35} This process results in the measurable turbidity of the solution above its transition temperature. This theory has been supported by a number of studies, especially by using circular dichroism (CD) measurements.^{3.2, 3.36} In a recent report, it was demonstrated that combining ELP with the trimer forming α -helical coiled coil motif resulted in the tail ends of the ELP coming into close proximity above the transition temperature, further supporting this model.^{3.37}

There are numerous protein folds that incorporate such a triple helix, including the collagen triple helix and the α -helical coiled coil motif. Another instance is in bacteriophage T4 fibritin protein, which forms a fibrous trimer. Unlike the collagen and α -helical coiled-coil, the fibritin fold is not stable alone, but requires a terminal trimer folding domain to stabilize it.^{3.38} This domain, called foldon, can be expressed as a 27 amino acid peptide which forms a homotrimer in a large range of pH, with the notable exception of very low pH.^{3.39-40} At neutral pH, the domain is negatively charged and thermally stable up to 75°C.^{3.38} These features have been used to stabilize short triple helices of collagen for structural studies using recombinant methods to incorporate foldon.^{3.41} With the 27 amino acid sequence at either the N- or C-terminus, a collagen triple helix can be stabilized at higher temperatures and lower concentrations than one without the foldon sequence.^{3.38}

We report here the design and biosynthesis of a new elastin-like polypeptide construct that incorporates the foldon domain at its C-terminal end. We demonstrate that the foldon domain folds, producing a trimer resulting in a three-armed star ELP. The thermal transitions of this construct are compared to linear ELP at different polypeptide and salt concentrations. Additionally, we show that this construct behaves quite differently at low salt concentrations by forming nanoscale micellar aggregates at temperatures above the ELP transition temperature. We describe generally the conditions that lead to the formation of these micelles.

3-3. Materials and Methods

3-3-1. Gene Design and Preparation

The synthesis of the elastin-like polypeptide gene was based on the methods described by Meyer and Chilkoti.^{3,27} In short, complementary oligonucleotides (Invitrogen) encoding five repeats of amino acid sequence GVGVP were designed to have appropriate overhangs to insert into pUC19 cloning vector (Novagen) digested with *EcoRI* and *HinDIII*, as well as internal cut sites for restriction enzymes *BglII* and *PflMI*. These two oligonucleotides were annealed together by heating to 95°C and slowly cooling in a thermocycler. An aliquot of pUC19 vector was double digested using *EcoRI* and *HinDIII* and then purified using a DNA extraction kit (GenScript). The annealed DNA was ligated into the double digested vector in a three to one molar ratio using Quick Ligase (New England Biolabs) for 15 minutes. A 10 µL aliquot of the ligation product was combined with 50 µL of chemically competent *E. coli* cells and transformed by heat shocking (30 min at 4°C followed by 2 min at 37°C) and left overnight on solid agar plates supplemented by 100mg/L of ampicillin. Colonies were grown overnight and screened using PCR screening techniques. The sequence was ultimately confirmed by DNA sequencing.

To increase the length of the ELP encoding DNA, we double digested the pUC19 vector containing (GVGVP)₅ with *NdeI* and *PflMI* restriction enzymes. A separate sample of the same plasmid was also double digested by using *NdeI* and *BglII* restriction enzymes to produce the insert. Using double digestions minimizes the number of false positives on the plates after transformation and does not require dephosphorylation of the vectors. This process resulted in DNA encoding ten GVGVP repeats. This process was

repeated twice more to reach (GVGVP)₄₀. This (GVGVP)₄₀ encoding DNA was transferred to a pET20b expression vector modified as described by Meyer and Chilkoti^{3,27} to have one *Sfi*I recognition site that leaves an overhang that is compatible with the *Pfl*MI cutsite in the pUC19 construct. The (GVGVP)₄₀ DNA resulting from the digestion of the pUC vector with *Nde*I and *Pfl*MI was inserted into the pET vector digested with *Nde*I and *Sfi*I. The resulting gene encodes a 206 amino acid polypeptide: MGH(GVGVP)₄₀GWP.

To prepare DNA encoding the foldon domain, oligonucleotides were designed so they would anneal into a double stranded DNA cassette with overhangs compatible with the *Sfi*I cut site, and leaving a *Pfl*MI recognition site. The codons were selected for optimal expression in *E. coli*.^{3,42} This construct was ligated into the modified pET20b vector described above digested with *Sfi*I. A (GVGVP)₄₀ encoding DNA was then inserted as above resulting in the 233 amino acid polypeptide that includes a C-terminal foldon domain sequence: MGH(GVGVP)₄₀GWPGYIPEAPRDGQAYVRKDGWVLLSTFL.

3-3-2. Protein Expression and Purification

Expressions were started by preparing a 5 ml overnight starter culture from frozen stock in LB medium supplemented with 100 µg/ml ampicillin (LBA) at 37°C. The starter culture was added to 1 L of LBA medium in a 2 L Erlenmeyer flask and shaken at 300 rpm and 37°C to an OD₆₀₀ of between 0.9 to 1.0, at which point expression was induced by adding 0.1 mM IPTG. The cells were harvested after 4 to 5 hours by centrifugation for 30 min at 3000×g. To lyse the cells they were resuspended in 20 ml of bacterial protein extraction reagent (B-PER, Pierce) by vortexing vigorously for several minutes. The lysed cells were then centrifuged at 4°C for 20 min at 20000×g to separate the soluble

protein from insoluble cell lysate. The soluble supernatant, which contained our polypeptide, was then purified using inverse transition cycling method.^{3,43} We utilized warm centrifugation of the protein solution for 15 min at about 40°C at 15000×g to pellet out the aggregated proteins. The isolated pellet was resuspended by using 5 ml of chilled PBS buffer pH 7.4 (Fisher Scientific). The resuspended solution was then centrifuged at 4°C for 15 min at 15000×g. This process was repeated two times to purify the ELP.

3-3-3. Protein Characterization

Purified ELP was characterized first by using 10-20% gradient Tris-Glycerol SDS-PAGE (Lonza) to confirm the purity and molecular weight. The samples were prepared in loading buffer containing 0.1% SDS and heated for 5 minutes prior to loading on the gel at temperatures ranging from room temperature to 100°C. The concentration of the proteins was quantified using UV absorption at 280 nm on a Biomat3 (Thermo Scientific). The extinction coefficient used to convert absorbance to concentration was calculated based on the tyrosine and tryptophan content of the peptides.^{3,44} To observe the folding of the foldon domain, circular dichroism spectra were obtained using an Aviv 215 CD spectropolarimeter.

Transition temperatures of ELP solutions were determined by solution turbidity measured using a Cary 50 Bio UV-visible spectrophotometer equipped with a temperature-controlled cell (Varian). Spectra from 800 nm to 200 nm were obtained at 0.5°C steps with an average temperature ramp of 1°C/min. The transition temperature is defined as the temperature corresponding to the midpoint of the baseline and maximum absorbance (approximate maximum slope) of the 350 nm absorbance curve

Particle size measurements were performed using 90 Plus Particle Size Analyzer (Brookhaven Instruments), which is a fixed angle (90°) dynamic light scattering instrument equipped with peltier temperature control. The samples were carefully filtered using a 0.22 µm filter prior to loading in 1 cm x 1 cm quartz cuvettes. The measurements were made in 2 minute runs and repeated at least twice and were analyzed by BIC software (Brookhaven Instruments). The results are the mean diameter of multimodal size distribution (MSD), being calculated by the software based on the non-negatively constrained least square (NNLS) algorithm.

3-4. Results

ELP with and without foldon were successfully expressed and purified with yields between 50 and 100 mg/L of culture. The foldon sequence is expected to fold as a homotrimer resulting in a three-armed star polypeptide (Figure 3-1). An SDS PAGE of the (GVGVP)₄₀-foldon illustrates the formation and stability of the trimer (Figure 3-2). Samples heated to different temperatures in loading buffer containing 0.1% SDS prior to loading on the gel resulted in different band patterns. Since the foldon domain is stable up to concentrations of 2% SDS⁴⁰ the foldon domain in the sample which was not heated remained folded and the construct was observed at about 60 kD, three times the weight of a single polypeptide chain. By heating the sample at temperatures of 65°C or above, the foldon domain was completely denatured and the individual polypeptide chains are observed at 20 kD. This temperature is about 10°C lower than the reported stability of the foldon domain, which is reasonable considering the presence of SDS in the loading buffer which will destabilize it. At intermediate temperatures (e.g. 45°C) partial unfolding is observed where bands appear at both 20 and 60 kD. Circular dichroism spectra of the

(GVGVP)₄₀-foldon constructs further confirmed trimer formation by a peak at 228 nm which is characteristic of the folded foldon domain^{3,38} (Data shown in Chapter 2).

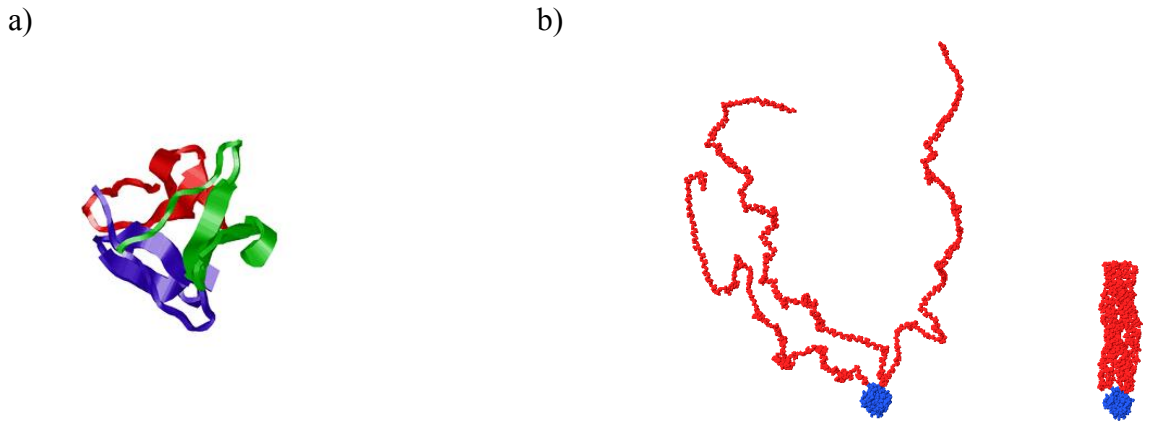


Figure 3-1. a) Ribbon diagram of the foldon domain. This homotrimeric oligomerization domain is stable up to 70°C in water (PDB accession number 1RFO).^{3,39} Elastin-like polypeptide is added to the N-terminal residues to make the ELP trimer. b) The three-armed star elastin-like polypeptide (GVGVP)₄₀-foldon before and after thermal transition. The foldon domain holds the three chains together below and above the ELP transition temperature. The contour length of the folded ELP reduces to about 20% of the unfolded one. The collapsed twisted filament ELP model^{3,35} was created using Swiss PDB viewer based on reported backbone dihedral angles for the β -spiral structure.³⁴

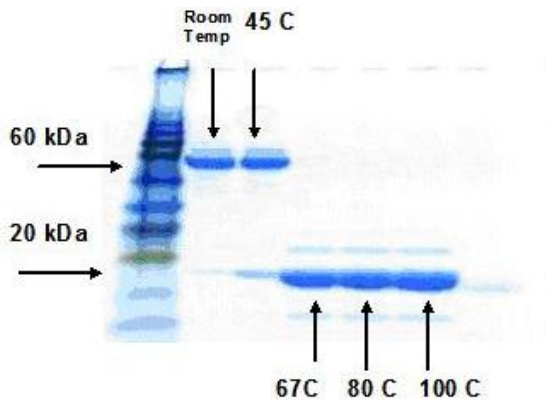


Figure 3-2. An SDS-PAGE gel of (GVGVP)₄₀-foldon with different degrees of heating prior to loading. The sample in lane 2 was not heated prior to loading on the gel and it runs around 60 kD, which is three times the size of a single (GVGVP)₄₀-foldon. The other lanes were heated for five minutes at the designated temperatures prior to loading on the gel. For the 45°C sample, most of the polypeptide shows up as the trimer, but a small

fraction of it runs as monomer (~20 kD). The samples which were heated to temperatures above 65°C resulted in the complete disruption of the trimers.

The defining characteristic of the environmental response of an ELP is the transition temperature (T_t) above which the protein solution becomes turbid, most frequently measured by absorbance in a UV-visible spectrometer. Representative data for obtaining the T_t of ELP and ELP-foldon constructs (Figure 3-3a) show a relatively sharp change in the 350 nm absorbance as a function of temperature for three different constructs. A linear relationship is observed between the T_t values of these ELP constructs and the logarithm of molar concentration of GVGVP pentapeptides (Figure 3-3b). Using the molar concentration of pentapeptide normalizes the samples to the total amount of ELP in solution. This represents an equivalent molar concentrations for the (GVGVP)₄₀-foldon trimer and linear (GVGVP)₁₂₀, whereas the (GVGVP)₄₀ is at three times the molar concentration of the others. Even at a higher molar concentration, the T_t of linear (GVGVP)₄₀ is higher than that of (GVGVP)₄₀-foldon. The transition temperature of ELP-foldon trimer is higher than a linear ELP with the same molecular mass indicating that the molecular geometry affects the T_t . The similar slopes of the two samples indicate that the three-armed geometry results in a constant increase in T_t at equivalent concentrations.

The linear dependence of the transition temperature on salt concentration, a well-described phenomenon,^{3,45-46} is observed for the three-armed ELP. The transition temperature decreases linearly as a function of the sodium chloride concentration (Figure 3-4). This relationship suggests the zero salt concentration transition temperature for a (GVGVP)₄₀-foldon solution with 25 μM protein should be 38°C. This is not observed. At salt concentrations below 50 mM, no turbidity is observed up to the temperature of the

foldon stability ($\sim 70^\circ\text{C}$), well above the expected transition temperature. Even when the solutions are held at these temperatures for extended times no phase separation is observed. This is in contrast to the linear $(\text{GVGVP})_{40}$, which exhibits a T_t even at zero salt concentration. The near identical slopes of the curves in this figure suggest that the effect of salt in decreasing the transition temperature is independent of geometry and molecular weight of the constructs.

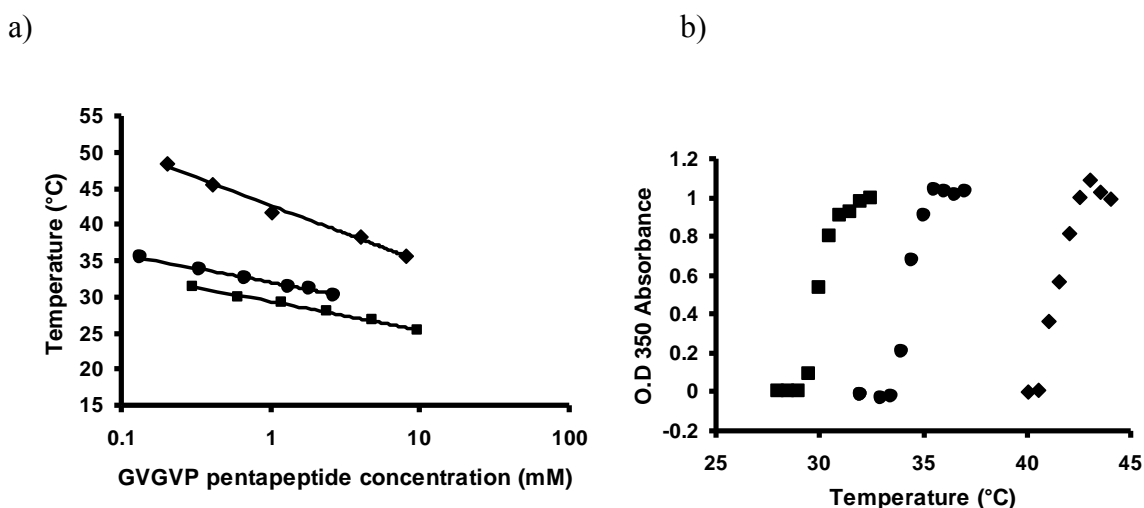


Figure 3-3. a) Determination of the ELP and ELP-foldon transition temperatures. Transition temperatures are characterized for 25 μM solutions of $(\text{GVGVP})_{40}$ (diamonds), $(\text{GVGVP})_{40}$ -foldon (circles) and $(\text{GVGVP})_{120}$ (squares) in PBS by measuring UV absorbance at 350 nm as a function of temperature with a temperature ramp of $1^\circ\text{C}/\text{min}$. The transition temperature is defined as the temperature at the midpoint between the baseline and maximum turbidity (approximately the steepest slope). For these samples, the transition temperatures are 41.5°C for $(\text{GVGVP})_{40}$, 34.5°C for $(\text{GVGVP})_{40}$ -foldon, and 30.0°C for $(\text{GVGVP})_{120}$. b) Transition temperatures of $(\text{GVGVP})_{40}$ (diamonds), $(\text{GVGVP})_{40}$ -foldon (circles) and $(\text{GVGVP})_{120}$ (squares) as a function of molar concentration of GVGVP pentapeptides (i.e. constant ELP mass concentration). A linear relationship between T_t and $\log C$ is observed for each construct. Comparing

(GVGVP)₄₀-foldon and (GVGVP)₁₂₀, which have the same ELP molecular weight, the slopes are the same, but the former has higher T_t values.

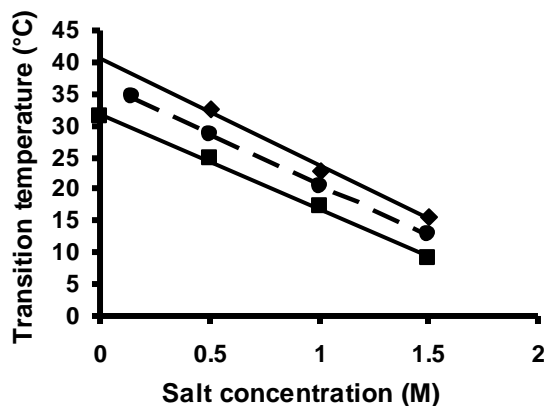


Figure 3-4. Effect of salt on the transition temperature of (GVGVP)₄₀ (diamonds), (GVGVP)₄₀-foldon (circles), and (GVGVP)₁₂₀ (squares). There is a linear relationship between salt concentration and the transition temperature of ELP-foldon solutions, except at low salt concentrations. At salt concentrations below 45 mM, a transition temperature is not observed by turbidity measurements. This contrasts the typical ELP, which exhibits transitions even without salt. The slopes for the three samples are equivalent, indicating that the geometry does not affect the salt dependency of the T_t for the ELP molecules. The (GVGVP)₄₀-foldon transition temperatures for all salt concentrations fall between (GVGVP)₄₀ and (GVGVP)₁₂₀.

