Investigation into the phase separation behavior of concentrated elastin-like polypeptide solutions

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ABSTRACT

Elastin-like polypeptides (ELPs) are environmentally responsive biopolymers that respond to various forms of external stimuli such as temperature, light, and pH. Several factors can influence the transitioning behavior of ELPs. These factors include the amino acid composition of the ELP, the protein concentration in solution, the salt concentration of the solution, and the polymer chain length.

Elastin-like polypeptides are soluble in water below a critical solution temperature, however, above this temperature the ELPs become insoluble and phase separate. This point of temperature triggered phase separation is referred to as the transition temperature. This process is completely reversible and ELPs will redissolve once the solution temperature is decreased below the transition temperature.

In this study, the phase transitioning behavior for several different concentrations of a three-armed ELP, (GVGVP)_{40}-foldon, was investigated. After expression and purification of this ELP construct, characterization was performed starting at a highly concentrated protein solution then diluting stepwise. Transition temperature were determined using UV-Visible spectrophotometry then several
calculation methods were used to analyze the transition temperature data, resulting in multiple potential phase diagrams. Finally, to further investigate the phase transitioning behavior of ELPs microscopy was used to observe the phase transitioning process for several samples of different ELP concentration.
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1.1 Introduction of Elastin-like Polypeptides

Elastin is a type of extracellular protein matrix that is highly crosslinked and insoluble in water. It mainly exists in the connective tissue of arteries, skin, and ligaments in mammals. Elastin gives reversible deformation and expansion ability to those tissues and organs, which can allow tissues and organs to go through huge deformations without fracture. Tropoelastin is a water soluble precursor of elastin composed of alternating hydrophobic and hydrophilic domains. Hydrophilic domains are composed of many lysine and alanine residues, and hydrophobic domains are rich in valine, proline, alanine and glycine \(^{[1-2]}\). Elastin contain a large number of the repeated pentapeptide amino acid sequence “VPGVG” \(^{[3-4]}\). Due to their structure, hydrophilic domains are highly crosslinked and insoluble in water, making natural
elastin difficult to separate. However, water soluble tropoelastin can be synthesized from elastin after being treated with trypsin.

Scientists have synthesized a novel class of artificial protein polymers, called elastin-like polypeptides (ELP). ELP amino acid sequences are composed of repeated pentapeptide sequences of \((G\alpha G\beta P)_n\), where \(\alpha\) can be any of the 20 natural amino acid, \(\beta\) can be any amino acids except for proline, and \(n\) is the number of pentapeptide repeats in the ELP molecule\(^5\). The composition and length of amino acid sequences can be precisely designed, if the ELP is synthesized using protein engineering. It does not cause an immune response in the body and degrades into non-toxic amino acids, which gives ELP very broad prospects in research, especially in the field of biomedical materials application, such as tissue engineering and drug delivery systems\(^6\).

1.2 Inverse Transition Temperature, \(T_t\)

1.2.1 Properties and Mechanism

An important characteristic of ELPs is their display of a reversible phase transition in response to temperature. The temperature at which this transition begins is called the solution’s transition temperature, \(T_t\). The phase separation behavior of this transition is of the lower critical solution temperature (LCST) type where, when the solution temperature is below the \(T_t\) the ELP is completely soluble and phase separation occurs when the solution temperature is raised above the \(T_t\) (Figure 1 A, B). Below the \(T_t\) ELPs exist in solution in a fully hydrated random coil structure (Figure 1 C). In this soluble hydrated state, water molecules surrounding nonpolar or hydrophobic moieties within the ELP chain are in a more ordered state and
conformational restricted when compared to bulk water. This water, called the water of hydrophobic hydration, is of such low entropy that authors have referred to it as “iceberg-like”, not due to its material nature but because of it being fixed or frozen in position.\(^7\) When the solution temperature is raised above the transition temperature the ELP folds and assembles into an ordered structure, called a beta-spiral, where hydrophobic regions within the ELP associate both intra- and intermolecular (Figure 1 C). Due to this hydrophobic folding and assembly, nearly all of the water of hydrophobic hydration is released to become bulk water and the ELP aggregates then separates from solution as a second phase. While the entropy of the protein is decreased during this transition this is far offset by the increase in entropy associated with the water lost from hydrophobic hydration. Therefore, the phase transitioning process is consistent with the second law of thermodynamics\(^{8-9}\).
Figure 1. Transition from soluble solution to insoluble phase separated solution.