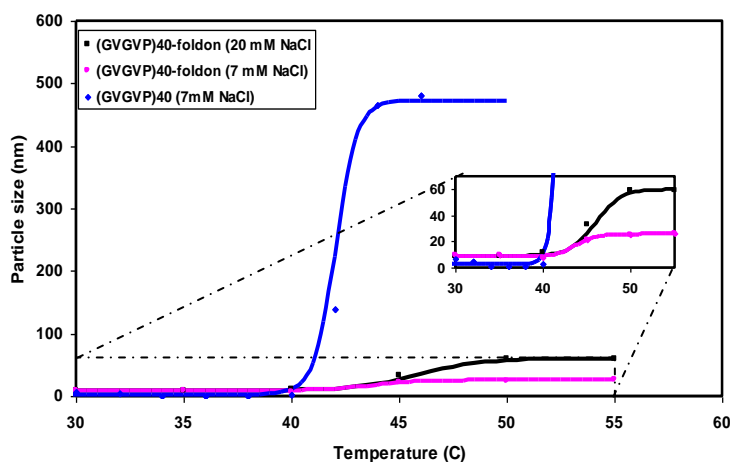
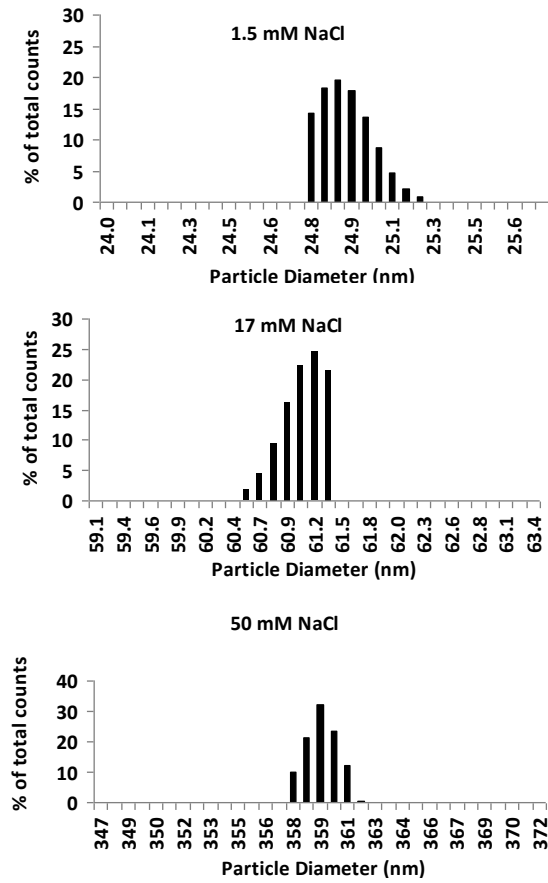


Figure 3-5. Dynamic light scattering particle size determination as a function of temperature for (GVGVP)₄₀ and (GVGVP)₄₀-foldon. The solutions are in low salt concentration and the graphs show very clear difference between the sizes of the particles above the transition temperature. For (GVGVP)₄₀ the particle size changes from below 10 nm to about 500 nm resulting in turbidity of the solution. In the case of (GVGVP)₄₀-foldon, the diameter of particles does not exceed 30 nm when the salt concentration is 7 mM and 60 nm for a salt concentration of 20 mM. These solutions remain clear and the particle diameter does not change over time. This is also a reversible process as no difference in particle size as a function of temperature is observed when the sample is cooled. The inset is a magnification of the graph illustrating the region where small particles form.

Dynamic light scattering particle sizing was used to further investigate aggregation, particularly in low salt solutions (Figure 3-5). Below the transition temperature, the linear (GVGVP)₄₀ is soluble as observed by a polymer chain conformation size of less than 10 nm. Above the transition temperature, it aggregates into particles of about 500 nm in size. This particle formation is consistent with the turbidity observed for these solutions. For the (GVGVP)₄₀-foldon trimers, a distinctly different behavior is observed. At salt concentrations below 15 mM, when heated from below to above its expected transition temperature (based on the linear relationship to salt concentration), the size changes from below 10 nm to about 30 nm. The small aggregate size explains why no turbidity is observed by UV absorption under these conditions. For salt concentrations between 15 mM and about 45 mM, when the solution is heated to temperatures above the transition temperature, the size of the particles change to about 60 nm. The formation of the small particles at low salt is a reversible process as is observed by cooling down the solution and observing no hysteresis between warming and cooling experiments.

a)



b)

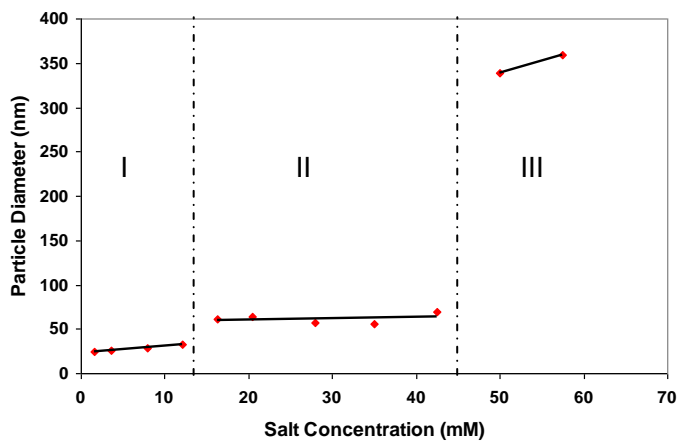


Figure 3-6. Dynamic light scattering of (GVGVP)₄₀-foldon in different NaCl concentrations. Dynamic light scattering was performed on 10 μ M polypeptide solutions at 50°C, which is above the polypeptide

transition temperature. The measurements show very narrow distribution of particle sizes over a wide range of salt concentrations (a) and that the size of the aggregates varies as a function of salt concentration (b). Three zones are observed. For salt concentration below 15 mM, the particles are less than 30 nm and below. For salt concentrations between 15 mM and 45 mM the size of the particles are in the range of 60 to 65 nm. Increasing the salt concentration to values above 45 mM the aggregates are greater than 300 nm and the solution becomes visibly turbid.

Further measurements demonstrate that the size of the aggregate is dependent on salt concentration, with three distinct regimes (Figure 3-6). In Regime I, at salt concentration below 15 mM, the aggregates are 30 nm or smaller and appear to linearly increase with salt concentration. For salt concentrations between 15 and 45 mM (Regime II), the size of the aggregates is approximately constant in the range of 60 to 65 nm, and in Regime III, for salt concentrations above 45 mM, the particles are large and turbidity is observed which is consistent with the UV turbidity measurements. The particle size distributions in all three regimes are quite narrow having peak widths less than 1% of the particle diameters (Figure 3-6a).

The addition of SDS surfactant to the trimer solution generally led to a decrease in the stability of aggregates in both high and low salt conditions. SDS was added to two different (GVGVP)₄₀-foldon solutions with different salt concentrations. In one solution, SDS was changed from 0 to 0.05% while the salt concentration was kept constant at 7 mM (Figure 3-7). In this case, the particles were stable up to about 0.02% of SDS and then the diameter of the particles dropped sharply in to what is expected to be a random coil polypeptide. In the case of the second solution with about 150 mM salt (Figure 3-7),

the aggregates were not stable in the presence of SDS and even at SDS concentration of 0.02% there was no turbidity.

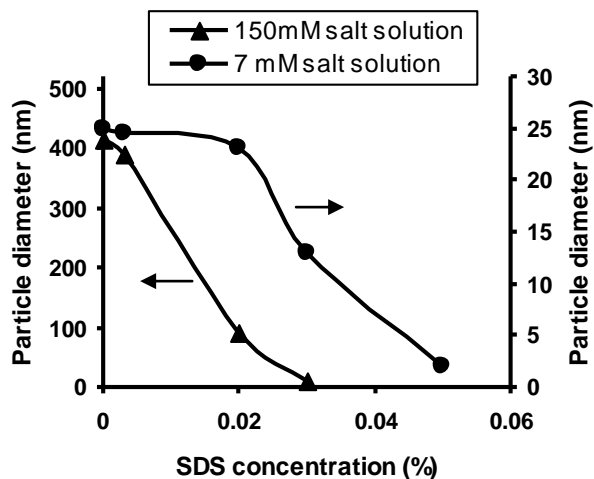


Figure 3-7. The effect of SDS on the (GVGVP)₄₀-foldon micelles and aggregates. At 150 mM salt (left axis), (GVGVP)₄₀-foldon aggregates of several hundred nanometers form above the transition temperature. SDS is able to disrupt the formation of these aggregates. SDS can also disrupt polypeptide micelle formation in low salt conditions (right axis), but requires higher concentrations.

3-5. Discussion

Typical ELP chains heated above their transition temperatures go from a random coil to a more ordered hydrophobic state and phase separate into a coacervate containing 63% water and 37% polypeptide.^{3.1} Previous reports have shown that the initial formation of the coacervate phase is followed by aggregate growth and coalescence over time.^{3.5,3.47} We would not expect significant differences in the behavior of the ELP when attached to the foldon domain, since aggregation above its transition temperature has been reported when ELP was used as a fusion protein for protein purification^{3.43} and in the coiled-coil trimer of Fujita et al.^{3.37} Like typical ELP, our data shows that the three-armed ELP in

moderate salt concentrations does exhibit a transition temperature that is dependent on salt and ELP concentrations, as well as an aggregate size that increases with time.

However, at low salt concentrations (<45 mM), the three-armed ELP, formed by ELP-foldon, behaves differently in comparison to linear ELP. Under these conditions, stable nano-scale aggregates form. Under further scrutiny, it is observed that there are two regimes of stable aggregates with considerably different size distributions (Figure 3-6b). We propose that each of these two regimes consists of stable micellar particles that do not coalesce over time, which is notably different from the higher salt regime. Below we interpret the size of these stable aggregates by considering the geometry of the ELP-foldon and the intermolecular interactions.

The foldon domain has a net negative charge and so can behave as a hydrophilic group to stabilize the coacervate aggregates formed by the ELP chains above their transition temperature. In essence, the ELP-foldon is a surfactant with three hydrophobic tails and a charged head group, which forms micelles at salt concentrations below 45 mM (Regimes I and II). The salt concentration plays a key role in determining the size and type of aggregation that occurs in charged surfactant systems. The primary effect of salt in solution is to moderate the electrostatic interactions between charged species; however, salt can also modify hydrophobic interactions as is observed by the dependence of the transition temperature of ELPs on salt.

The geometry of any surfactant determines the size and shape of micellar aggregates^{3,48-49}, based on the dimensionless packing factor:

$$v/a_0l, \tag{3-1}$$

where v and l are the volume and length of the hydrophobic tails, respectively, and a_0 is the effective area of the hydrophilic head group. If this dimensionless number is less than 1/3, spherical micelles will form with a size such that the diameter of the micelle will be

$$d = 6 v/a_0. \quad (3-2)$$

For spherical micelles, the maximum diameter of the micelles is twice the length of the hydrophobic tail. Based on the ELP folding into a beta-spiral and forming a triple strand^{3,1}, the length of a (GVGVVP)₄₀-foldon in its collapsed state is around 15 nm (Figure 3-1), corresponding to a maximum diameter of 30 nm for a spherical micelle. For (GVGVVP)₄₀-foldon below its transition temperature, a micelle would have a maximum diameter of 150 nm. Based on the micelle size as a function of salt concentration, this indicates that Regime I is consistent with spherical micelles with folded ELP chains. Regime II diameters are greater than 30 nm, which suggests that the micelles are either non-spherical or spherical micelles with unfolded or partially unfolded ELP chains.

We expect that the hydrophobic tails phase separate in the interior of the micelle into an immiscible coacervate-like phase, as in typical ELP solutions. Since the coacervate contains 37% protein and 63% water^{3,1}, and is slightly more dense than water, it is estimated that the volume occupied by the 120 pentapeptide repeats in a (GVGVVP)₄₀-foldon trimer is approximately 200 nm³. This is comparable with an estimation based on the molecular model of the collapsed ELP conformation occupying a cylinder of diameter 4.8 nm and length of 11 nm (Figure 3-1). Assuming a spherical micelle, this volume together with the micelle diameter can be used to calculate the head group areas. It is notable that Regime I exhibits a slight increase in diameter with increasing salt. Based on equation 3-2, the apparent head group diameter is between 8 and 9 nm with a slight

decrease as the salt concentration increases. Such a decrease in the head group size is not unexpected. The apparent area of the foldon domain, which has a net negative charge, will be affected by the electrostatic shielding of the additional salt.

In Regime II, the micelle size remains approximately constant, corresponding to a head group diameter of about 5 nm if it were a spherical micelle. This value is slightly larger than the 2 to 3 nm diameter of the foldon domain, which suggests that they are not spherical micelles. Nevertheless, the constant size in this region suggests that the ion shielding is large enough that increasing the salt concentration does not result in any further reduction in head group area. This is consistent with a reduction in the Debye length to 2.5 nm at 15 mM NaCl. However, since the micelles are stable, the overall electrostatic interaction between micelles is apparently strong enough to prevent coalescence. As the salt concentration increases above 50 mM and we move into Regime III, the charge on the foldon domains is shielded to a greater extent so that large aggregates will form.

The effect of increasing SDS concentration for both micellar and large aggregates of (GVGVP)₄₀-foldon results in a rather sharp decrease in the particles size, but the micellar aggregates shows greater stability, since the large particles disaggregate at lower SDS concentration (Figure 3-7). These patterns give us criteria that can be used together with the salt dependency of the ELP-foldon micelles to predict the size of the aggregates. There is a limited range of salt and SDS concentration in which micelles can be made. The size of these particles for a (GVGVP)₄₀-foldon can be controlled and be kept as small as 20 to 30 nm. It is potentially possible to have even smaller micelles using shorter ELP chains.

Several other ELP micelles have been reported, most using a diblock polymer configuration. In recent work by Fujita et al. a hydrophobic block of different lengths of ELP was combined with a C-terminal hydrophilic polyaspartic acid block resulting in micellar particles.^{3,24} They showed the formation of micelles only for ELP molecules with at least 80 pentapeptide repeats and no micelle formation was observed for (GVGVP)₄₀. In another work by Dreher et al., two different ELP molecules with total length of 124 to 184 repeats of pentapeptide was used as the building block for micelle formation.^{3,5} These two ELP molecules with two different transition temperatures were used to make micelles in the temperature range between their transition temperatures. These resulted in micelles of 35 to 45 nm.

Since our system uses a small hydrophilic head group, short ELP molecules can be used to make these micelles and quite small aggregates have been achieved. Additionally, the second hydrophilic block does not restrict the responsiveness of the micelles. In a very recent study it has been shown that the transition temperature of block copolymers is also a function of the way that polarity is arranged and distributed along the ELP chain.^{3,50} In an ELP block copolymer this can add to the complexity of controlling the transition temperature. For our system this is not an issue and we are able to control the characteristics of the particles in a more precise way. This study demonstrates the possibility of making small micellar particles without the need of using block copolymers. Even though this approach relies on non-physiological salt concentrations, there is no reason that modification of the head group cannot produce similarly small particles in physiologically applicable salt concentrations. This will make these particles appealing for applications such as targeted drug delivery.

3-6. Conclusions

We have designed and constructed a new responsive three-armed star elastin-like polypeptides and by using a trimer forming oligomerization domain. This new construct exhibits typical behavior of ELP molecules, except at low salt concentrations where it responsively forms micellar particles above its transition temperature. The process of micelle formation is thermally reversible and the particles are shown to be stable at temperatures above the transition temperature and can be made as small as 20 to 30 nm in diameter. The size of these micelles is among the smallest ELP particles that have been reported. Salt and surfactant were observed to affect the stability and size of the micelles, and by adjusting these two parameters the size of these particles can be controlled.

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Chapter IV

Size and Shape Characterization of Thermoreversible Micelles of Three-armed Star Elastin-like polypeptides

4-1. Abstract

Three-armed star elastin-like polypeptides have been shown to form micelles in certain solution conditions. Here, a thorough study of size distribution, shape, and molecular weight of these micelles at different salt concentrations and pH values was done. Multi angle dynamic light scattering was used to study the formation, reversibility, and size of the micelles under different conditions. As a function of the solution salt concentration, two distinct regimes based on micelle size and shape, as well as a transition region between them, were identified. Static light scattering was done to study the molecular weight and anisotropy of the micelles in each regime. The anisotropic behavior and the elongation of the particles was then studied by polarized and depolarized dynamic light scattering and a model for the micelles at each regime was proposed. The size of the micelles were verified independently using viscosity measurements. The results of this study suggest that there is big jump in size and molecular weight of the micelles from the

first to the second regime, and the shape of the micelles transition from spherical to cylindrical micelles with more than 10:1 axis ratio. The micelles were then crosslinked into responsive nano-particles that are stable and responsive in physiological conditions.

4-2.Introduction

Self-assembly of macromolecules into well defined shapes and geometries has been studied for many years but during the past decade it has received particular attention with the need for developing nano-scale materials for delivering drugs, genes, or other agents in the rapidly growing field of nanomedicine and targeted delivery.^{4.1-5} The use of these well-defined self-assembling systems in minimizing premature drug release, maximizing drug circulation time, decreasing systemic toxicity, and increasing drug availability to the targeted organs have made them the focal point of many studies. Most of these nano-scale self-assembled particles are based on either liposomes or large polymeric molecules in the form of polymer conjugates or polymeric micelles.^{4.6} Among these polymeric materials, micelles have been shown to have great potential for drug loading and administration.^{4.7, 4.8} They were introduced as potential drug delivery vehicles in the 1980's,^{4.9, 4.10} particularly for hydrophobic drugs with low solubility in water, they have superior characteristics in comparison with other nano-scale carriers based on their small size, relative ease of control over their shape and size, and high drug loading capacity. It has been shown that using micelles as drug delivery vehicles not only maintains the drug concentration at higher levels in the system but also gives the opportunity to modify the surface of the micelle with ligand binding receptors for targeted delivery.^{4.6} It has also been shown that micelle shape, in addition to its size has a direct effect on the drug

delivery efficiency,^{4.11} and so a thorough understanding of size and shape of the micelles in each system has practical importance in designing drug delivery systems.

In general, the structure of polymeric micelles is based on the spontaneous self-assembly of amphiphilic polymer molecules, called unimers, under certain solution conditions.^{4.12} In most cases the core of the micelle, consisting of the hydrophobic blocks, can act as a depot for hydrophobic molecules. The size of the micelle is usually dictated by the kinetic and thermodynamic stability of it based on the geometry of the unimer. While the block length would affect the disassembly rate of the micelles, the thermodynamic stability of the system is affected by the micelle interactions in solution. But since the thermodynamic stability of micelles are generally high above the critical micelle concentration (a concentration at which formation of micellar particles is energetically favorable), the length and chemical characteristics of blocks are usually the most effective parameters in determining final shape and geometry of the micelles.^{4.6}

The same concept of critical micelle concentration can apply to a solution at a constant concentration in which increasing the temperature would push the solution towards the aggregation of the monomers into micelles at a temperature called the critical micelle temperature (CMT).

The critical micelle temperature for polymer micelles can be attributable to lower critical solution temperature (LCST) behavior observed, where in some polymeric systems the polymer begins to phase separate at high temperatures. One of the most well-known polymeric systems possessing LCST behavior is poly(N-isopropylacrylamide) (PNIPAm) which has a LCST of 32°C and has been used in developing many temperature responsive polymeric systems.^{4.13-16} It is a relatively flexible system and has

been shown that the blocks of the copolymers can be chosen such that PNIPAm would act as either core or shell of the micelle.^{4.15, 4.17}

Another approach to make responsive polymer micelle systems is by using environmentally responsive peptide-based polymers. The best known family of responsive polypeptides is elastin-like polypeptides (ELP).^{4.18} These polymers consist of repeats of (Val-Pro-Gly-Xxx-Gly) in which Xxx can be any of the naturally occurring amino acids except for proline.^{4.19} The chemical identity, length and ultimately transition temperature of ELP constructs can be precisely controlled from the gene level and their transition temperature is affected by the length, sequence, and architecture of the polypeptides.^{4.20-22} The fact that they can be designed to respond to many environmental stimuli, including temperature, pH, light, and ionic strength makes them ideal for developing responsive biomaterials.^{4.23-25}

Molecular self-assembly of ELP molecules to micro and nano-scale constructs has been an area of active research in the past few years and many research groups have tried to use this highly controllable system to develop medically applicable micelle systems by constructing di- or tri-block copolymers of hydrophobic and hydrophilic blocks.^{4.26-28}

Recently, we have designed and constructed a three-armed star ELP molecule consisting of a trimer forming oligomerization domain (foldon) and repeats of GVGVP which self-assembles into micellar particles.^{4.29} Instead of having a bulky hydrophilic polymer block, this system utilizes the compact charged foldon domain as the hydrophilic head group. This increases the fraction of the micelle consisting of the hydrophobic core. Micelles of this system were shown to be as small as 20 nm in diameter and the size could be controlled by adjusting the salt concentration in the solution. The initial

characterization of this system was primarily based on data from UV-vis spectroscopy and single-angle dynamic light scattering particle size analysis. Our results suggested the existence of spherical particles in salt concentrations up to 15 mM and two other regimes at higher salt concentrations, one between 15 to 45 mM and the third one above 45 mM.

Although our previous study provided insight into this new system and its ability to form micellar particles with predictable sizes, the single angle dynamic light scattering did not give us enough information to characterize the shape of the particles and, at times, their wide distribution.^{4,29} Other limitations of our previous study were the low laser power and insufficient quality of the optics in the particle-sizer used and the lack of capability for adjusting the optical parameters of the system.

The scope of the current study is to fully describe the size and shape of the micelles in each regime. We use data from multi angle dynamic light scattering to confirm the sizes of particles in each regime and to better understand the geometry of them. Static light scattering measurements along with polarized and depolarized dynamic light scattering were performed at different salt concentrations to study the shape of the particles and to determine the molecular weight of the particles in each regime.

4-3. Materials and Methods

4-3-1. Polypeptide Preparation

The genes encoding (GVGVP)_n-foldon were designed and constructed using standard molecular biology methods as previously described.^{4,29} The exact sequence encoded by the gene as confirmed by DNA sequencing (Cleveland Clinic) is MGH-(GVGVP)₄₀-GWPGYIPEAPRDGQAYVRKDGWVLLSTFL. The (GVGVP)₄₀-foldon gene was expressed and purified with yields between 100 to 200 mg/ml as previously described.^{4,21}

The purity of the sample and its molecular weight were confirmed by SDS-PAGE gel and ion-spray quadrupole/time-of-flight mass spectroscopy (AB/Sciex), respectively.

4-3-2. Sample Preparation

Samples for measurement were made by diluting the PBS stock solutions in reverse osmosis purified water (Millipore, 18.2 M Ω -cm). Salt concentration was adjusted by adding NaCl solution from a 5 M stock solution to the desired concentration. The pH of the samples was then adjusted using 1 M NaOH solution. To make sure that pH had stabilized, samples were left at room temperature after initial pH adjustment for at least half an hour and the pH was measured again and, if necessary, adjusted to the desired pH value. The samples were then filtered using 0.22 μ m Millipak filters (Millex-GV, 0.22 μ m, Millipore) into borosilicate glass cuvettes. The cuvettes were cleaned prior to use by soaking the cells for 30 minutes in concentrated sulfuric acid and then for 10 minutes in a strong oxidizing solution (containing 2 molar NaOH and 0.2 molar KMnO₄). The cells were then rinsed by water and dipped into a dilute HCl solution for several times before a through rinse with deionized water and dried using ultra high purity Nitrogen gas (99.9%). For each measurement a fresh sample was loaded into a newly cleaned cuvette.

4-3-3. Light Scattering

Static and dynamic light scattering experiments were done using an Ar⁺ Spectra Physics 2017 laser with wavelength of 514.5 nm and maximum power of 2 W. The system was optically isolated to eliminate the background noise. A TSX-1A variable neutral density filter (ORIEL) and a VPH-4 optical iris (NRC) were used to control the power of the laser reaching the sample when necessary. The laser beam was directed to the sample in a BI-200SM Goniometer (Brookhaven Instruments) using a series of

mirrors. The sample cell was held in a decalin-filled vat and the temperature of the cell was controlled using a HAAKE-A81 refrigerated water bath ($\pm 0.2^\circ\text{C}$). Dynamic light scattering (DLS) experiments were carried out at scattering angles from 40° to 145° with a step of $5\text{-}15^\circ$. Scattered light was detected by BI-DS2 photomultiplier and analyzed with BI-9000 digital correlator.

DLS analyzes fluctuations in intensity (I) of the scattered light through measured intensity-intensity correlation function $S(q, \tau)$,

$$S(q, \tau) = \int_0^T I(t)I(t + \tau)dt \quad (4-1)$$

Where q is the scattering vector, τ is the delay time, and T is the duration of the experiment. The magnitude of scattering vector is,

$$q = \frac{4\pi n}{\lambda_0} \sin\left(\frac{\theta}{2}\right) \quad (4-2)$$

Here n is the refractive index of the solvent, θ is the scattering angle and λ_0 is the wavelength of the light in vacuum. The measured spectra $S(q, t)$ were analyzed by line shape analysis method^{4.30}, in which intensity correlation function is converted to a field correlation function $g^{(1)}(q, t)$ by Siegert relation. $g^{(1)}(q, t)$ was then fit to a sum of stretched exponentials using nonlinear-least-squares simplex algorithm.^{4.31}

$$g^{(1)}(q, t) = \sum_{i=1}^N A_i \exp(-\theta_i t^{\beta_i}) \quad (4-3)$$

Here N is the number of observed relaxation modes labeled by i , θ_i is the decay pseudorate, β_i is the stretching parameter and A_i the amplitude. It was found that most of the data could be fit with either one or two modes. $g^{(1)}(q, t)$ can also be expressed as a Laplace Transform of the normalized distribution of decay rates $A(I)$,

$$g(t) = \int_0^{\infty} d\Gamma A(\Gamma) \exp(-\Gamma t) \quad (4-4)$$

The analysis of the correlation function can yield mean relaxation times ($\langle \Gamma^l \rangle$) of the most prominent modes. The spectral time moment analysis was applied in this study to determine the zeroth time moment for i^{th} mode obtaining the mean decay rate Γ_i for that mode.^{4.31} An apparent mean diffusion coefficient D_i corresponding to each mode can then be calculated from each mean decay rate Γ_i as

$$D_i = \Gamma_i / q^2. \quad (4-5)$$

The spectral time moment analysis allows for calculation of mean diffusion coefficient of each mode from an accurate fit of a field correlation function. The approach considers the properties of each observed mode in detail to identify physical processes corresponding to this mode. Specific methodology, applications, and advantages of this method over Inverse Laplace Transform based methods are described elsewhere.^{4.30, 4.32}

Depolarized DLS measurements were done using the optical system described above with a Newport RSP 2T polarizer inserted between the scattering cell and PMT. The polarizer was rotated 90° between vertical and horizontal measurements. The laser light in the all experiments was vertically polarized and inserting the polarizer into the system resulted in a 3-5% decrease in the intensity of the light.

To determine the mean translational (D_i) and rotational (θ) diffusion coefficients from polarized and depolarized dynamic light scattering, the measured spectra were analyzed using spectral time moment analysis described above to obtain the mean decay rates ($\langle \Gamma_i \rangle_{\text{VH}}$ and $\langle \Gamma_i \rangle_{\text{VV}}$) of the correlation functions for the horizontally (VH) and vertically scattered light (VV).

The mean diffusion coefficient (D_i) and mean rotational diffusion (θ) were then determined from plotting corresponding $\langle \Gamma_i \rangle$ vs. q^2 from VV and VH and data using

$$\langle \Gamma_i \rangle_{VV} = D_{Ti} q^2 \quad (4-6)$$

and

$$\langle \Gamma_i \rangle_{VH} = D_{Ti} q^2 + 6\theta_{Ri} , \quad (4-7)$$

in which D_{Ti} is the slope and θ_{Ri} is the intercept of the plot from Equation 4-7.

In static light scattering (SLS) experiments the angular dependence of the reduced time-average scattering intensity, known as excess Rayleigh ratio $R_{vv}(q)$ was measured. SLS measurements were carried out with the same optical system as DLS measurements but with a smaller angular step and only samples of 10 μ M concentration were used. SLS data was analyzed in terms of Berry equation for a case of dilute solution with concentration C .

$$\left(\frac{K.C}{R_{vv}(q)} \right)^{\frac{1}{2}} = \left(\frac{1}{M_w} \right)^{\frac{1}{2}} \left(1 + \frac{1}{6} \langle R_g^2 \rangle q^2 + \dots \right) \quad (4-8)$$

Here, $K=4\pi^2 n^2 (dn/dc)^2 / (N_A \lambda_o^4)$, n is solvent refractive index, dn/dc is the differential refractive index increment of the solution, N_A is the Avogadro number, M_w is the weight average molecular weight, $\langle R_g^2 \rangle$ is z-average root mean square radius of gyration, and A_2 is the second virial coefficient. In cases of significant nonlinearity of $Kc/R_{vv}(q)$, the Berry plot equation was used for the extrapolation. It should also be noted that the concentration of particles in our study was low, making A_2 negligibly small, which makes extrapolation to zero concentration unnecessary. The extrapolation to zero scattering

angle yielded the apparent M_w while the fit of the angular dependence of $Kc/R_{90}(q)$ produced an apparent R_g .

In order to reliably obtain a molecular weight from static light scattering measurements the differential refractive index increment (dn/dc) of the solutions had to be directly measured. Bruce-Phoenix differential refractometer with sample cell controlled by Thermo RTE-7 refrigerated bath/circulator was used for these measurements. The values of the measured dn/dc under different were then used in the calculations.

4-3-4. Micelle Crosslinking

To crosslink the micelles, a 5 μM solution of (GVGVP)₄₀-foldon in 10 mM PBS solution and pH of about 10.2 the solution was heated up to 55°C. Different concentrations of GTA solutions (2.5%, 5%, 10%) were then added to the solution drop by drop and the solution was stirred at 55°C for about 18 hours. The final product was then filtered using 0.22 μm filters.

4-4. Results

4-4-1. Dynamic light scattering

We performed single angle dynamic light scattering using a 10 μM (GVGVP)₄₀-foldon solution with salt concentration of 10 mM (Figure 4-1) to confirm the temperature induced formation of micelles, including the reversibility of the process and reproducibility of the data, as previously observed^{4,29}. Using the more powerful light scattering apparatus, significantly bimodal correlation functions were measured. The faster mode (with $D = 5-9 \times 10^{-7} \text{ cm}^2/\text{s}$), was found to have small standard deviation and

no dependence of apparent diffusion coefficient on scattering angle, indicative of small diffusive particles with an apparent hydrodynamic radius (R_h) of about 3 to 5.5 nm in agreement with our previous findings^{4,29}. An additional slower mode which was not seen previously was also observed below the transition temperature was observed. This mode, with a typical D of $1-10 \times 10^{-8}$ cm²/s, was not seen previously, but had a significant contribution to the measured correlation function (with amplitude of 75-85%) and had a stretching parameter $b=0.6-0.7$ which indicates significant polydispersity of the scatterers. The estimates of apparent hydrodynamic radius for this mode (appearing on Figure 4-1) are somewhat misleading as this mode was found to have a strong dependence of D on scattering angle (atypical of center of mass diffusion of a particle). This mode is indicative of slower processes in the system such as relaxation of the unfolded trimers. It should be noted that the different angular dependence of the observed two modes argues against averaging the two processes into one. The faster mode ($D = 5-9 \times 10^{-7}$ cm²/s), was found to have smaller standard deviation and diffusive in multi-angle experiments showing no dependence of apparent diffusion coefficient on scattering angle.

Above the transition temperature, only a single mode was observed for solutions with 10 mM of salt. Using a second mode to fit measured correlation function could not be justified in this case as the RMS error did not change significantly with the addition of the second mode to the fit (see supporting information). The single observed mode was found to have a D which was independent of a scattering angle, corresponding to particles with an apparent hydrodynamic radius of 12 to 15nm.

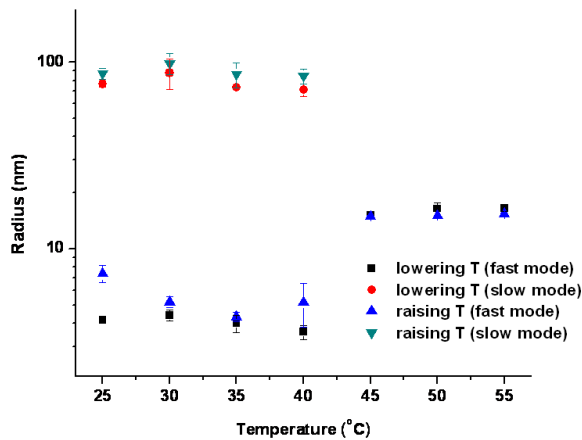


Figure 4-1. Micelles radius as a function of temperature for 10 μM solution of $(\text{GVGVP})_{40}$ -foldon at pH of 10.2 and 10 mM salt concentration. Below the transition temperature the correlation functions can be fit to two modes and a two-mode distribution of particle sizes was observed. Above the transition temperature only one population of particles with fairly narrow size distribution was observed.

The experiments were repeated at least three times and the data points are shown to be in very good agreement from one experiment to another. Also, the reversibility of the micelle formation process was confirmed by showing formation of micelles with increase in temperature and disassembly of micelles with decrease of temperature.

The pH dependency of micelle formation process was investigated by measuring the size of the micelles formed as a function of pH at constant salt concentration. A series of 10 μM protein samples with 40 mM salt ranging from pH 9.6 to 11.5 were characterized (Figure 4-2). The results show a gradual decrease in micelle size from pH 9.6 to 10.2, above which a fairly constant size was observed. Based on these results all of the following measurements were done after adjusting the protein solutions between pH 10.2 and 10.4.

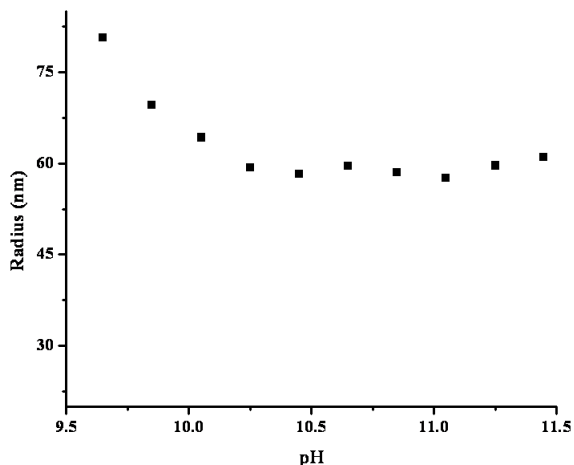


Figure 4-2. Effect of pH on micelle size. The micelles were seen to only exist in pH values above 9.6. A gradual decrease in the size of the particles was observed from this pH value to about 10.2 and then the micelle sizes reach a plateau.

The correlation functions of micelles formed above the transition temperature were carefully studied at different scattering angles to further investigate the size and shape of micelles. The experiments were performed in the range of salt concentrations from 5 to 60 mM. At all salt concentrations at 50°C (above the transition temperature), the normalized correlation functions of (GVGVP)₄₀-foldon solutions measured at scattering angle of 90° cover two to two and half orders of decay, signifying a good quality of the experiments (Figure 4-3). Comparing the normalized correlations functions, it is apparent that the lower salt concentrations (below 20 mM) display faster decays of the correlation functions corresponding to particles of smaller apparent size. These correlation functions are comparable to each other indicating a constant apparent size in this salt regime. The increase of salt concentration to values above 30 mM yields a significant change in shape of correlation function. Higher salt data display much slower decays corresponding to particles with larger apparent sizes.

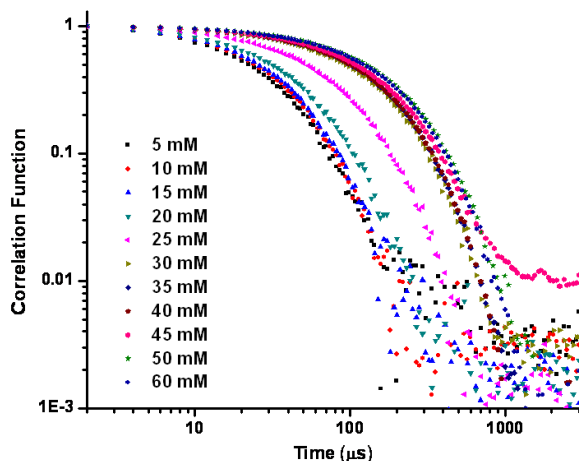


Figure 4-3. Dynamic light scattering correlation functions of 10 μM solution of $(\text{GVGVP})_{40}$ -foldon at different salt concentrations. The two and half orders of magnitude decay of correlation function shows the good quality of the data. In the low salt regime (below 15 mM), all are nearly identical fast decaying correlation functions. A similar behavior is observed for high salt concentration regime (above 30 mM) except for much slower decay rate. In between the two regimes an area of transition is seen.

The estimates of the diffusion coefficients for different salt concentrations were obtained in two different ways (Figure 4-4): from single angle experiments at 90° and from multi-angle measurements, in both cases using either one- or two-mode fits. For the single angle (90°) measurements were analyzed with two modes, and two apparent diffusion coefficients are reported. For the multiple angle measurements, Figure 4-4 reports only the diffusion coefficients of the angular-independent (diffusive) modes.

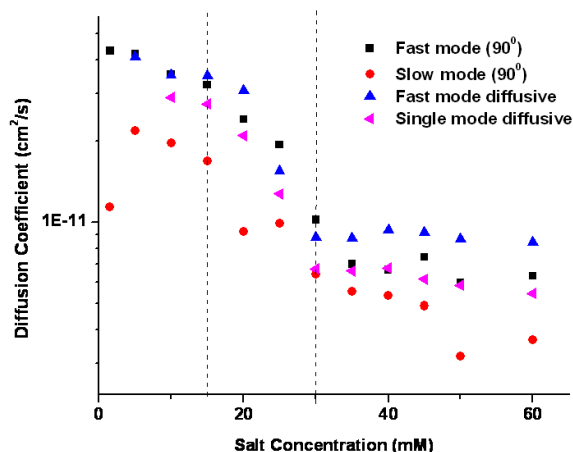


Figure 4-4. Diffusion coefficients as a function of salt concentration for a 10 μM solution of $(\text{GVGVP})_{40^-}$ foldon. The diffusion coefficients show the two salt regimes and a transition region. The first regime with higher diffusion coefficients corresponds to particles with smaller diameters while above 30 mM of salt a clear decrease in the diffusion coefficients is observed. Fitting the correlation functions to a single mode (circles) or using the diffusive mode (square) of a bimodal fit result in very similar trends but some small differences in the diffusion coefficients.

Plotting the diffusion coefficients above the transition temperature versus the salt concentration shows the two distinct regimes where micelles are formed as previously reported^{4,29}, but with improved concentration resolution, a transition region is observed between them (Figure 4-4). The first regime is up to about 15 mM of salt and the second regime is between 30 and 60 mM salt where fairly constant diffusion coefficients are observed. A significant decrease in diffusion coefficients (an increase in the micelle sizes) is observed in the transitional region between the two regimes. The relative agreement between values of D for two modes of 90° measurements and D from angular measurements for low salt indicates relative monodispersity and isotropic nature of scatters. On the other hand at high salt, there is a significant difference not only between

D for the two observed modes of 90° measurement, but also between those and the angular results. This is indicative of either significant polydispersity of the sample or/and anisotropy of the sample.

The calculated diffusion coefficient (D) of each apparent mode of these correlation functions was then used to calculate corresponding hydrodynamic radius of the particles R_h at each salt concentrations using Stokes-Einstein equation,

$$R_h = \frac{kT}{6\pi D\eta} \quad (4-9)$$

where k is Boltzmann's constant, T is temperature, and η is the solvent's viscosity. This relationship assumes a suspension of dilute, spherical, monodisperse particles in a small molecule solvent. Our system is composed of dilute particles in a small molecule solvent. However, one cannot *a priori* assume the monodispersity of the system or the spherical shape of the micellar particles. Therefore, measurements at various scattering angles are needed to ascertain the possibility of using Equation 4-9 in the analysis. If the decay rate of a correlation function scales linearly with the scattering wave vector q with zero intercept, then the particles are considered to be spherical and Equation 4-9 can be used to estimate their hydrodynamic radius.

A representative angular dependence for the first salt regime is presented as a plot of the decay rate as a function of scattering wave vector (Figure 4-5a). The two-mode fit of the correlation functions in this regime yields two linear dependences of Γ vs. q^2 with small intercepts corresponding to apparent radii of ~ 9 nm and ~ 18 nm. The one mode fit also gives a linear Γ vs. q^2 dependence with a small intercept corresponding to an apparent radius of ~ 14 nm. As mentioned before the addition of the second mode to the one-mode fit in analyzing the data does not change the RMS significantly. In addition,

the obtained two mode parameters have similar angular dependence to the one mode fit results yielding the apparent sizes that average to a value similar to a single mode fit radius. This indicates that the observed particles have somewhat broad distribution of sizes but still on average behave as spherical micelles with a radius of ~ 14 nm. These sizes are very well in agreement with our previously reported sizes for this regime^{4,29}. The spherical shape of these particles is also confirmed by depolarized dynamic light scattering in which no significant signal was detected from the particles at low salt concentrations.