(A): Protein solution at room temperature (single phase). (B): Turbid solution transitioned from soluble to insoluble. (C): The schematic diagram of ELP phase transition.

When the solution temperature is greater than the solution $T_\text{r}$, Urry $^7$ has proposed that the protein structure consists of a $\beta$-turn at the Pro-Gly position within the (VPGVG) unit. The repeat $\beta$-turns form a right-handed helix $\beta$-spiral that
aggregates together (Figure 2)\textsuperscript{[7]}. Also, above the phase transition temperature, it was observed that a certain amount of amino acids formed β-sheets in the ELP chain, which further promoted the interaction and aggregation between different ELP chains\textsuperscript{[10]}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{(A): Schematic representation of the polypeptide chain with the Pro-Gly sequence inserting a repeating β-turn. (B): β-spiral structure of (VPGVG)\textsuperscript{n}. (Reprinted with permission from reference [7]. Copyright 1997 The Journal of Physical Chemistry).}
\end{figure}

Previous studies have described the phase transition mechanism of (GVGVP)\textsuperscript{n} from the aspect of energy change. The overall ELP transitioning process is
endothermic, but this is actually the combination of two different phenomena possessing different kinetic behavior \[^{[11]}\]. During the ELP transitioning process, some steps are endothermic while others are exothermic. Upon heating, the transitioning process begins with the restructuring of water molecules associated with hydrophobic hydration, a process that is known to be endothermic. Once the ELP has lost much of its hydrophobic hydration protein chains associate and fold due to van der Waals cohesive interactions, a process that is exothermic.

### 1.2.2 ELP Transition Temperature

The transition temperature of ELPs can be affected by ELP amino acid composition, sequence length, ELP concentration, salt concentration, and solution pH. The substitution of hydrophobic or hydrophilic amino acids within the ELP chain has an effect on the $T_t$ of ELPs. When more hydrophobic amino acids are incorporated into the ELP, the $T_t$ is lowered, while a more hydrophilic substitution results in an increase in the $T_t$\[^{[7]}\].

The transition temperature of ELPs depend on their molecular weight. For a given pentapeptide repeating unit, as the molecular weight of the ELP decreases the transition temperature increases and vice-versa. This effect of molecular weight on transition temperature is of greater influence at lower numbers of pentapeptide repeats and becomes less significant as the number of pentapeptide repeats increases. \[^{[12]}\]
For an ELP of known sequence and molecular weight, its transition temperature also depends on its concentration in solution. Previous experiments show that within a certain range of concentration the transition temperature significantly decreases with increasing protein concentration \(^{[13,14]}\). However, for high concentration ELP solutions, when the concentration is higher, the transition temperature is increased \(^{[13]}\).

The addition of NaCl to an ELP solution can significantly reduce the transition temperature because the existence of ions will increase the ionic strength of the solution, increase the polarity between water molecules and hydrophobic regions, increase the interfacial tension of the ELP-water interface, and weaken the energy associated with hydrophobic hydration. \(^{[15]}\) For these reasons, the addition of NaCl to an ELP solution is often used as a simple method of decreasing the solution’s transition temperature when necessary, such as during ELP purification. There is a linear relationship between NaCl concentration and transition temperature; moreover, the higher the NaCl concentration, the greater the effect on transition temperature \(^{[16]}\).