For the transitional region the plot of Γ vs. q^2 shows linear dependence of decay rate on scattering angle for the first mode (with a small intercept) and somewhat different behavior for the second mode (Figure 4-5b). In fact, for the second mode, there is some deviation from the linear Γ vs. q^2 dependence at high angles (large q), which is an indication of some micelle anisotropy in this regime. The nonlinearity of the second mode is noticeable but not as strong as at higher salt concentrations

For the second regime, with the single mode fits, the obtained Γ vs. q^2 shows even more non-linearity at high angles (above 100°) (Figure 4-5c). This non-linearity increases with increased in salt concentration. Therefore the data at high salt concentrations had to be analyzed with two modes due to significant bimodality of measured correlation functions. The faster of the two modes is somewhat diffusive (linear Γ vs q^2 with significant non-zero intercept) yielding micelles with apparent hydrodynamic radius of about 45-50 nm. This mode does not change with increasing of salt concentration (Figure 4-4). On the other hand, the slower mode is completely non-diffusive, as seen from a lack

of strong angular dependence of the decay rate (Figure 4-5c) and the values of the stretching exponent ($\beta=0.6-0.85$).

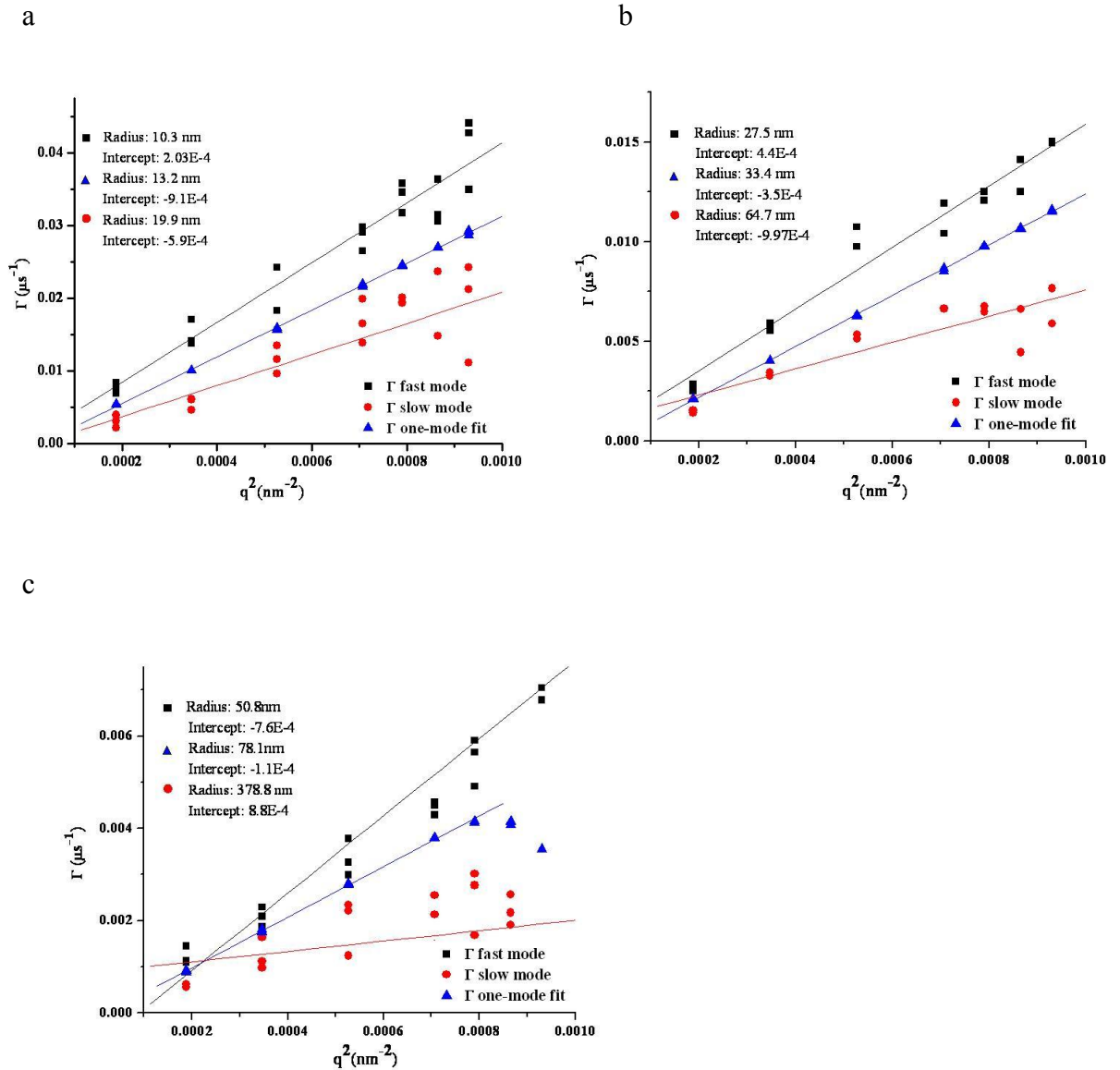


Figure 4-5. Decay rate (Γ) as a function of scattering vector (q^2) from dynamic light scattering at different incident angles. For low salt regime at 10 mM salt concentration (a), the single-mode fit or bimodal fit result in linear dependencies. The two-mode fit results in two ranges of radii which can be averaged to the radius from a single mode fit and based on the RMS values, a single-mode fit seems to be sufficient for this region. For the transition region at 20 mM salt concentration (b), the two-mode fit results in a non-linear

deviation in the slow mode at high angles that might be the effect of some anisotropy in the particles shape. The high salt regime at 40 mM salt concentration (c), shows a non-diffusive slow mode from the two-mode fit which can be an indication of significantly elongated particles.

4-4-2. Static Light Scattering (SLS) Measurements

To further investigate the size and shape and molecular weight of the micelles a series of static light scattering (SLS) measurements were done at different salt concentrations. The average intensity of scattered light (I) was measured at a wide range of angles and compared with the solvent scattering under identical conditions. The angular dependence of the reduced intensity I was analyzed (Figure 4-6), and the radius of gyration, R_g , was obtained from the Berry fits of the data.

The dependence of I^l vs. q^2 above the transition shows very little (if any) angular dependence at low salt (below 20 mM) which indicates that particles present under those conditions are too small ($< \lambda/20$) to diffract light. Both in transitional region and in the second salt concentration regime the angular dependence is strong indicating the presence of large particles.

The radius of gyration of micelles was calculated using the Berry formula (Equation 4-8) due to significant curvature seen in I^l vs. q^2 dependence at higher salts. The obtained values for R_g are listed in the Table 4-1. The salt concentration dependence of R_g is similar to salt concentration dependence of R_h . Both apparent sizes slightly increase with salt concentration up to 15 mM, change fourfold in the transition region (15<C<30mM), continue to slightly increase with concentration in the second concentration regime, and show significant increase in size above 45 mM. The ratio of R_g to R_h was also calculated for different salt concentrations. The ratio R_g/R_h overall increased from 1 at low salts to

1.45 at high salt indicating some elongation of the observed particles. However, this result has to be taken with caution as most of the analyzed DLS data was bimodal yielding two values for R_h while SLS data produced one value of R_g .

Salt(mM)	R_g for 90° angle	R_g/R_h for 90° angle
5	14.5	1.06897
10	15.4	1.11688
15	16.1	1.03727
20	20.6	0.99029
25	36.4	1.31044
30	63.5	0.99213
35	67.2	0.98512
40	67	1.00746
45	72	1.22222
50	83.6	1.26435
60	82	1.42805

Table 4-1. R_g and R_g/R_h at different salt concentrations for (GVGVP)₄₀-foldon in 10 μM solution

Extrapolation to zero scattering angle, measurement of the scattering by the standard (toluene), and the direct measurement of the differential refractive index increment (dn/dc) of the solutions permits the calculation of the molecular weight, M_w , of the micelles (Figure 4-7). M_w reveals a salt concentration dependence reverse that of the salt concentration dependence of D (Figure4-7). Namely, in the first concentration regime, M_w increases slightly from 4,500 to 6,500 kDa with salt concentration increase. In the transitional region, M_w increases to about 70,000 kDa. In the second concentration regime, M_w is relatively constant up to 45 mM of salt.

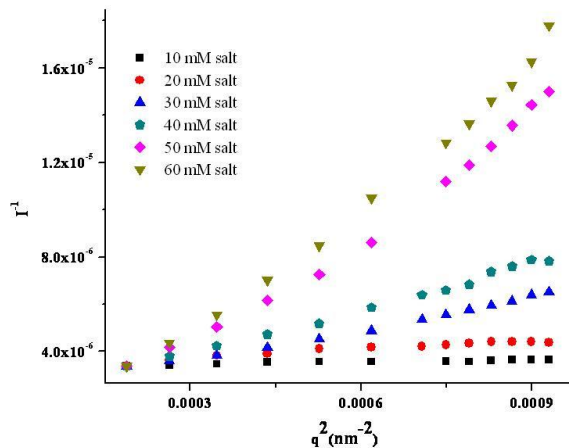


Figure 4-6. Average intensity of scattered light as a function of scattering vector at different angles. At low salt concentrations (10 mM) no angular dependency is observed which indicates the presence of very small particles. At high salt concentration an obvious and somewhat non-linear angular dependence points toward more elongated particles.

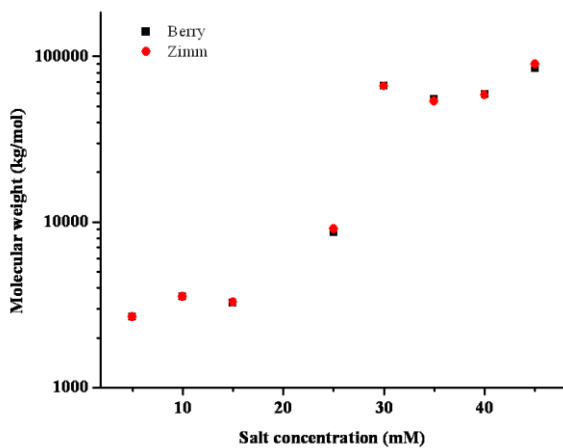


Figure 4-7. Molecular weight as a function of salt concentration. The same two high and low salt concentration regimes and the transition region are observed from both Zimm and Berry plots. In the low salt regime a fairly constant molecular weight distribution around 3000 to 4,000 kDa is measured while above 30 mM of salt a considerable increase in the molecular weight is measured. There is also a jump in the high molecular weight region above the 45 mM salt concentration.

4-4-3. Polarized and Depolarized Dynamic Light Scattering

Polarized (VV) and depolarized (VH) dynamic light scattering experiments were carried out to study the extent of elongation of the micelles in each salt regime under the same conditions that unpolarized dynamic light scattering experiments were carried out. In general, VV showed a much greater intensity than VH measurements with the same optical settings yielding depolarization ratio of 0.01 or lower. This means that the entire unpolarized DLS scattering, which consists of VH and VV components was in essence due to VV scattering. Still VH signal was strong enough for reliably measuring correlation functions (Figure 4-8). The relationship between Γ and q^2 for both VH and VV scattering was studied and showed a linear dependence with a similar slope in both cases. The slopes of VV and VH decay rates and the intercept of VH decay rate were used to calculate transitional and rotational diffusion coefficients of the micelles based on Equations 4-6 and 4-7 (Figure 4-9).

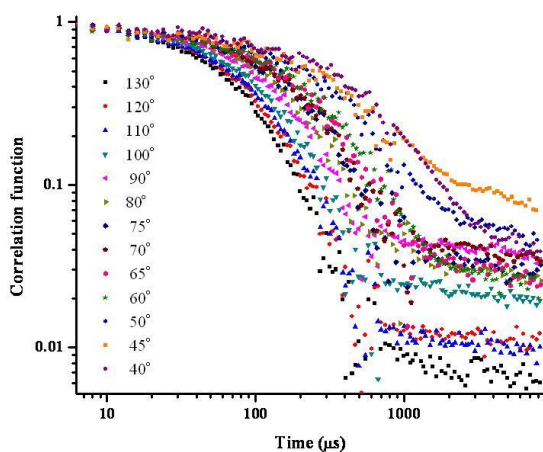


Figure 4-8. Correlation functions from depolarized light scattering at different angles. The two orders of magnitude decay shows good quality of the data while the obvious angular dependence of the correlation functions points towards the elongation of the particles.

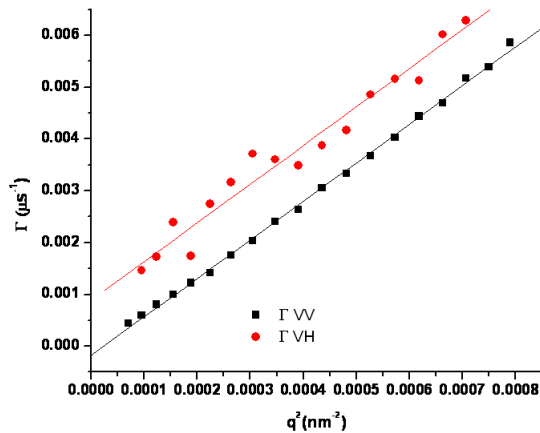


Figure 4-9. Decay rate (Γ) as a function of scattering vector (q^2) from polarized and depolarized dynamic light scattering. The two lines are appeared to be parallel. The polarized light data set (squares) have zero intercept while depolarized light data (circles) has a positive intercept corresponding to the rotational diffusion coefficient.

4-4-4. Viscosity Measurements

Based on the viscosity measurements done of (GVGVP)₄₀-foldon solutions in a range of concentrations from 200 μM to 600 μM at 40°C, the intrinsic viscosity is calculated to be $1.92 \times 10^{-4} \mu\text{M}^{-1}$. (Data from Sumit Kambow Master's thesis, Cleveland State University, 2012)

4-5. Discussion

4-5-1. Effect of pH

Micelles of (GVGVP)₄₀-foldon are observed to form only above pH 9.6. This is above the pK_a of a typical N-terminal amine (about pH 9), and it is presumes that at lower pH values, the positive charges on the ELP tails are sufficient to disrupt micelle formation by

giving a net charge to the ELP core. By neutralizing a higher fraction of the N-termini at higher pH values, the phase separation of the ELP in the core of the micelle becomes energetically favorable.

The mechanism with which pH changes affect the micelle size is not clear but one explanation might be the effect of end charges preventing the three arms from folding together which then results in more extended chains of ELP in the micelle core. It should be noted that the size of the micelle reaches a minimum at about pH 10.2 when the majority of N-terminal amines are deprotonated. At a pH of the pK_a , about 50% of all the N-terminal amines are deprotonated and so there are still considerable charged N-terminal amines but above pH of about 10, we expect most the N-terminal amines to be neutralized.

4-5-2. Micelle Formation and Characterization

Based on our findings from the dynamic and static light scattering in this work and our previously reported data^{4,29}, the micelle formation in this system can be studied in different regimes based on the total salt concentration of the solutions. Here a thorough study of the micelles size and shape in each regime is presented.

4-5-2-1. Regime I. This regime occurs in solutions with up to 15 mM salt, where a fairly constant diffusion coefficient is observed above the transition temperature (Figure 4-4). Below the transition temperature in this salt concentration regime DSL reveals two distinguishable modes. The diffusive mode was found to correspond to particles with hydrodynamic radius (R_h) of about 3 to 5.5 nm. This mode corresponds to (GVGVP)₄₀-foldon trimers in the form of random coils. The second mode observed below the

transition was found to be non-diffusive and corresponded to a much slower process in the system such as relaxation of the unfolded trimers.

The single mode observed for the 10 mM salt above transition (or the average of the two similar diffusive modes) was found to be diffusive (Figure 4-5a) and corresponds to particles with an apparent hydrodynamic radius of 12-15 nm, which corresponds to micelles formed from folded (GVGVP)₄₀-foldon trimers.

To better understand the geometry of the micelles at each regime static and dynamic light scattering measurements should be considered together. In the first concentration regime (up to 15 mM) a slightly increasing molecular weight from about 3000 to 4000 kDa was measured from static light scattering. Although the protein content of these micelles cannot be measured directly, if we consider that the aggregation in the micelles is the same process as the bulk aggregation, there should be about 37% protein in the micelles.^{4,18} That translates to about 20 to 24 trimers in each micelle at the low salt concentration considering that (GVGVP)₄₀-foldon trimer has a molecular weight of about 60.4 kDa. If we consider 3500 kDa as the average molecular weight of the micelles in this salt regime, the density of the spherical micelle of such molecular weight and a 30 nm diameter can be calculated to be 0.42 g/cm³. This value is of course much less than the approximate density of pure ELP which is estimated to be about 1.1 to 1.2 g/cm³ and so there should be a considerable amount of water associated with the micelle that is moving along with the particle and is measured in DLS as a part of the micelle size but is not measured in SLS and so is not included in the molecular weight.

The actual hard core sphere of the micelles and the extent of water shell can be calculated based on the ELP mass in each micelle. The mass of the average number of

trimers, 24 of (GVGVP)₄₀ with monomer mass of 17 kDa is calculated to be 2.08×10^{-18} g. Considering that the ELP specific gravity of about 1.1, the volume of this mass of ELP is about 2290 nm³. Since the core of the micelle should only contain about 37% protein, the total volume would be about 6020 nm³. The radius of a sphere with such volume is then calculated to be 11.3 nm. This is the core of the micelle which consists of aggregated ELP chains only and the foldon head groups should be added to this sphere to calculate the actual size of the micelles. From protein data base, foldon in its folded state is a globular protein which has about 3.1 nm in diameter and so the radius of the micelle should be about 14.4 nm. This is very close to 15 nm from DLS measurements and the difference can be contributed to the outside water shell on the surface of the micelles. We believe this water shell is closely organized around the micelles outer surface and is behaving differently from the bulk water in that it can move along with the particles so it is measured in correlation functions of DLS measurements yet the nature of this water is not tightly associated to the protein like the water trapped inside the micelle, and so it cannot be seen from SLS measurements (Figure 4-10).

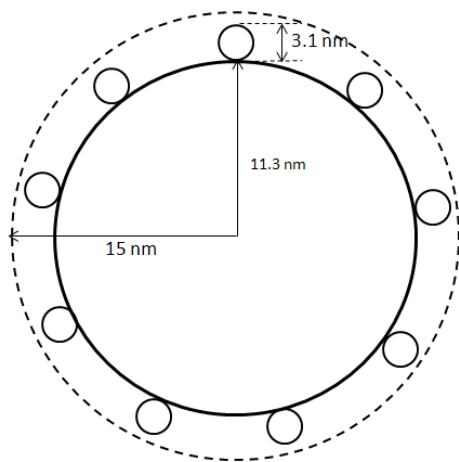


Figure 4-10. Schematic of the spherical micelles at low salt regime. The coacervate core interior hydrophobic sphere of the micelle which consists of folded ELP trimers is calculated to be close to 11.3 nm in radius. Foldon head group is about 3.1 nm in diameter which gives a total of about 14.4 nm in radius to the micelle. There is a small water shell surrounding the micelle that makes it to appear slightly larger in dynamic light scattering.

Viscosity measurement data was used to independently verify molecular weight and size of the micelles in the first salt regime. The intrinsic viscosity of (GVGVP)₄₀-foldon at 40°C was measured to be $1.92 \times 10^{-4} \mu\text{M}^{-1}$. Using this value and Einstein equation,

$$\rho = \frac{2.5M_w}{[\eta]} \quad (4-10)$$

the density of the micelles is calculated to be 0.262 g/cm^3 with $\pm 10\%$ error. This density, together with the volume of a spherical micelle with 30 nm in diameter, results in $3.71 \times 10^{-18} \text{ g}$, as the total protein mass (ELP, foldon, and water) in each micelle. This mass, translates to 73 ELP molecules or about 24 trimers in each micelle. This number is essentially the same as what was calculated based on light scattering data analysis and confirms the micelle core size.

To further study the consistency of the results from different measurements in our experiments including molecular weight from SLS, hydrodynamic radius from DLS, and density of micelles from viscosity measurements, each of these values can be calculated using the other two measured values. Comparison between these values shows fairly good agreement between calculated and measured values (Table 4-2)

	experimental	calculated
M_w (g/mole)	3482-3603	1707-4182
R_h (nm)	12-15	12.41-13.56
ρ (g/cm³)	0.236-0.296	0.409-0.827

Table4-2. Comparison of measured and calculated values of M_w, R_h, ρ at low salt regime.

This spherical shape at low salt is also consistent with the molecular geometry based on theory that ELP assume a more ordered structure of β-spirals above their transition temperature.^{4.19} The β-spiral of the (GVGVP)₄₀ polypeptides, which acts as the hydrophobic tail of the construct is considered to have the maximum length of about 15 nm. Since the maximum radius of a spherical micelle cannot exceed the length of the hydrophobic tail, micelles of up to about 15 nm in radius are expected. This is consistent with the size that is observed in this regime and we speculate that these folded trimers are the unimers of these spherical micelles at these low salt concentrations. It is noticeable that the micelle core size is observed to be somehow smaller than the expected length of 15 nm and we speculate that the actual folded construct of ELPs in the micelles are in a more compact form than what was calculated based on the molecular model.

4-5-2-2. Transitional region. The transition region starts around 15 mM salt, where the micelles begin to gradually increase in size, and continues to about 30 mM salt when the size plateaus (Figures 4-3 and 4-4). The decay rates of correlation functions in this regime are slower than in the first regime and the correlation functions can be fit to two distinct modes. This region was not observed previously using single angle particle size analyzer and above 15 mM salt concentration a jump in the micelle size was reported.^{4.29} The depolarized dynamic light scattering measurements for samples in this region did not

result in meaningful and reproducible data which might be an indication of unstable and transitional particles that are constantly changing in size and shape. SLS experiments in this region shows significant linear angular dependence indicating that scattering species are big enough to diffract light and that, on average, there is no significant anisotropy in the system. In this region, the particles get larger and the molecular weight increases up to about 70,000 kDa which is the start of the second regime (Figure 4-7).

We think inconsistencies between the new results and our previous study can be explained by the fact that our previous data was based on single angle measurements and the results were compiled and analyzed by the instrument that had no ability to assess possible non-spherical nature of the particles. Here we have expanded our experiments to multi angle light scattering done with more powerful laser and better optics that allowed us to actually probe the entire breadth of particulates in tested solutions, their apparent size and their shape.

4-5-2-3. Regime II. This regime starts from about 30 mM salt concentration and very similar decay rate of correlation functions from one salt concentration to another is observed (Figure 4-3). But these decay rates are much slower in comparison to regime I. The slower decays for the correlation functions correspond to bigger particles. This is in general agreement with our previous findings but more accurate results are obtained by fitting the correlation functions to a two-mode model. Based on the two-mode fit analysis, the slow mode is consistently present in our analysis in this concentration regime and constitutes about 50-60% of correlation function. The presence of this mode either indicates significant anisotropy of micelles or is a probe of other slower processes in a sample such as diffusion of random coils. In the case of this salt regime the two

modes should not be averaged as they exhibit a very different nature as apparent from different angular dependences. The anisotropy of the micelles in this regime can be seen from significant and somewhat non-linear angular dependence of I^l which is an indication of bigger and possibly elongated particles (Figure 4-6).

The huge increase in the molecular weight in this regime is in agreement with observations of particle becoming anisotropic in the high salt regime. In the regime of highest salt concentrations the apparent molecular weight was found to increase somewhat with salt concentration but an average of about 70,000 to 80,000 kDa can be observed.

We believe that at higher salts the head groups of the trimers, which consist of negatively charged foldon domain, become smaller. The shape of the micelles is directly affected by the packing factor

$$V/a_0l, \quad (4-11)$$

Where V is the volume and l is the length of the hydrophobic tail and a_0 is the surface area of the head group. So the smaller head group means a larger packing factor, and when it becomes larger than $1/3$, the micelles begin to become non-spherical.^{4,12} Consequently in our system higher salt concentrations push the system towards non-spherical particles that can accommodate many more of the trimeric monomers leading to the much higher molecular weight. These conclusions are in agreement with our SLS and DLS data and especially polarized DLS data in which non-zero VH signal indicates that the micelles in the second regime are elongated and anisotropic. It is possible that these particles have distribution of different lengths of elongated micelles and consequently a

distribution of different molecular weights because one can expect very little energy barrier between lengths of elongated particles.

There is also a jump in the size deduced from the second mode in this regime around 45 mM of salt, which is consistent with where the start of the third regime was reported previously.^{4,29} This might be attributed to the fact that at these higher salt concentrations the micelle head group is very small and bigger aggregates start to grow.

4-5-3. Study of the Particles Elongation

The polarized and depolarized dynamic light scattering at different salt concentrations provided us the best tool to study the shape of the micelles under these conditions and the non-zero VH signal in the second salt regime proved the existence of anisotropic particles in this regime. The correlation function from VH measurements showed some angular dependency (Figure 4-8) and the Γ vs. q^2 plot of VV and VH resulted in linear dependency for both of the measurements, while VH measurements resulted in an intercept and the two lines are observed to be parallel (Figure 4-9). This is in agreement with what is expected from equations 4-8 and 4-9, in which the slope of this linear dependence should be identical and can be used to calculate the rotational diffusion coefficient. The intercept from VH measurement is related to the translational diffusion coefficient. Based on the data from this plot, we fit the data to cylindrical models to calculate the elongation of the particles in each salt concentration. In this system we considered a cylinder with radius r which is equivalent to the maximum radius of the spherical micelles and length of L and calculated D_T and θ_R using the Broersma equations:

$$D_T = \frac{K_B T}{3\pi\eta L} \left[\delta - \frac{1}{2} (\gamma_{\parallel} + \gamma_{\perp}) \right] \quad (4-12)$$

$$\theta_R = \frac{3K_B T}{\pi\eta L^3} (\delta - \xi) \quad (4-13)$$

In which

$$\delta = \ln \frac{2L}{d}, \quad (4-14)$$

$$\gamma_{\parallel} = 0.807 + \frac{0.15}{\delta} + \frac{13.5}{\delta^2} - \frac{37}{\delta^3} + \frac{22}{\delta^4} \quad (4-15)$$

And

$$\gamma_{\perp} = -0.193 + \frac{0.15}{\delta} + \frac{8.1}{\delta^2} - \frac{18}{\delta^3} + \frac{9}{\delta^4} \quad (4-16)$$

$$\xi = 1.14 + \frac{0.2}{\delta} + \frac{16}{\delta^2} - \frac{63}{\delta^3} + \frac{62}{\delta^4} \quad (4-17)$$

where k is the Boltzmann constant, T is the temperature and λ is the viscosity.

By fitting the data from our DLS and polarized and depolarized light scattering experiments to this model the extent of elongation of the particles in each regime was calculated (Figure 4-11).

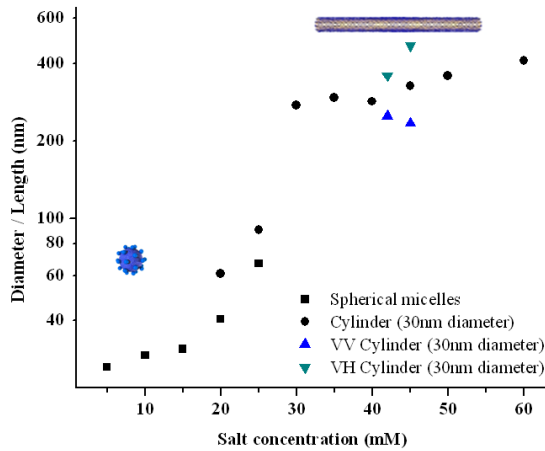


Figure 11. The extent of elongation of particles as a function of salt concentration from dynamic light scattering and polarized and depolarized dynamic light scattering. The data from dynamic light scattering was fit to ellipsoidal model and up to about 20 mM the particles are consistently spherical (squares). The result of this fit for data above 20 mM results in particles with a much more elongated nature (spheres). The polarized and depolarized light scattering data in the second regime results in elongated ellipsoidal particles as well (triangles) which can be as long as 800 nm to 1000nm in length.

Based on this model, the two regimes and the transition region can still be identified. The micelles in the first regime are shown to be spheres of about 30 nm in diameter which is again consistent with all other measurements and analysis so far and give us the final piece of data as a proof of the existence of spherical micelles in low salt concentrations. But interestingly the micelles at the second regime which starts around 30 mM salt concentration show very large elongation up to about 300 to 350 nm. This can explain the very different behavior of these particles from DLS and SLS measurements. We also used an ellipsoidal model to fit the data and quite similar results were obtained which is expected considering the large length to diameter ratio of the micelles which makes the ellipsoidal model very close to the cylindrical one.

Considering the elongated particles at higher salt concentrations with a diameter around 30 nm and lengths of 300 to 350 nm, the number of trimers that are housed in these micelles is calculated to be about 1000 to 1200. From SLS measurements molecular weights of micelles in this regime are calculated to be around 70,000 to 80,000 kDa and considering about 60 kDa as the molecular weight of each trimer, the number of trimers is calculated to be about 500 to 600 in each micelle. The difference between the two calculations can be attributed to the fact that the DLS measurements result in Z-averaged

values which means a higher than actual lengths for the elongation particles might be observed.

4-5-4. Crosslinking of Micelles and Responsive Particle Formation

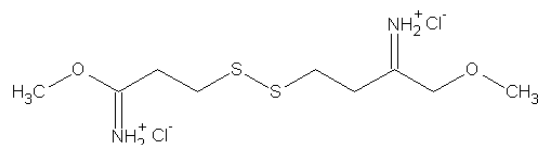
Up to this point we studied the micelle formation of ELP-foldon constructs at low salt and high pH conditions. Although responsive micelles have been used in different applications as discussed in the first chapter of this manuscript, it is always an advantage to have environmentally responsive particles which are stable in any salt or pH conditions so that they can be utilized especially in physiological conditions. For that reason many efforts have been made in crosslinking micelles into particles using different techniques such as UV irradiation^{4.33}, chemical reaction with bifunctional crosslinking agents such as carbodiimide coupling^{4.34}, 1,2-bis (2'-iodoethoxyl) ethane (BIEE)^{4.35}, Glutaraldehyde^{4.36} activated esters^{4.37}, disulfide-based crosslinkers^{4.38}, and many other techniques.^{4.39}

In our system, since the micelles are formed in non-physiological conditions, it would be necessary to crosslink them especially if they are to be potentially considered as drug delivery or theronostic particles.

The first attempt to crosslink these micelles was done by our collaborators in Technical University of Munich by using radioactive source and irradiation of a 10 μ M sample of (GVGVP)₄₀-foldon in 10 mM PBS solution for an hour. The results were mixed and although some potentially crosslinked particles were observed by atomic force measurement (AFM) imaging, there was not enough evidence to convince us that the

particles are still responsive and they can go through a change in their size by passing the transition temperature (data not shown).

The next approach was to do chemical crosslinking of the micelles by using the available amines on the N-terminal of the ELP constructs. Dimethyl 3,3-dithiobispropionimidate (DTBP) is a homobifunctional imidoester that contains imidoester group at both ends and can react to the amine groups in the pH range of about 7 to 10 and produces amidine linkages between them.^{4,40}



The crosslinking was done by reacting 10 μ M solution of (GVGVP)₄₀-foldon (3 mM PBS and pH 9.5) for 2 hours at 55°C using 300 μ M DTBP. The reaction was quenched by adding 50 mM Tris.HCl. The solution was then dialyzed against 3 mM PBS to remove reactants and reduce the pH. The resultant solution was dried on a mica surface covered with ZnCl and was then imaged at room temperature by AFM (tapping mode) at room temperature. The results show the existence of the particles with diameter around 30 to 40 nm which is in agreement with the expected size of the micelles in this salt concentration (Figure 4-12). This experiment was a proof of concept that we were able to crosslink the micelles into particles which are stable at room temperature.

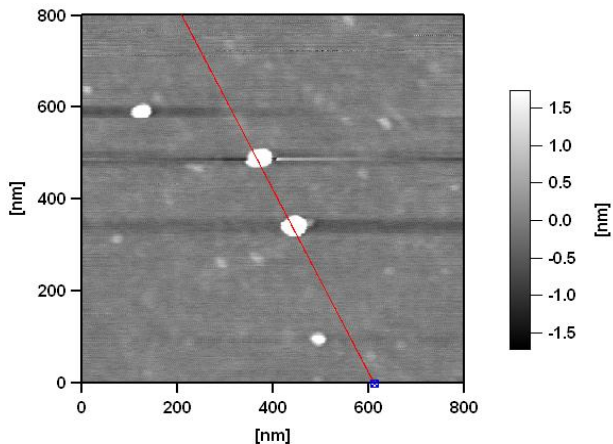


Figure 4-12. AFM image of crosslinked particles at room temperature. The micelles were made from a 10 μM solution of (GVGV P) $_4$ -foldon at 10 mM salt concentration and pH of above 10. The micelles were then dried on a mica surface and AFM images were obtained in tapping mode. The size of the micelles seem to be consistent with the measured size from light scattering.

Glutaraldehyde (GTA) is the most commonly used homobifunctional crosslinker and the reaction of this molecule with amines proceed through the formation of Schiff bases while subsequent reactions with some reducers results in stable secondary amines.^{4.40} Although GTA can be polymerized in either acidic or basic conditions, it has been shown that the polymerization and subsequently crosslinking is usually most effective in higher pH values. That in fact makes it a good candidate for our system in which micelles are formed only in alkaline conditions.

With all the three different GTA concentrations reasonable size particles were observed by single angle dynamic light scattering although the best results were obtained using higher concentrations of GTA. The particles were shown to be stable at room temperature

and had a mean diameter of about 60 to 65 nm. When heated, the size of the particles was observed to become smaller with mean diameter of around 45 to 50 nm in diameter (Figure 4-13) with narrower size distribution. These crosslinked particles were salt and pH independent and unlike their micelle counterparts they kept all of their characteristics when the solution conditions were changed to physiological conditions (PBS).

In addition to temperature responsive particles, other constructs with pH sensitivity were constructed and crosslinked. One such construct was ((GVGVG)₆(GHGVP))₄-foldon. In this construct four of the Valine residues are substituted with Histidines to give the construct pH responsiveness. The crosslinked particles from this construct were shown to shrink and expand by changing the pH below and above the pK_a of Histidine.

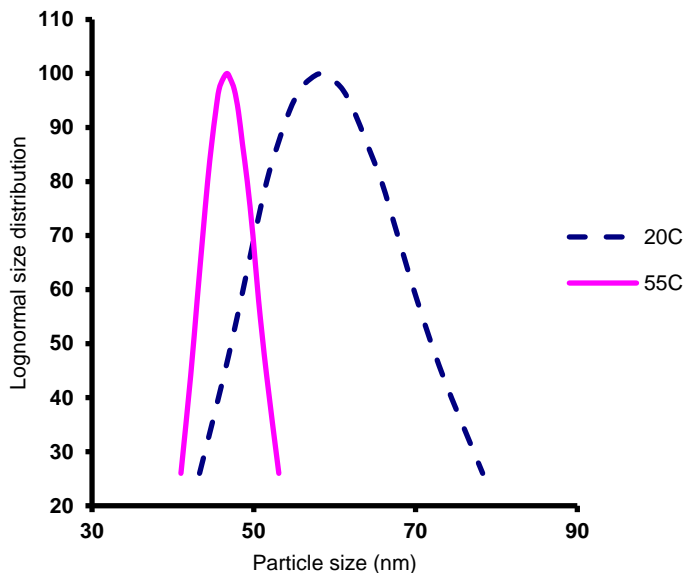


Figure 4-13. Particle size distribution of crosslinked (GVGVG)₄₀-foldon micelles above and below the transition temperature. The particles are generally swelled at temperatures below the transition temperature but by going through the transition a shift in the size distribution of particles is observed and the mean

diameter of particles decreases from around 65 nm to below 50 nm which is close to 50% change in the volume.

4-6. Conclusions

In this work, which is a complementary study to our previously reported data, the geometry and size of thermoresponsive micelles made from three-armed star elastin-like polypeptides were investigated as a function of temperature, pH, and salt concentration. The studies were mainly based on using different light scattering techniques and were also verified by viscosity measurements. The temperature was shown to effectively drive the reversible micelle formation above the transition temperature of ELP. The solution pH was shown not only to be essential in micelle formation, with the micelles only forming at pH values above 9.6, but also has a measurable effect on the size of the particles, which reach to a constant, minimum size above pH 10.2. Increasing the salt concentration affected the size and shape of the micelles, which exhibited different behaviors in a low salt regime (below 15 mM) and a high salt regime (above 30 mM) with a transitional region between them. The micelles in the low salt regime are spherical and consist of about 24 unimers while the micelles in the high salt regime are elongated cylindrical particles with high molecular weights. The intrinsic viscosity was measured at temperatures above the transition temperature to independently verify total mass and volume of the micelles. We also were successful in crosslinking the spherical micelles into responsive nano-particles which are responsive in the physiological conditions and stable for a long time at room temperature.

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Chapter V

Mixtures of Elastin-like Polypeptides with Different Architectures

(Part of this chapter was modified from publication with N.B. Holland, Biomacromolecules 2011, 12, 4022-4029)

5-1. Introduction

We have shown previously that ELP systems can be designed such that they self-assemble into micellar aggregates above the transition temperature,^{5.1} but the size of these micelles could only be controlled at certain plateaus. Although these micellar particles have great potentials for further applications, a more defined control over their size such that micelles with any size would be achievable is very desirable. Mixtures of elastin-like polypeptides of different lengths and chemical identities with other polypeptides and with each other have been studied especially for protein purification purposes,^{5.2} or for making micro-scale particles with different properties.^{5.3} It has been reported that mixing solutions of ELP molecules with different lengths and molecular weights, but the same amino acid sequence repeats, results in a single population of aggregates and a single transition temperature.^{5.3} Additionally, it has been shown that a single transition would take place for mixtures of ELP and ELP fusion proteins when the ELP component of each has the same molecular weight and sequence.^{5.4} Therefore, if different architectures

of the ELP molecules (e.g. making trimer constructs) behave similar to the linear molecules, i.e. the foldon is just acting as a fusion protein for these new construct and not affecting the folding and aggregation of ELP molecules, a single transition temperature for their mixtures would be expected. In addition, we hypothesize that, for a mixture of linear and non-linear ELP molecules in conditions similar to that of the micelle formation, the aggregates of the linear ELPs can be stabilized by ELP-foldon trimers forming micelles with different sizes and that by adjusting the ratio of linear to trimer constructs virtually any size of the micelles could be achievable. This would give us higher flexibility in controlling the size and shape of these micelles and we would be able to tailor the ELP molecules and force them to make micelles with a specific size and shape. To test these hypotheses, we performed a series of experiments with linear and trimer mixtures at different ratios and concentrations and characterized them using UV-vis spectrometer and particle size analyzer.

5-2. Materials and Methods

(GVGVP)₄₀ and (GVGVP)₄₀-foldon were expressed and purified as described previously.^{5.1, 5.5} The mixtures were made either in PBS or by diluting the samples in pure water and then adjusting the salt to the desired concentration. The UV absorbance measurements were performed at 350 nm on a Shimadzu 1800 spectrophotometer equipped with a temperature-controller sample holder unit. The spectra were obtained with a temperature ramp of 1°C collecting data at 0.1°C steps.