For ELPs containing amino acids capable of accepting or donating a proton, the solution’s pH value will affect the transition temperature because the degree of protonation will impact the hydrophobic hydration of the peptide chain. \(^{[17,18]}\) This behavior is of greater influence when the solutions pH is around the amino acid p\(K_a\) \(^{[17]}\). If the residue amino acid is charged, the charge reduces the amount of hydrophobic hydration thus, the transition temperature will increase. According to Urry’s work, whether an amino acid is charged or neutral will lead to a large difference in the ELP transition temperature \(^{[7]}\).
1.3 Synthesis of Elastin-like Polypeptides

ELP can be synthesized through chemical synthesis or from protein engineering methods. Chemical synthesis can insert some unnatural amino acid at the specific site in a sequence, but this method suffers from high cost, slow production, and low yield. \cite{19,20} To circumvent the problems associated with chemical synthesis protein engineering is often used for the synthesis of ELPs \cite{20}.

Recursive directional ligation (RDL) is an important genetic recombinant synthesis method used to design ELP genes for the manufacture of ELPs (Figure 3). \cite{21} First, a synthesized DNA oligomer which codes for an ELP sequence with several repeats is ligated into a plasmid cloning vector. For the next cycle, two restriction endonuclease (RE1 and RE2) are used to cut out the ELP block, then only restriction endonuclease RE2 is used to cut the plasmid, thus, the plasmid becomes a new cloning vector containing an ELP block. After purifying the ELP block and the vector, they are ligated together. This process is repeated until the desired length of ELP gene has been designed.
1.4 Applications of Elastin-like Polypeptides

ELPs have many advantages compared to synthetic materials including good biocompatibility, biodegrading into amino acids, non-toxic, no immune response\textsuperscript{[22]}, excellent pharmacokinetic behavior\textsuperscript{[23]}, ability to precisely design the amino acid sequence and molecular weight,\textsuperscript{[24]} and high yield and quick purification.\textsuperscript{[25]} Therefore, ELPs have very broad applications in biomedical fields. Some of more popular applications include drug delivery and tissue engineering.
1.4.1 Elastin-like Polypeptides in Drug Delivery Systems

Under certain environmental conditions, ELPs can self-assemble into particles, nanoparticles, or micelles. In the ELPs phase transition temperature region, they possess the ability to encapsulate drug molecules and release them as a function of external temperature. This behavior makes ELPs an effective drug carrier [26,27]. Bessa et al. [28] used ELPs dissolved in phosphate buffer solution, and added proteins BMP-2 and BMP-14, then incubated 30 minutes at 37 °C. This resulted in the formation of spherical nanoparticles with a diameter of approximately 237 nm. The entrapment efficiency of BMPs was over 90%. According to in vitro release experiments, these nanoparticles released approximately 20% of their BMP payload in 24 hours, then slowly released up to 14 days.

Drug delivery systems play a vital role in the treatment of cancer because they can increase the amount of drug at the tumor site, and reduce the side effects of drugs on the normal tissue and organs [29]. The mechanism used to increase the amount of drug at the tumor site is called the enhanced permeability and retention (EPR) effect. The EPR effect is a phenomenon by which certain sizes of particles (typically liposomes, nanoparticles, and macromolecular drugs) tend to accumulate in tumor tissue much more than normal tissues [30]. It has been shown that in the treatment of cancer drugs can be delivered to a tumor site through systemic or topical ways [31,32]. According to the difference between transition temperature and physiological temperature (T_p), ELP drug delivery systems have four potential methods for tumor treatment (Figure 4) [33].
In Figure 4 A, when $T_t < T_p$ the hydrophobic drug bound ELPs will self-assemble to form micelles. Systemic drug delivery of the ELP-drug micelles accumulates at the tumor site by the EPR effect and aggregate in that position. This reduces the drugs toxicity while increasing its effectiveness in treatment. When $T_p < T_t < 42 \, ^\circ C$, (Figure 4, B) ELP-drug conjugates form insoluble aggregates at the external tumor site using mild thermal therapy. When heating is discontinued, ELP at the tumor site will redissolve producing a large concentration gradient of ELP bound drug. This promotes ELPs with drugs to diffuse into cells and achieve the targeted drug delivery. Besides, when the physiological temperature is higher than $42 \, ^\circ C$, the cells in human body will be severely damage and even death, that is why the transition temperature of ELPs should not exceed $42 \, ^\circ C$. Another approach to drug delivery, called multivalent targeting drug delivery, (Figure 4, C) involves attaching a targeting ligand to the ELP-drug conjugate. After topically heating the tumor site, ELP micelles are formed through self-assembly. Since these micelles have a targeting ligand attached they possess high affinity for their programmed target site and result in strong cellular uptake. When the ELPs $T_t$ is less than $T_p$, (Figure 4, D) a common therapeutic approach is to directly inject the ELP-drug conjugate into the tumor site. After injection, they immediately aggregate and can remain at the tumor site for an extended period of time. Using this method to extend the contact time between the drug and the tumor site sufficiently inhibits the growth of tumor tissue.
Figure 4. Four delivery strategies for ELPs to deliver drugs to a solid tumor in vivo.