Particle size measurements were performed using a 90 Plus particle size analyzer (Brookhaven Instruments) equipped with temperature control unit at 90° fixed angle. All the samples were filtered prior to light scattering measurements (Millex 0.22 µm,

Millipore). The measurements were made in 2 to 5 minutes runs and were repeated at least twice. Analysis of the data was done by the instrument using BIC software (Brookhaven Instruments) and the results are reported as the mean diameter of multimodal distribution.

5-3. Results and Discussion

To test the differences between transition of pure ELP solutions and mixtures of ELP constructs with different architecture, mixtures of (GVGVP)₄₀ and (GVGVP)₄₀-foldon in a ratio of 20 to 1 were examined over a wide range of dilutions. The UV absorbance of these mixtures exhibit two distinct behaviors depending on the total concentrations (Figure 5-1a). At high concentrations, a single transition, similar to the pure species, is observed. However, at lower concentrations two distinct transitions are observed. The temperature of the first transition is very similar to the transition temperature corresponding to the concentration of the pure (GVGVP)₄₀-foldon (Figure 5-1b). This is interesting considering the fact that the presence of linear molecules at much greater concentration, does not affect the trimer T_c . The trimers presumably aggregate independently at first, however, the linear molecules may to some extent become incorporated prior to the second transition since the turbidity is greater there than for the pure trimer solution. This dual transition phenomenon is only observed at lower concentrations while at higher concentrations a single transition is observed at temperatures below both the pure (GVGVP)₄₀ and (GVGVP)₄₀-foldon solutions (Figure 5-1c).

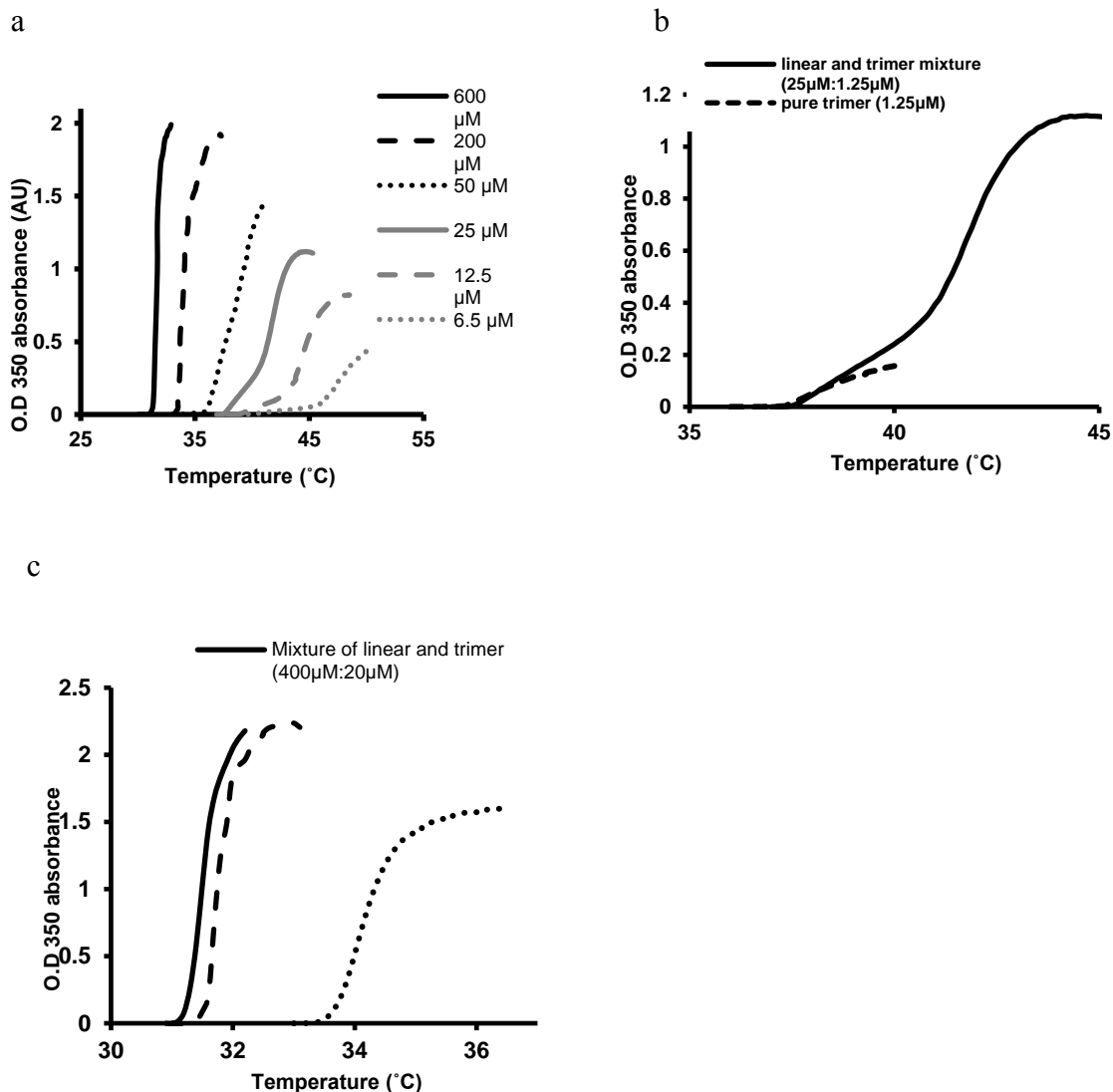


Figure 5-1. Turbidity measurements for mixtures of (GVGVP)₄₀ and (GVGVP)₄₀-foldon at constant ratio of 20:1. a) UV absorbance at 350 nm was measured for each solution mixture, labeled by concentration of the (GVGVP)₄₀ component. At high concentrations a single transition is observed, while at lower concentrations two transitions are observed. b) The mixture of linear (GVGVP)₄₀ at 25 μM and the trimer (GVGVP)₄₀-foldon at 1.25 μM concentrations (20:1 ratio) results in two transition temperatures, where the first transition occurs at the same temperature as the pure 25 μM ELP trimer solution. c) For the UV absorbance of the mixture of (GVGVP)₄₀ and (GVGVP)₄₀-foldon at higher concentration (400 μM and 20 μM, respectively) only a single transition was observed, which is at a lower temperature than the pure solution of either component.

The transition temperatures of the mixtures have been plotted together with the corresponding transition temperatures for the pure components (Figure 5-2). The transition temperatures of the pure components cross each other because of the difference in their concentration dependence. This clearly illustrates that the transition temperatures of the mixture at high concentrations are lower than those of either of the pure components.

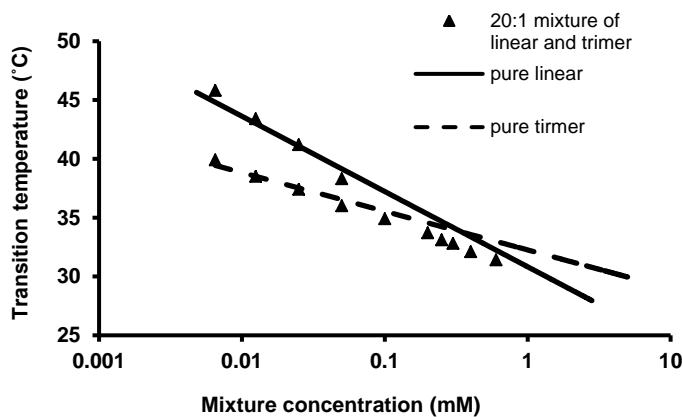


Figure 5-2. The comparison between the transition temperatures of pure (GVGVP)₄₀ and (GVGVP)₄₀-foldon and their 20:1 mixtures as a function of mixture concentration. The mixture at high concentrations shows a single transition temperature that is lower than the transition temperature of pure linear ELP. In this region pure linear ELP (solid line) has a lower transition temperature than that of the trimer (dashed line). But at lower concentrations, where the pure ELP-foldon has a lower transition temperature, two transition temperatures are observed. The first one is the same as the pure ELP-foldon transition temperature and the second one approaches that of the linear ELP.

At lower concentrations, where the mixture exhibits two transition temperatures, the lower transition temperature follows the transition temperature of a pure (GVGVP)₄₀-foldon construct while the second transition temperatures approaches that of the pure (GVGVP)₄₀. It appears that if the concentrations of the linear and trimer constructs are

such that they have a minimum of 2°C difference between the transition temperatures of their pure solutions, two transition temperatures are observed in the mixture.

The dual transition behavior of ELP and ELP-foldon can be explained by the difference in the nature of the folding and aggregation of the two constructs, which is contributed to the difference in their architecture. For either of these constructs the folding of individual chains would proceed by the maturation step^{5,6} in which a few of the folded chains come together and to stabilize the more hydrophobic conformation. The concentration dependence arises because this step needs a sufficiently high concentration of folded polypeptide chains to proceed. The architecture of ELP-foldon may modify this maturation process. When temperature rises, the three ELP chains that are connected by the foldon domain, will associate with each other first because of their local proximity, enhancing the maturation process. Consequently in a mixture of linear and trimer constructs, the maturation of trimer constructs occur at some lower temperature in comparison to the linear ones which leads to the independent aggregation of the trimers. In the case when linear constructs have a lower transition temperature than the pure trimer in the mixture, there is no preference for the folded linear polypeptide to aggregate with itself compared to the trimer, so both linear and trimer molecules aggregate together at a temperature below the transition temperature of the pure linear ELP. These data suggest that folding and aggregation occur differently for the trimer constructs than for the linear constructs. Further experimentation will be necessary to fully elucidate the differences.

The micelles that form from ELP-foldon constructs grow in size by transitioning from spherical to nonshperical shapes, as was discussed in detail in Chapter 4 of this

dissertation. At low salt concentrations (below 15 mM NaCl) there is a fairly constant size distribution of particles in the solution, attributed to the length of the folded ELP portion of the construct. Although this is an important finding that can be used in preparing small responsive particles, it might be interesting to have a system in which the size of the particles can be changed to any desirable size without the need of changing the structure of the molecules (i.e. changing the molecules to longer or shorter ELPs). In fact the reason for having a range of salt concentrations in which a constant size distribution of particles is achieved can be contributed to the fact that the particles in the system do not attain different sizes unless a change in geometry occurs (based on their packing factor) and as we have shown in previous chapter, this geometrical change can push the particles to go from very small particles to large micelles with different geometries and much higher molecular weight. We have shown that this transition from one regime to another happens in a fairly narrow transition region and the particle sizes show some kind of a plateau at each salt concentration

To overcome this limitation, we modified the system by making mixture of linear and trimer ELP molecules with different linear to trimer ratios with the intent that the interior of the micelles can be filled with more ELP molecules and gradually expand to any size for a given salt concentration and instead of transitioning to other geometries keep their spherical shape. The mixtures were made based on the fact that ELP-foldon and ELP molecules can either go through the transition independently or simultaneously depending on the mixing ratios as discussed in this chapter.

Based on the results from Figure 5-2 the experiments can be done in two different regimes. If the mixture ratio is below the crossing point of the pure linear and trimer

transition temperatures, we expect to have two transitions in which the trimers form the micelles prior to the aggregation of linear constructs which means we should expect two transition temperatures and consequently two populations of particles. This was studied by a mixture of (GVGVP)₄₀ and (GVGVP)₄₀-foldon with 20:1 ratio in which the trimer had much lower concentration than the linear construct. The results from particle size analyzer confirm the existence of two different sizes based on the temperature at which the measurements were done (Figure 5-3). The first transition was shown to be above the transition temperature of the trimer construct and the second transition just above that of the linear. But interestingly there was not two populations of particles above the second transition temperature and the size of the particles were smaller than what is expected from aggregation of the linear ELP molecules which is in the range of 500 to 800 nm.

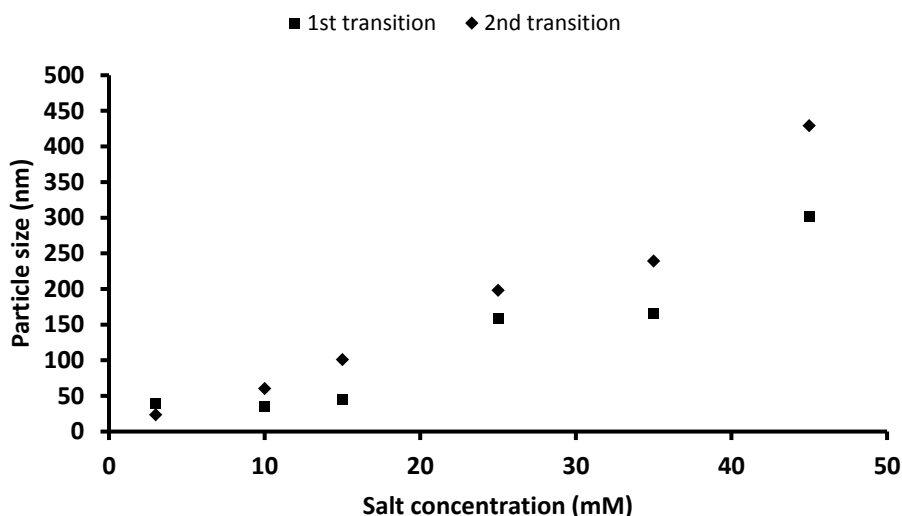


Figure 5-3. Micelle formation at different salt concentration for a mixture of (GVGVP)₄₀ and (GVGVP)₄₀-foldon at a ratio of 20:1 at total concentration of 10 μM. The two transitions show a two-step particle formation in which the trimer first goes into particles but above the second transition temperature a single population of particles with sizes well below the expected aggregates of linear ELP is observed.

The smaller size of the aggregates can be attributed to the stabilization of aggregates during the growth period of the particles by ELP-foldon construct. The fact that only a single population of particles are observed in the mixture at any temperature also can indicate that in fact the linear ELPs start to separate into the already formed micelles of ELP-foldon. This makes sense especially for higher salt concentrations in which the ELP-foldon micelles are less stable in their spherical form and start to transition to elongated micelles. But the presence of linear ELP molecules above their transition temperature which can be housed in the core of these micelles would provide enough core volume to maintain their spherical geometry and remain relatively small.

5-4. Conclusion

The effect of architecture on the behavior of ELP transition was studied by using mixtures of ELP and ELP-foldon constructs. It was shown that the existence of the trimer changes the aggregation behavior of ELP molecules such that these molecules can in fact aggregate independent of the linear constructs in certain concentrations. The conditions in which this independent aggregation can happen were investigated by UV-vis spectroscopy.

The mixtures of linear and trimer ELPs were also studied with the aim of controlling the size of the ELP-based micelles in a practical way. It was shown that the mixtures can be made such that they go through two transitions but in each transition only a single population of particles was observed. The first transition is attributed to the trimer transition with particles of small sizes as it was expected from a pure trimer solution but

the second transition is considered to be the aggregation of linear ELPs into the ELP-foldon micelles or decoration of ELP aggregates by ELP foldon constructs.

Although this study on its own is not yet conclusive enough to give us a complete map of different sizes of ELP-based particles at any given mixing ratio, it demonstrates that the very good control over the size and shape of these ELP-based micelles can be achieved by this method. Further investigation of micelle formation at different salt concentration and mixing ratios are necessary to complete this study.

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Chapter VI

Study of ELP Folding and Aggregation Using Different Molecular Architectures

6-1. Introduction

Understanding how molecules assemble into supramolecular architectures requires careful consideration of the inter- and intra-molecular interactions which control the molecular associations. This is also true for the self-assembly of elastin-like polypeptides and their derivatives but as discussed in the first chapter of this dissertation, the theory of aggregation of elastin-like polypeptides is still debated and there are different points of view as to how these molecules assemble and come out of solution above their transition temperature. Understanding the aggregation process of these molecules and ultimately controlling this process at the molecular level would give us insight into how to design them for a desired measurable response.

There are at least four different suggested pathways for the molecular transition of ELP constructs from below to above the transition temperature and each of them are supported by a number of experimental or theoretical studies. Here an overview of these theories is presented and later based on our experimental data, our theory of folding and aggregation of ELPs will be discussed.

Pathway of no order to homogeneous order through folding and association

This is the most discussed and the most widely accepted pathway suggested by Urry three decades ago as discussed in the first chapter.^{6.1} Based on this theory, which is supported by a number of circular dichroism, NMR, and dielectric relaxation studies, mostly below the transition temperature, the ELP chains are in random coil conformation when they are well below the transition temperature and by increasing the temperature they start to go into more internally ordered structures consisting of β -turns.^{6.2, 6.3} A few of these molecules then come together and make a “matured” intermediate construct which has some exposed hydrophobic residue.^{6.4} These intermediate molecular units then come together to form more stable and energetically favorable aggregates which is the macroscopic outcome of the transition process. In this theory there are in fact three different stages involved in the process of aggregation including a conformational change, an association step and finally the aggregation.^{6.4} The first step is shown to be gradual but the next two are supposed to be happening at the same time when the solution gets to the transition temperature especially because the result of the maturation process is small aggregates of a few molecules (perhaps a triple helix^{6.1}) with exposed hydrophobic surface.

Pathway of no order to homogeneous order without well-defined chain associations

The difference between this theory and the last one is that here no association step is proposed and although the constructs go through some internal ordering, they aggregate out of solution with no intermediate folded unit.

Based on molecular simulation of (VPGVG)₃, Krukau et al. suggested the existence of some structured elements on the peptide chains close to and even above the transition

temperature but could not conclude the existence of specific folding pattern.^{6.5} Li et al. molecular simulations showed that above T_t the ELP monomer can be described as a compact and amorphous structure which contains disordered β strands. They suggested some ordering in each of the chains but a random aggregation overall.^{6.6}

Based on this theory the aggregation is a continuous process which starts at lower temperature by some internal ordering of the chains and finishes at the transition temperature by random aggregation of these chains.

Pathway of no order to heterogeneous order

Ohgo et al. suggested a simultaneous presence of different conformations at the transition temperature of ELP constructs.^{6.7} These conformations include type II β -turns, type I β -turns and unordered structure which makes the ELP a structurally heterogeneous biopolymer. Using TEM they also observed twisted-rope aggregates which might be in agreement to the Urry's theory.

Kumashiro et al. did NMR studies on the ELP constructs and concluded that the best describing scenario for these polypeptides is a "conformational ensemble" which includes a number of different conformations including type II β -turn and type I β -turn structures at the same time.^{6.8} This heterogeneity in conformation was also reported by Ahmed et al. using CD, UV and Raman spectroscopy for cyclic polypeptides in which populations of unordered, type II β -turns and type III β -turns were reported along with noticeable increase in type II β -turns at temperatures above the transition temperature.^{6.9}

Based on this theory increasing the temperature would increase the ordering of the chains but there are different conformations that the chains fold into and so a heterogeneous ensemble of conformations is expected. This theory would not necessarily

be in contradiction to Urry's twisted filament theory but it suggests no clear pathway to folding and aggregation of the chains.

Pathway of no order to no order

Based on this theory, regardless of the chemical identity and length of ELP constructs they are always unordered and there is no folding, ordering or intermediate state between the chains at low temperature and the ones or above the transition temperature. This theory is almost solely backed by molecular simulations and not much experimental results.^{6.10, 6.11}

Comparing these four different pathways, it is clear that in all of them except for the last one some kind of chain ordering or folding is expected when the temperature of the solution changes from below up to the transition temperature. But these theories are very different when it comes to predicting the intermediate stages between random chains and ordered aggregated ones.

Developing a new method to study the transition pathway

In this study we probed the existence and stability of the intermediate steps by making constructs that should increase the stability of a folded stage while still far from aggregation. This can give us a tool to understand the aggregation process and the theory behind it. If Urry's theory of folding and aggregation is valid, it should be possible to design ELP constructs such that the matured constructs would stay stable at temperatures below the transition temperature of ELP. To examine this we expanded our investigation of new architectures of ELP molecules by capping N-, C- or both termini of the trimer constructs and also designed molecules such that the exposed surface of them after folding according to the Urry model would consist of hydrophilic residues, stabilizing

them in the solution without aggregation. These different architectures were investigated using mainly UV-vis and CD spectroscopy to probe their molecular behavior and the relationship between their microscopic and macroscopic transitions.