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1.4.2 Elastin-like Polypeptides in Tissue Engineering

ELPs in tissue engineering are used as a type of injectable biomedical material. They can form a hydrogel through chemical or physical crosslinks in the body, thus, ELPs can be used as a tissue scaffold. Before gelling, the ELP liquid precursor is easy to handle and can be directly injected at the site of injury. Triggered by environmental stimuli such as temperature, pH value, or ionic strength, the ELP will then self-assembly into a hydrogel \([34]\). After injection and application of the desired environmental stimuli, ELPs rapidly form a hydrogel that can be used as a three-dimensional artificial extracellular matrix. This extracellular matrix has the capability to provide cells with the structure and function necessary for tissue repair. In addition,
for other applications in tissue engineering, ELPs can be formed into hydrogels, films, and fibers through different methods, which further improve its properties [35-37]. Currently ELP hydrogels have been successfully applied to tissue repair of cartilage and vascular transplant [38-40].

For instance, Betre et al [38,39] showed that chondrocytes can be loaded into a hydrogel capsule formed by ELPs, and these chondrocytes maintain their unique morphology in vitro for 15 days, which indicates that the coacervate layer of ELP can support the growth of chondrocytes. In addition, in the absence of extra chondrocyte supplement, ELP coacervate can also promote adipose-derived adult stem cells to differentiate into chondrocytes because cartilage tissues suffer from stress, and the scaffold that is used in the damaged cartilage site also needs to be able to withstand the pressure. Lim [41] developed an ELP containing lysine with propanoic acid crosslinking. After 5 minutes of reaction, these crosslinked ELPs have 3 orders of magnitude higher mechanical properties than ELPs without crosslinking. In the application of in vitro tissue engineering, the ELPs crosslinked by this method can serve as an injectable biomaterial, and it can be used as scaffold for cartilage tissue regeneration.

1.5 Foldon Domain

In the head of bacteriophage T4, there is an elongated and trimeric protein fibritin [42], which possesses a small C-terminal globular domain. This small globular domain plays a key role in the correct folding of the protein and hence has been
named foldon\textsuperscript{[43]} (Figure 5)\textsuperscript{[44]}. The foldon domain is composed of 27 amino acids and forms an isolated trimeric structure at temperatures of up to 75 °C. The foldon domain can be used as an artificial trimerization inducer or enhancer because of the trimer’s extreme stability\textsuperscript{[43]}.

Figure 5. Ribbon diagram of the C-terminal foldon domain. (Reprinted with permission from reference [44]. Copyright 2004 Elsevier).

In previous studies, the foldon domain was used to stabilize short triple helices of collagen.\textsuperscript{[45]} Even at low concentration and high temperature and independent of whether the foldon sequence was at the C-or N-terminus, the collagen triple helix formed from foldon oligomerization was much more stable than the one without the presents of the foldon domain.

Using the behavior of collagen as inspiration, in our lab, a new kind of elastin-like polypeptide construct with a foldon domain incorporated at the C-terminal end was designed and biosynthesized. The foldon domain in these new constructs folded as a homotrimer resulting in three-armed star ELPs (Figure 6)\textsuperscript{[46]}. The transition temperature of ELP-foldon is lower than the ELP linear structure because in this new
structure, the distance between ELP chains is relatively short, and they do not need to diffuse a long distance to aggregation, even at low concentration\[^{16}\].

![Figure 6](image)

**Figure 6.** (A): ELP-foldon is shown as an unfolded state below transition temperature. (B): ELP-foldon is shown as a folded state above transition temperature. (Reprinted with permission from reference [46]. Copyright 2011 American Chemical Society).

**1.6 Previous work on the ELP Phase Diagram**

When a part of a system has the same composition and the same physical and chemical properties, this uniform part is called a phase. The transformation of a thermodynamic system from one phase to another is governed by heat transfer. This process is called a phase transition. It is the result of order and disorder competition with each other. Phase equilibrium is “state of balance”, which means rate of transfer of matter or heat from one phase to the other, equals the rate of transfer in the
reverse direction at equilibrium. Under the equilibrium condition, a phase diagram is a type of chart used to show the relationship between each phase and external conditions, such as temperature or pressure, which is usually used in physical chemistry, engineering, and materials science fields.

In previous work, Reguera et al. [11] considered the phase diagram curve of polypentapeptide-water system (Figure 7A). When the protein concentration was lower than approximately 38%, the protein-water system was completely soluble as a single phase when the temperature was lower than 25 °C (region A). As the temperature was higher than 25 °C (the LCST), the solution became two phases from one phase (region D). One was the protein rich phase containing nearly 40% protein, and the other one was water rich phase existing poor protein molecular. When the protein concentration was higher than 38%, the solution was one phase before the temperature reached 25 °C. The protein molecular combined the water molecular forming coacervate when the temperature was higher than 25 °C. Reguera et al. [11] considered that when the protein content was almost or higher than 40%, the solution formed coacervate completely. Therefore, both the structure transition region (region B) and coacervate region (region C) were one phase which was protein rich phase, and no water rich phase appeared. However, when the temperature exceeded 60 °C, the protein molecular denatured by high temperature and could not hold the water molecular anymore. Therefore, the water molecular escaped from coacervate forming to the water rich phase, and the solution became two phases again (region E). Scientists proposed this phase diagram of the polypentapeptide-water system in this study. However, this phase diagram does not match with all ELP systems, particularly in the high protein concentration.
In Otto’s thesis \cite{13}, a (GVGVP)$_{40}$ solution phase diagram shows the relationship between transition temperature and concentration of solution (Figure 7B). Three different methods to determine the transition temperature in his study, it shows the trend of each different transition temperature with the increasing concentration protein solution concentration, which provided the basis data for studying the properties and applications of (GVGVP)$_{40}$ solution with different concentrations. However, it is not clear which method is better, and which phase diagram curve is the closest to the actual phase diagram curve. It is because of those limitation, we continue to study the phase diagram of ELP system in this research.
1.7 Scope and Significance of the Thesis

The phase transition behavior is the most important characteristic of ELPs, and brings more and more attention to ELPs. To study the relationship between the transition temperature and ELP solution concentration, and to better understand the process transition process, we designed these experiments and methods.

In this investigation, we expressed and purified (GVGVP)$_{40}$-foldon. Using UV-Visible spectrophotometry the transition temperatures were measured at several ELP concentrations spanning a range in concentration from 423 to 1 mg/ml. Phase diagrams were constructed relating $T_t$ to ELP concentration that were then used to better understand the phase transitioning behavior of ELP-foldon, especially at high ELP concentration and in pure water. These diagrams were also useful in the identification of special areas within the phase plane where further experimentation should be explored. To gain further insight into the behavior of the ELPs phase transition process, the transitioning process was observed under high magnification using a temperature controlled microscope.
CHAPTER II

MATERIALS AND METHODS

2.1 Overview

This chapter explains the methods and materials that are used in expression, purification, and characterizations of the ELP protein. The ELP expressed and purified for this research is the trimer forming (GVGVP)_{40}-foldon. After completion of the concentration measurements, the ELP samples were ready for the further analysis.