6-2. Materials and methods

All the genes were constructed with the same molecular biology techniques that were described previously.^{6.12, 6.13} A complete list of all different constructs which were made for this research can be found in appendix A but the genes which are discussed here are (GVGVVP)₄₀, (GVGVVP)₄₀-foldon, foldon-(GVGVVP)₄₀, foldon-(GVGVVP)₄₀-foldon, and foldon-(GLGVPGQGVP)GQGVVP)₁₂-foldon. The exact amino acid sequence of these constructs are reported in Appendix A.

Protein expression was in general similar to what described in previous chapters. One notable difference was for the purification of the constructs with foldon on both ends because with the standard method of heat and cold cycling which is used for other ELP constructs most of the protein ended up staying in the pellet from the first cold centrifugation. To overcome this problem, after the first cold centrifuge of the disrupted cells, the pellet was brought back up into the solution by adding PBS and keeping the solution at 4°C for 3 to 4 days. This solution was centrifuged at 4°C again and the supernatant was then heated up in the presence of 1 molar NaCl and the pellet was then resuspended again by adding cold PBS and was left at 4°C for another 3 to 4 days. The solution was vortexed a few times each day. The solution was then centrifuged at 4°C and the supernatant was separated and the cycle was repeated again if necessary.

Characterization of the constructs were done using SDS-PAGE gel electrophoresis to confirm their molecular weight and purity and UV-vis spectroscopy (Shimatzu 1800) was used to measure the transition temperatures. Circular dichroism (CD) spectroscopy (Aviv 215) was used extensively for this study in order to study the conformational changes in each of the constructs at different temperatures. The CD samples were prepared the same way as the UV samples by diluting a PBS stock solution either in PBS or water. The samples were loaded in either 1 mm or 0.1 mm pathway cuvettes, depending on the concentration of the sample and the salt concentration, and the spectra were measured from 190 nm to 250 nm.

6-3. Results and discussion

Our goal in this study was to modify the architecture and surface decoration of ELP molecules such that it becomes energetically favorable for them to stay in the matured or folded state if such intermediate step actually exists. Here the results of characterization of each of the constructs are discussed separately and then they will be compared.

(GVGVP)₄₀

This construct was used as our reference molecule. It is linear and is very well characterized both in the literature and in our lab.^{6,13} Turbidity measurements of solutions of this ELP molecule with many different ranges of concentrations were studied and discussed in detail in previous works.^{6,13} UV absorbance curves from these solutions as a function of temperature at have a sharp absorbance maxima of around 1 (except for very low protein concentrations)(Figure 6-1).

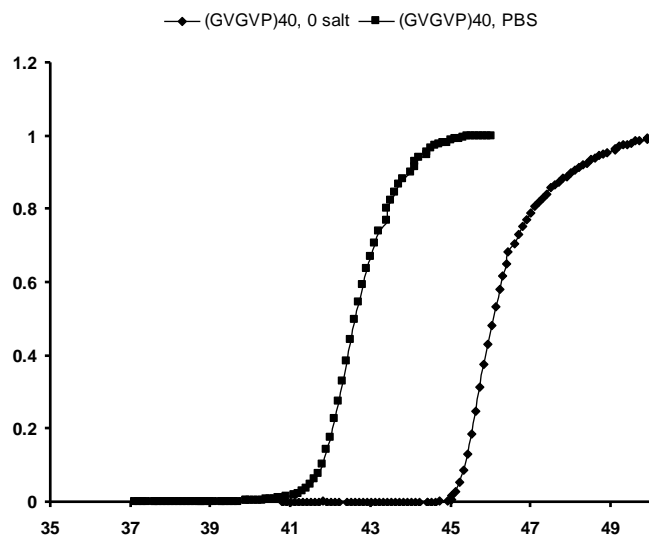


Figure 6-1. UV-absorbance of (GVGVP)₄₀ at 25 μM protein concentration dissolved in PBS (square) and zero salt concentrations (diamond). The behavior is very similar in both salt concentrations except for the different transition temperatures.

The circular dichroism (CD) spectroscopy of (GVGVP)₄₀ in PBS shows gradual changes in the conformation (Figure 6-2). The low temperature spectra have the well-known characteristic minimum of a random coil around 195 accompanied with a weaker one at 220. These minimums then start to become less negative by increasing the temperature. The decrease is accompanied by a slight red shift of the bands which is an indication of the presence of at least some β-turn structures at higher temperatures. The third peak at 212 nm is fairly constant and can be considered as the isodichroic point. The presence of this point is an indication of the presence of two main conformational states at the same time and the fact that one changes to the other by changing the temperature.^{6.14, 6.15}

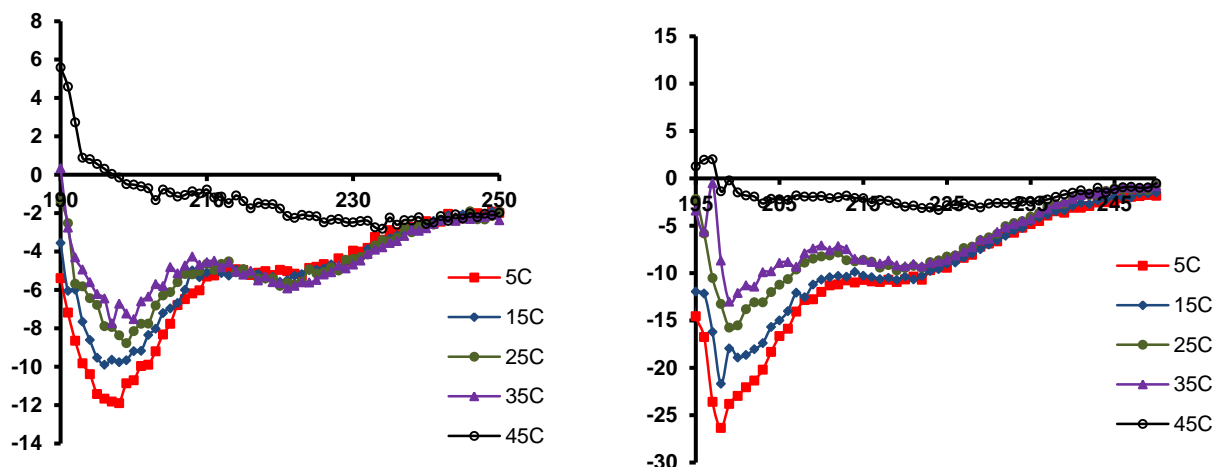


Figure 6-2. CD spectroscopy of (GVGVP)₄₀ (a) at 10 μ M protein concentration in PBS from below to above transition temperature and (b) at 25 μ M protein concentration in water from below to above transition temperature

An important aspect of this spectrum is the lack of an expected positive band between 200 and 210 nm which is also a characteristic of type II β -turn. This can be explained by the strong presence of random coils even at higher temperatures. But it appears that the construct starts to become more ordered and shifts towards a combination of random coil and type II β -turn structure. This is in agreement with previously reported CD spectra of different ELP constructs.^{6.14, 6.16} Above the transition temperature, the aggregation causes the loss of the signal for this construct.

The CD spectrum of the same polypeptide diluted in water (low salt) also exhibits similar general behavior, but it appears that the 212 nm peak does not behave as an isodichroic point anymore as it shifts to higher values with increasing temperature. This might be an indication of tendency towards β -turn structure and less random coil in the solutions at higher temperatures. But nevertheless there is still a considerable amount of

random coil conformations at or close to the transition temperature together with this ordered structure.

(GVGVP)₄₀-foldon

As shown in previous studies^{6,12}, this construct is a three-armed star elastin-like polypeptide which also has different aggregation properties than linear ELP. However, the measurable response of these molecules, when studied in PBS, was shown to be similar to the linear ones, with a sharp change in UV absorbance at the transition temperature and similar features at low or high salt concentrations.

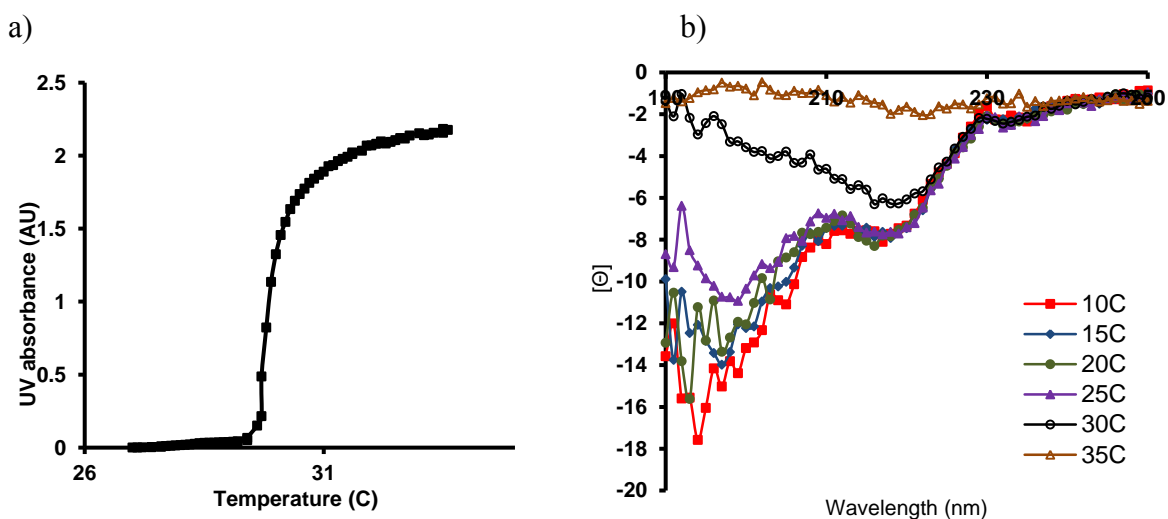


Figure 6-3. UV absorbance (a) and CD spectrum (b) of $(GVGVP)_{40}$ -foldon at 100 μ M protein solution in PBS using 1mm pathway cuvette for CD measurement and 10 mm pathway cuvette for UV measurements.

UV spectroscopy of a 100 μ M solution of $(GVGVP)_{40}$ -foldon in PBS shows a sharp transition below 30°C (Figure 6-3a). But without losing the signal, the CD spectrum of the same sample at 30°C shows clear sign of more ordered structure above the transition temperature (Figure 6-3b). In general the behavior of the ELP-foldon molecules in PBS at

low temperatures is similar to that of the linear ELP, but seems to show more order close to the transition temperature. This can be an indication of the effect of foldon on inducing order in the ELP molecules especially when the solution is close to the transition temperature.

Foldon-(GVGVP)₄₀

This construct has very similar geometry to (GVGVP)₄₀-foldon but instead of having the foldon on the C-terminus of the ELP, it is located on the N-terminus. It has been shown previously that ELP conformation can be affected by adding a self-assembling domain to either of the two termini and that there is a difference between adding this domain to C- or N-terminus,^{6,17} so we believe there could be molecular and conformational differences between adding the foldon to the ELP N- or C- terminus.

UV-absorbance was used to measure the transition temperatures of foldon-(GVGVP)₄₀ as a function of concentration and the results were compared to the (GVGVP)₄₀-foldon (Figure 6-4). The two curves have very similar slopes but foldon-ELP transition temperatures appear to be consistently about 3°C lower than the ELP-foldon constructs at the same concentrations. This is interesting considering the similar geometry of the two constructs. It is possible that foldon-ELP trimers fold into their matured stage (with the assumption that such stage actually exists) at lower temperatures compared to the ELP-foldon constructs and this causes them to aggregate out of the solution at lower temperatures. This is a clear sign of different molecular behavior of these two constructs.

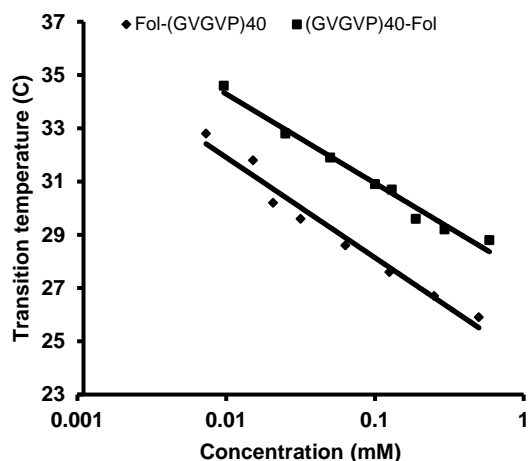


Figure 6-4. Transition temperature as a function of concentration for (GVGVP)₄₀-foldon (squares) and fol-(GVGVP)₄₀ (diamonds). The foldon-ELP construct shows the same linear dependency between the transition temperature and logarithm of concentration but is about 3°C lower in transition temperature compared to the same concentration of ELP-foldon construct.

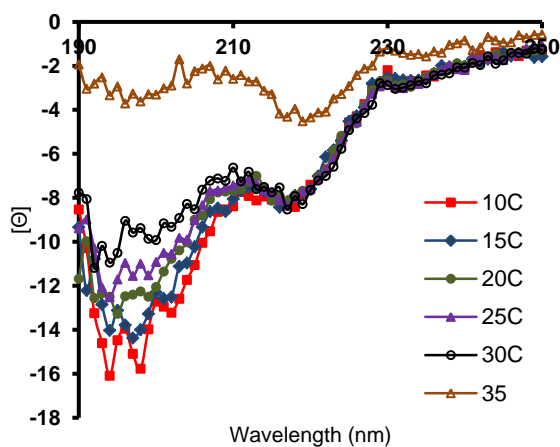


Figure 6-5. CD spectra of fol-(GVGVP)₄₀ at 100 μM in PBS and different temperatures. The spectra show similar behavior as linear or ELP-foldon at low temperatures but above the transition temperature (33°C) a red shift at 210 nm peak and decrease in random coil conformation is observed.

To study the conformational changes of these two constructs, the CD spectra of fol-ELP (Figure 6-5) were compared to those of the ELP-foldon (Figure 6-3b). The spectra

in general show similar behavior at low temperatures as linear or ELP-foldon but at 35°C, which is above the transition temperature, the signal is not lost in contrast to the linear construct and conformational ordering is observed. Also interestingly, for foldon-ELP, there is not a considerable change in the CD signal even at 30°C which is above the transition temperature for the 100µM solution while for ELP-foldon the 197 nm minimum is lost at the transition temperature. Another interesting difference between trimer constructs and the linear ones is the absolute value of the CD signal. For a 25 µM linear (GVGVP)₄₀, the 195 minimum is about -25 deg cm² dm⁻¹ at 10°C while for a 100 µM solution of ELP-foldon this signal is only -18 deg cm² dm⁻¹ and for 100 µM fol-ELP the signal is at -16 deg cm² dm⁻¹. If the three construct contained the same amount of random coil constructs we would expect to see a much higher negative value for ELP-foldon and foldon-ELP at concentrations 10 times the linear. The fact that the peak is in fact less negative, is a good indication of much less random conformation in the solution even at low temperatures. We believe this is the orientation of the chains as a result of adding the foldon domain. Between the two trimer constructs, foldon-ELP exhibits less random coil content as well. This can be an indication that folding of the ELP molecules may preferentially start from the N-terminal and holding a few of the ELP chains together from this end, gives them better chance to come together even at low temperatures.

To further investigate the conformation of trimer ELPs in different environments, the CD spectra of foldon-ELP was studied in low salt solutions. The CD spectra of fol-(GVGVP)₄₀ in water show very different behavior compared to that of the linear construct (Figure 6-6). There is no 195 nm minimum even at very low temperatures and instead there is a minimum at 218 nm which becomes more visible at higher temperatures

and close to transition temperature shifts to about 220 nm. This spectrum has the characteristics of protein with high population of β structure especially at wavelengths above 205.^{6.18} But for it to be all β a positive maximum around 197 is expected. The reason for not observing this peak could be the presence of random segments in the conformation of the polypeptides even at high temperatures. This specially might be the case for segments of ELP far from the foldon domain that might not be affected by the foldon or have less ordered conformations in the presence of C-terminal ends of the other chains. At higher temperatures (45°C) a weak positive peak at 210 nm is observed. This band together with negative band around 220 nm is an indication of the existence of type II β -turns. The small red shift in the two peaks in comparison to the expected type II β -turn spectrum might be an indication of existence of other types of β turn in the conformation.^{6.19} Regardless of the exact conformation of this construct, the fact that a noticeable difference between the conformation of this construct and linear ELP is a good reason to believe that the architecture of these molecules actually affects their conformation and that there is in fact a folding process occurring prior to aggregation. The reason for not having the same type of behavior for fol-ELP when it is in PBS is not completely clear but it might be attributed to the salting-out effect of NaCl.

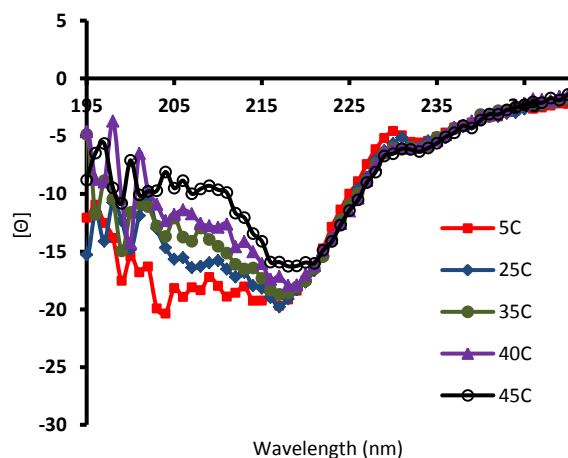


Figure 6-6. CD spectra of fol-(GVGVP)₄₀ at 25 μM in water. The spectra show ordered structure at low temperature. Increasing the temperature results in more β-like conformations.

Up to this point we at least partially answered the question of whether or not the ELP molecules fold (or have the potential to fold) into more ordered structures and if this ordered structure can be stabilized independently from the aggregation process. To continue our investigation, we synthesized new constructs which are capped at both ends. Our goal was to induce the folding into a homogenous population at any environmental condition. By capping both ends of the ELP trimer we would avoid random folding or aggregation of free chains and answer the question of if ELP aggregation is inherently heterogeneous or can be forced to be a homogeneous population.

Foldon-(GVGVP)₄₀-foldon

This construct is very similar to the ones just described except that both ends of it are capped by foldon domain. The UV spectra of this construct at different protein concentration show some different general behavior in comparison to other linear or trimer ELP constructs (Figure 6-7).

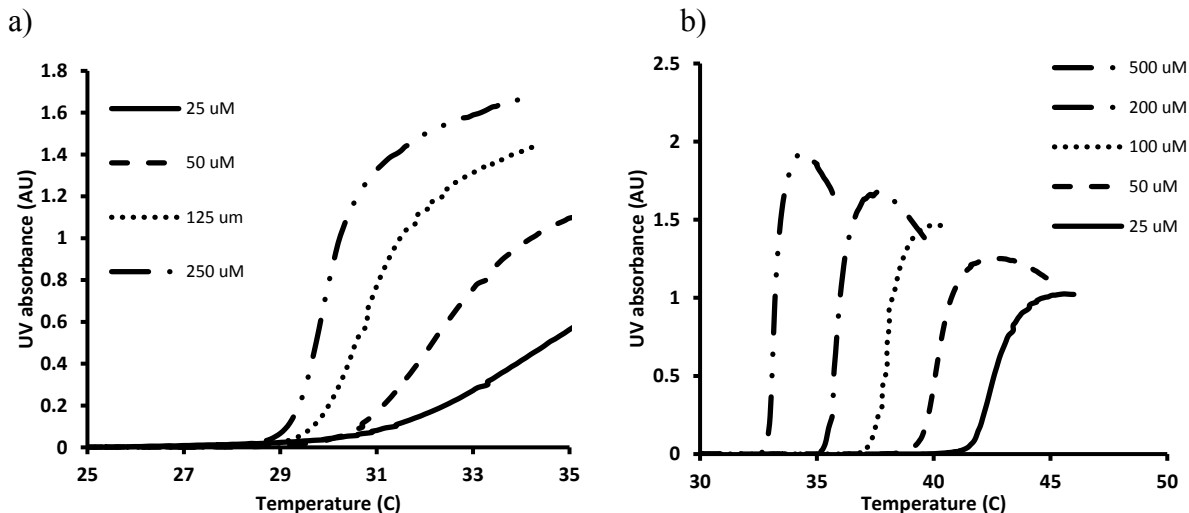


Figure 6-7. UV absorbance of (a) foldon-(GVGVP)₄₀-foldon and (b) (GVGVP)₄₀ at different total protein concentrations in PBS. The construct with both ends capped shows different transitional behavior than the linear construct

It is noticeable that the concentration change of one order of magnitude (from 25 μ M to 250 μ M) changes the transition temperature only by less than 2°C (Figure 6-7a). This is of course very different from linear ELP constructs in which a change of about 8°C for the same change in the concentration is observed (Figure 6-7b). The smaller change in the transition temperature can be attributed to smaller concentration dependency of this construct. From a molecular point of view this might be the result of matured trimers that are made in a more orderly process. For a typical ELP, the chains are randomly distributed and it is expected that they also find other chains randomly and so each chain might associate with a number of different chains as the temperature start to rise and that means the process of aggregation is more dependent on the number of available chains in the solution which is directly proportional to the solution concentration. But for foldon-ELP-foldon construct the three chains are already in close proximity and the maturation

process is less dependent on the solution concentration and more related to the chemical identity of the ELP and the hydrophobicity of the folded trimer.