2.2 Protein Expression and Purification

The elastin-like polypeptide used in this study is (GVGVP)_{40}-foldon and its molecular weight calculated from its amino acid sequence (Figure 8) is 20,143 g/mol. The methods of protein expression and purification is based on previously published work \(^{[20,21]}\).
MGH(GVGVP)_{40}GWP-GYIPEAPRDGQAYVRKGDGEWVLLSTFL

Figure 8. (GVGVP)_{40}-Foldon amino acid sequence.

2.2.1 Expression

Before expression, Luria-Bertani (LB) media was prepared for cell growth. Four ingredients (Table 1) were mixed in a 2 L Erlenmeyer flask then autoclaved for sterilization (121 °C, 2 hours). After the media cooled to the room temperature, 100 mg of ampicillin was added.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Pure RO H₂O</td>
<td>1 L</td>
</tr>
</tbody>
</table>

A total of 20 ml of media, which had been prepared previously, was transferred into two culture tubes, each tube containing 10 ml media. Cells containing the plasmid for (GVGVP)_{40}-Foldon were transferred from glycerol frozen stocks. Then the culture tubes were incubated at 37 °C while being shaken on an incubated shaker table at 300 RPM. After overnight growth, approximately 12-16 hours, two 10 ml starter cultures were added to each liter of prepared media.
When the starter cultures were transferred into the media, which means the expression process was initiated, the media with cultures were incubated at 37 °C and 300 RPM shaking speed. Growth progress was monitored according to cell density using a BioMate 3 UV-Visible spectrophotometer. A sample of LB media before adding starter culture was used as a blank sample for measuring the cell growth. When the cell density measurement OD\textsubscript{600} reached 0.8-1.0, 240 mg of Isopropyl-Beta-D-thiogalactopyronoside (IPTG) per liter of media was added to induce the culture, initiating protein production. Then the solution was incubated for 4 to 5 hours at 37 °C while shaking at 300 RPM. After induction the cell cultures were divided into 3 centrifuge tubes per liter of culture and centrifuged at 4 °C and 8000 x g for 25 minutes using a Beckman J2-21 centrifuge. The supernatant was discarded and the bacterial pellets were kept at -20 °C for later purification.

2.2.2 Purification

The frozen cell pellets were taken out from the freezer and thawed at room temperature for 20 minutes. Next 10 ml of RO H\textsubscript{2}O was added in each centrifuge tubes for resuspension. Upon full re-suspension, by shaking, three samples were transferred to a single conical 50 ml falcon tube, then sonicated using a Fisher Scientific 550 Sonic Dismembrator. For sonication, the falcon tube was placed into a beaker full of crushed ice, and placed under the sonicator tip. The re-suspended cell solution was lysed by pulse sonic disruption (Cycle time: 10 secs on 20 secs off, 100% power) for a total of 90 seconds on. After sonication, the sample was transferred into a high-speed round bottom centrifuge tube and placed into the crushed ice to allow cooling.
In order to purify ELPs, the inverse transition cycling (ITC) method was used. This method utilizes the inverse transition temperature of ELPs. During the purification process, the protein solution was selectively separated from other contaminants by raising and lowering the solution temperature. Adding NaCl or adjusting pH makes the procedure easier and quicker. When the solutions temperature was below the $T_t$ of the ELP solution, the ELP was collected in the supernatant by cold centrifugation (4 °C), when the solutions temperature was higher than $T_t$, the ELP was collected in the pellets using warm centrifugation (40 °C).

The cell lysate solution was cooled down in a crushed ice bath for approximately 120 minutes. The sample was removed from the ice and centrifuged at 4 °C and 14,000 x g for 25 minutes to remove insoluble cellular matter. The supernatant containing the ELP was immediately transferred into a new centrifuge tube. Then the supernatant solution was incubated at 55 °C for 120 minutes. Because the temperature was higher than the $T_t$ of (GVGVP)$_{40}$-foldon, the ELP became insoluble. After 2 hours, the supernatant was centrifuged in a warm cycle at 40 °C and 14,000 x g for 25 minutes. Upon completion of the cycle, the supernatant was removed and stored elsewhere. The pellet containing the ELP was re-suspended in 10 ml RO water and cooled in crushed ice. Once fully re-suspended and cooled, similar with previous steps, the sample was once again centrifuged at 4 °C and 14,000 x g for 25 minutes. The ELP solution was separated from the insoluble material by decanting the solution into a new centrifuge tube. Purification proceeded by incubating this solution at 55 °C for 120 minutes followed by centrifugation at 40 °C and 14,000 x g for 25 minutes. The final pellet was re-suspended in 5 ml RO water, cooled in an ice bath for 120 minutes,
and centrifuged at 4 °C and 14,000 x g for 25 minutes. The final protein solution was filtered through a 0.2 µm syringe filter and transferred to a 15 ml falcon tube, completing the purification process.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>RPM (xg)</th>
<th>RO H₂O (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold I</td>
<td>4</td>
<td>25</td>
<td>14,000</td>
<td></td>
</tr>
<tr>
<td>Incubate</td>
<td>55</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warm I</td>
<td>40</td>
<td>25</td>
<td>14,000</td>
<td></td>
</tr>
<tr>
<td>Resuspend (Cold)</td>
<td>4</td>
<td>120</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cold II</td>
<td>4</td>
<td>25</td>
<td>14,000</td>
<td></td>
</tr>
<tr>
<td>Incubate</td>
<td>55</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warm II</td>
<td>40</td>
<td>25</td>
<td>14,000</td>
<td></td>
</tr>
<tr>
<td>Resuspend (Cold)</td>
<td>4</td>
<td>120</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Cold III (Final)</td>
<td>4</td>
<td>25</td>
<td>14,000</td>
<td></td>
</tr>
</tbody>
</table>

### 2.3 Concentration Measurements

The method used to measure the ELP concentration was developed by Gill and Von Hippel [49]. These authors provide a method to calculate the molar extinction coefficient, at 280 nm, of a protein based on its amino acid composition (Table 3), the extinction coefficients are in units of M⁻¹cm⁻¹.
Table 3 Values of the molar extinction coefficients of \((GVGVP)_{40}\)-foldon at 280 nm

<table>
<thead>
<tr>
<th>Model compound</th>
<th>Extinction coefficient at 280 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetyl-tryptophanamide</td>
<td>5690</td>
</tr>
<tr>
<td>Gly-L-Tyr-Gly</td>
<td>1280</td>
</tr>
<tr>
<td>Cystine</td>
<td>120</td>
</tr>
<tr>
<td>((GVGVP)_{40})-foldon extinction coefficient</td>
<td>13490</td>
</tr>
</tbody>
</table>

Using the data presented in Table 3, the molar extinction coefficient can be calculated from equation 1:

\[
\epsilon_M = a\epsilon_{M,Tyr} + b\epsilon_{M,Trp} + c\epsilon_{M,Crs}
\]

Equation 1

where \(\epsilon_{M,Tyr}\), \(\epsilon_{M,Trp}\) and \(\epsilon_{M,Crs}\) are the molar extinction coefficients of tyrosine, tryptophan, and cysteine residues at 280 nm (Table 3), and a, b and c are the number of each residue in one protein molecule.