The CD spectra of foldon-(GVGVV)₄₀-foldon in PBS is also interestingly different from the other ELP constructs (Figure 6-8). At very low temperatures two minima, one at 205 nm and the other around 217 nm are observed. This spectrum can be interpreted as the existence of high population of β -structured proteins especially that a weak positive maximum around 197 nm to 200 nm is also observed in the spectrum even at low temperatures.^{6.18} In fact by increasing the temperature to the transition temperature of this solution, which is somewhere between 30 to 35°C, the shift towards a stronger positive peak at that range can be observed. The other way to interpret the data at low temperature is a slightly red-shifted α -helical conformation. But it has been shown that type II β -turn conformations from polypeptides containing “Pro-Xxx” might show similar CD spectra as helical structures and since there is no reason to believe these constructs make α -helical conformations, the best possible way of describing their spectra would be by assigning β structure conformation to them.^{6.20} In general there seems to be a high content of β -turn conformation at low temperatures which then change to more β strand conformation at or around transition temperature (Figure 6-8 dotted spectrum).^{6.21} Another interesting aspect of the spectra is that above the transition temperature the foldon peak starts to diminish while the positive peak at the 197 nm gets stronger and the minimum at 217 nm is not affected. This is a different behavior from all the other previously studied ELPs for which above the transition temperature the aggregation causes the loss or weakening of the whole spectrum. This might be an indication that in this case, the aggregation is not a random process among many different folded chains

and in fact the orderly folded chains start to aggregate into the β strand-like conformation above the transition temperature.

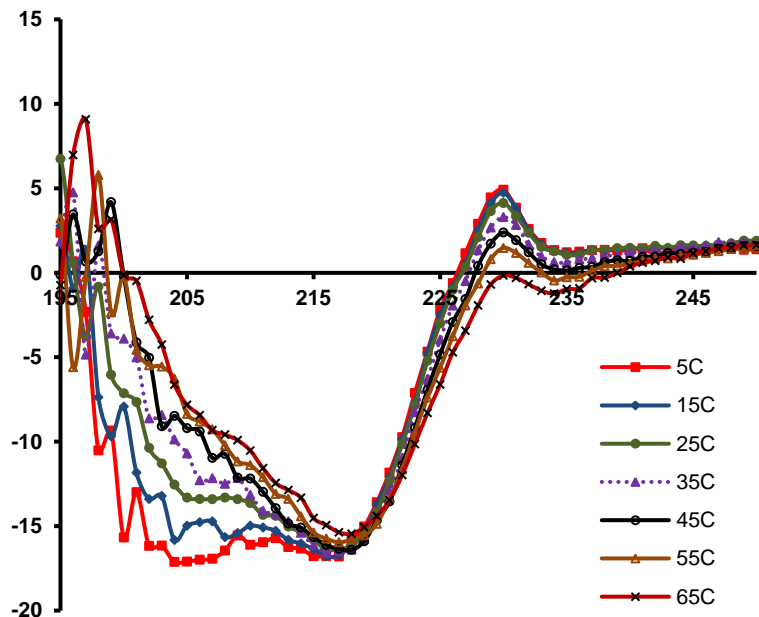


Figure 6-8. CD spectra of foldon-(GVGVP)₄₀-foldon at 10 μ M solution concentration in PBS. The spectra below the transition temperature (up to 35°C) shows signs of high β -turn content which then shifted towards more β strands conformation above the transition temperature.

Modifying geometry and chemical identity of ELP molecules

Up to this point we have shown that different molecular geometries modify the folding and aggregation of the ELP molecules. The next question is if the chemical identity of the construct can also affect the folding process, especially if it is combined with proper geometric design and also, if this can actually help the stabilization of the folded constructs. To answer this question we made a number of different constructs with the idea of giving the surface of the folded trimer more hydrophilic characteristics and burying the hydrophobic residues inside the folded trimer. To accomplish this goal, the

valines in the ELP sequences for each different architecture (linear, trimer or both ends-capped trimer) were substituted in a pattern to more hydrophilic or hydrophobic residues based on the molecular model of the folded ELP (Figure 6-9).

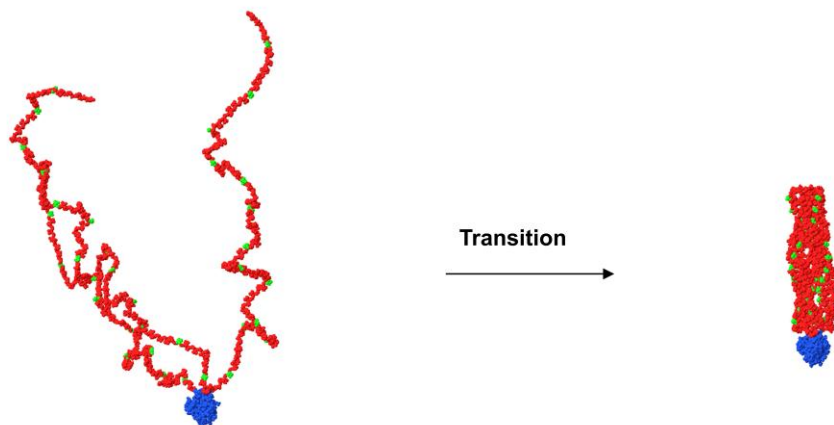


Figure 6-9. Molecular model of modified ELP trimer from unfolded to folded. The chemical identity of the folded trimer can be controlled by changing Valine residues which are going to be exposed to water (or hidden inside the folded trimer).

Foldon-(GLGVPGQGVGPGQGVP)₁₂-foldon.

Of the constructs synthesized with changed amino acid residues, most of the ones with only one end capped behaved more or less similarly to the previously described trimers, but the ones with both end capped showed some interesting characteristics. One of the most promising one is a construct that consists of 36 repeats of *GαGβP* in which α and β were substituted with valine, glutamine, and leucine and the arrangement was chosen to have the glutamine residues in contact with water after the folding of the trimer. The folding model was chosen to be β -spiral with repeating β -turns.

The UV spectrum of this ELP solution has an interesting feature that it shows much lower absorbance compared to other ELP solutions (Figure 6-10). This lower UV absorbance could be the result of less aggregation which might be a result of stable folded trimers that are energetically stable in their folded (matured) state without the need to aggregate out of the solution.

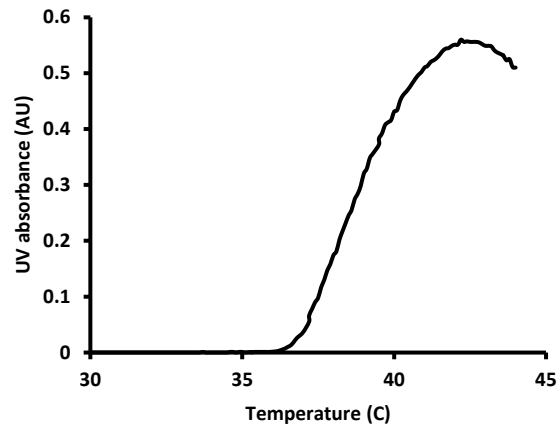


Figure 6-10. UV absorbance of foldon-(GLGVPGQGVGPGQGV)₁₂-foldon in PBS at 50 μ M concentration. The absorbance is close to one third of what is usually expected from a 50 μ M ELP solution.

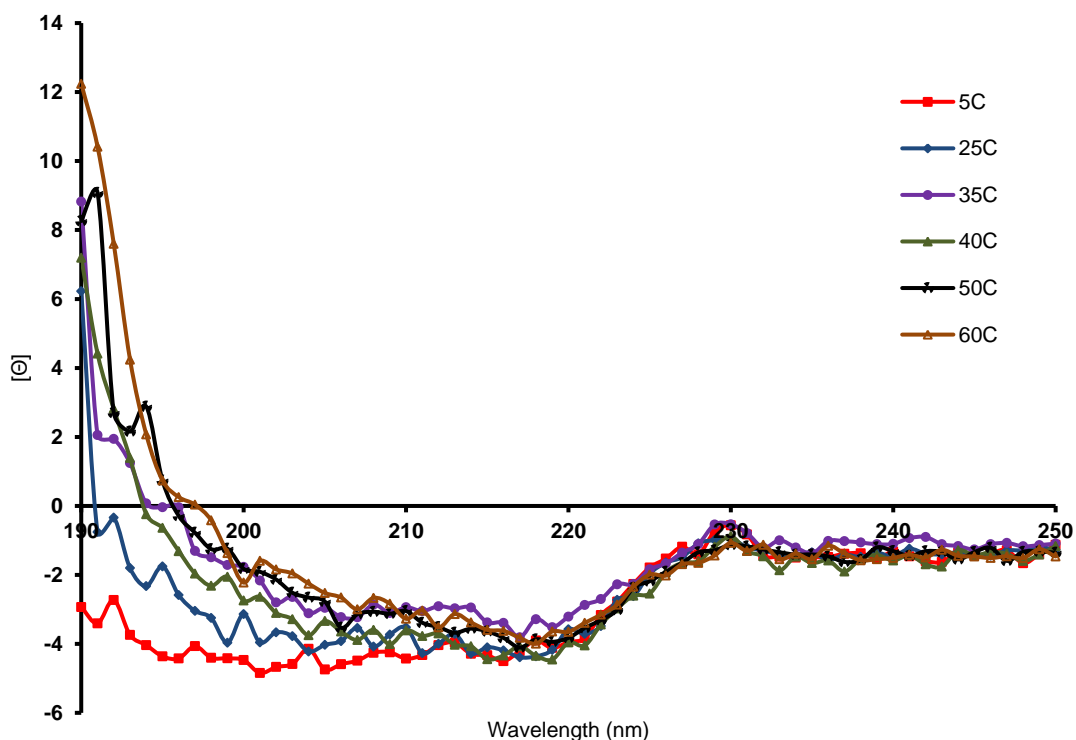


Figure 6-11. CD spectra of foldon-(GLGVPGQGVGPGQGVP)₁₂-foldon at 50 μ M in PBS at different temperatures. The spectra seem to be very similar from below to above the transition temperature. At high temperatures the strong positive peak around 190 nm might be an indication of β -strand structure while below transition temperature more β -turn structure is observed.

To study the conformation of this construct at different temperature, CD spectra of the same sample at different temperatures were measured (Figure 6-11). The spectra are in general similar to the ones for foldon-(GVGVPG)₄₀-foldon (Figure 6-8) but they show stronger β -turn content below the transition temperature. The signal is weaker than the other spectra which is a result of using cuvettes with 0.1 mm pathway instead of 1 mm. Above the transition temperature β -strand conformations are observed. In general, the construct above and below the transition temperature do not appear to be very different

and definitely no random coil conformation is observed. There is no loss of CD signal above the transition temperature.

It appears that this construct in fact is stabilized in its folded conformation and apparently it goes into this folded conformation at temperatures well below the transition temperature. But above the transition temperature there are some rearrangements of these folded units but not all of these folded constructs go into aggregates. This is very interesting because it shows that aggregation is not an inevitable result of folding or maturation of the ELP chains and they can be designed such that they stay soluble and stable even above their predicted transition temperature.

6-4. Conclusion

We have studied the folding and aggregation theories of ELPs by synthesizing different geometries and chemical identities of these molecules with the aim to gain better understanding of the process of folding. We have shown that depending on the geometry of the ELP molecules and the environment that they are studied at, their folding can be affected. We believe that there is definitely some kind of ordering of chains when the solution approaches its transition temperature but the folding starts at temperatures well below the transition temperature and it is a gradual process. It appeared to us that folding of the ELP chains starts most efficiently from their N-termini and constructs with their N-terminal capped show the ordering towards what can be interpreted as β conformations at lower temperatures but it is also observed that capping only one end of the ELP construct cannot induce the folding especially at higher salt concentrations. On the other hand, by capping both ends of ELP constructs into a trimer, well-defined conformations of β -turns

were observed at temperatures below the transition temperature. These β -rich constructs appeared to pack into some kind of β -strand conformation at temperatures above the transition temperature. Also, we have shown that the chemical identity of the chains can be designed together with the geometry, to result in a system of stably folded trimers. These trimers are the first ELP constructs to show clear folding without aggregation.

Based on these results, we believe that ELP molecules in fact fold into more ordered structures as the temperature rises to their transition temperature. But their final conformation is not a universal conformation, which means that depending on their molecular architecture, their chemical identity, and their environment they might take different conformations. It is possible to design them such that a separate folding and aggregation occur. It additionally seems that folding is necessary for the constructs to aggregate, but aggregation is not the inevitable fate of the folded constructs. This is especially interesting for the potential of utilizing these folded but not aggregated molecules for NMR structural studies and for certain applications in which a folded construct might be used as the modular unit to make responsive biomaterials.

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Chapter VII

Concluding Remarks

Elastin-like polypeptides have been shown to be an interesting class of environmentally responsive biomaterials and their molecular behavior and macroscopic response have been subject to many studies but the pathway of molecules from soluble to insoluble has not yet been clearly understood and also the effect of molecular architecture on their transitional behavior has not been explored. The aggregation of ELP molecules is concentration dependent and our hypothesis was to induce the folding of the chains at temperatures lower than the transition temperature by physically keeping them in close proximity. Our approach was to make use of a trimer forming oligomerization domain called foldon at N-, C- or both termini of the chains and essentially make new geometries of ELP molecules and study their behavior and also decorate the ELP chains by hydrophobic and hydrophilic residues in a way to increase the stability of the folded constructs.

The three-armed star ELP molecules are shown to fold into more extended rod-like structures at the transition temperature while the linear ELP keeps its random coil

conformation all the way to the transition temperature. A mathematical model was developed to enable us predict the critical transition temperature as well as understanding the molecular conformation at the transition temperature. Based on this model we predict that the ELP molecules, which are confined at their N-termini can fold into more extended conformation at the transition temperature compared to the linear or the ones which are capped at their C-termini. We also showed for the first time that capping both ends of the ELP molecules together with proper choice of amino acids in the chain induce stable folding of the trimers to β -rich conformations without aggregation even at temperatures above the transition temperature.

Using these new architectures and exploiting the slight negative charge on the foldon domain, we developed responsive micellar systems in which micelles are formed at certain salt and pH concentrations and their size can also be controlled from as low as 20 nm to few hundred nano meters by adjusting salt, pH and mixture ratios of linear and trimer constructs. We fully characterized these micelles at different salt regimes by dynamic and static light scattering. It appears that changing salt from below 15 mM to above 30 mM induce drastic changes in the size as well as the shape of the micelles. At low salt concentration, the trimers pack into spherical micelles of about 30 nm in diameter while at higher salt concentrations they start to expand and elongate into cylindrical micelles with lengths of about 10 times longer than the micelles diameter. This size increase is accompanied by a big jump in the molecular weight of the micelles. We were also able to crosslink these self-assembled construct to responsive nanoparticles that are responsive and stable at physiologic conditions

With these constructs we have shown how the design of new architectures can not only help us better understand the theory of folding and aggregation but it also give us a robust tool in developing self-assembling ELP systems.

The better understanding of the folding and aggregation of the ELP chains and the fact that we are able to have better control over the chain-chain interactions, together with self-assembling capabilities of these constructs give us the tools to design molecules in the future that can be used in applications such as responsive fibers in which a controlled directional response is desired.

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Appendix A

List of synthesized constructs

This is a list of the constructs that were made during the course of this research.

MGH(GVGVP)₅GWP

MGH(GVGVP)₁₀GWP

MGH(GVGVP)₂₀GWP

MGH(GVGVP)₄₀GWP

MGH(GVGVP)₄₀GWPC

MGH(GVGVP)₆₀GWPC

MGH(GVGVP)₈₀GWPC

MGH((GVGVP)₆(GKGV))₄GWP

MGH((GVGVP)₆(GKGV))₈GWP

MGH((GVGVP)₆(GKGV))₄(GVGVP)₂₀GWP

MGH(((GVGVP)₆(GKGV))₄(GVGVP)₂₀)₂GWP

MGH(GVGVP)₃(GVGVPGHGVP)₆GWP

MGH((GVGVP)₃(GVGVPGHGVP)₆)₂GWP

MGH(GLGVPGVGVPGQGVP)₁₂GWP

MGH(GLGQPGQGQPGQGLP)₁₂GWP

MGH((GLGQPGQGQPGQGLP)₃(GVGVP)₂₀)₂GWP

MGH(GLGQPGQGQPGQGLP)₆(GVGVP)₄₀GWP

MGH(GVGVP)₂₀(GVGVPGCGVPGVGVP)(GVGVP)₂₀GWP

MGH(GVGVP)₄₀(GVGVPGCGVPGVGVP)(GVGVP)₂₀GWP

MGH((GVGVP)₆(GEGVP))₂GWP

MGH((GVGVP)₆(GEGVP))₄GWP
(MGHGVTSTGTPQSSNDGNPDTSGQNNVP (GVGVP)₂₀)₈-
MGHGVTSTGTPQSSNDGNPDTSGQNNVPGWP
MGH(GVGVP)₂₀-GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
MGH(GVGVP)₄₀- GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
MGH(GVGVP)(GEGVP)(GVGVP)₂₁ GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
MGH(GVGVP)(GEGVP)(GVGVP)₄₁ GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
MGHGVTSTGTPQSSNDGNPDTSGQNNVPGWP(GVGVP)₄₀GWP
MGHGVTSTGTPQSSNDGNPDTSGQNNVPGWP(GVGVP)₂₀GWP
MGH((GVGVP)₆(GKGV))₄ GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
MGHGVTSTGTPQSSNDGNPDTSGQNNVPGWP((GVGVP)₆GKGV)₄-
GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
MGHGVTSTGTPQSSNDGNPDTSGQNNVPGWP(GVGVP)₄₀-GWPC
MGHGVTSTGTPQSSNDGNPDTSGQNNVPGWP(GVGVP)₄₀
GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
MGHGVTSTGTPQSSNDGNPDTSGQNNVPGWP(GVGVP)₄₀
GWPGYIPEAPRDGQAYVRKDGEWVLLSTFLC
MGH(GPP)₈-(GVGVP)₄₀ GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
(GLGVPGVPGQGV)₁₂GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
MGHGVTSTGTPQSSNDGNPDTSGQNNVPGWP-(GLGVPGVPGQGV)₁₂
GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
MGH((GVGVP)₆(GKGV))₄(GVGVP)₂₀ GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
(GLGVPGVPGQGV)₁₂GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL
((GVGVP)₃(GVGVP)(GKGV))₆₂GWPGYIPEAPRDGQAYVRKDGEWVLLSTFL