The concentration of the ELP sample was determined using a BioMate 3 UVVisible spectrophotometer to measure the absorbance at 280 nm. First, using a quartz cuvette, a blank was measured with 1 ml RO water. Then 50 μl of RO water was taken out from the blank, replaced with 50 μl of the ELP sample and gently mixed by pipetting up and down. Once adequately mixed the solutions absorbance was measured in triplicate to ensure measurement stability and then the measured absorbance value was recorded.
The protein concentration was calculated based on the Beer-Lambert Law, equation 2, where:

\[
c = \frac{A}{\varepsilon L}
\]

*Equation 2*

\(c\) is ELP concentration, \(A\) is the measured absorbance at a 280 nm wavelength, \(L\) is the light path length, and \(\varepsilon\) is the molar extinction coefficients. For (GVGVP)\(_{40}\)-foldon, the extinction coefficient is 13,490 M\(^{-1}\)cm\(^{-1}\) based on the content of tyrosine and tryptophan in the peptide (Table 3).

### 2.4 SDS-PAGE Gel

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the purity and molecular weight of the protein\(^{[50]}\). After completion of purification, the final protein solution was run on an SDS-PAGE gel to confirm purity. First, a clean gel cassette was loaded into the container full of tris-glycine SDS buffer. Molecular weight marker ladder (5 μl) was loaded in the first lane, and the samples were loaded in all remaining lanes. The final sample (15 μl) was mixed with 5 μl of 4X sample buffer dye. In order to keep the formation of trimer structure, the (GVGVP)\(_{40}\)-foldon sample was run and loaded by a pipette into the gel. Electrophoresis was performed at 100 volts for approximately 80 minutes, stopping when the blue dye line crossed the bottom of the gel. Next, the gel was carefully removed carefully and gently from the cassette to the container, and washed with RO water two or three times. After discarding the wash water, the gel was soaked in GelCode blue stain reagents for at several hours. Following staining, the gel was washed with RO water three times.
then placed in water for preservation. The gels confirmed that all samples used throughout the course of this study were of high purity.

### 2.5 Preparation of Coacervate

For this study highly concentrated protein samples were used. Coacervates of (GVGVP)$_{40}$-foldon consist of approximately 42% protein by mass with the remainder water. The required sample volume for the most concentrated sample used in the transition temperature measurement was at least 0.25 ml. In order to achieve the required quantity of protein, a minimum of 6 L of LB media was required. Once enough protein solution had been purified, multiple samples were combined into the one falcon tube at 5 °C. In order to form the coacervate, the samples temperature had to be increased above the transition temperature. Usually, coacervate formation takes several days because of the coacervates viscosity. To decrease the time required for coacervate formation centrifugation at high temperature was used.

All transition temperature measurements were made in a nuclear magnetic resonance (NMR) tube, the protein sample was transferred into the NMR tube (Figure 9 A), then put in the incubator at 55 °C. After the solution became cloudy (Figure 9 B), it was centrifuged at 55 °C for approximately 10 to 15 minutes. In order to protect the centrifuge from overheating, the centrifugation time was set for no more than 15 minutes. Constant heating and centrifuging alternately was used, requiring a few days. Since the centrifuged solution was divided into two obvious phases, the upper layer being a clear water solution and the bottom layer a clear but viscous protein solution
(Figure 9 C), the upper supernatant fluid was discarded (Figure 9 D) and more of the original final protein solution was added into the tube. The previous steps were repeated on the original protein solution until enough coacervated protein sample was left in the tube.

2.6 Transition Temperature Measurements

To measure the transition temperature of (GVGVP)₄₀-foldon, a Shimadzu 1800 UV-Visible spectrophotometer with temperature controller was used. The sample was
loaded into a glass NMR tube which was placed in a polymethyl methacrylate (PMMA) cuvette and surrounded with water. In order to hold the top and bottom of the NMR tube in the PMMA cuvette, a rubber stopper and resin were used (Figure 10). An NMR tube cap was used to seal the tube and prevent sample evaporation.

Figure 10. PMMA cuvette with NMR glass tube used in transition temperature measurements. Resin to hold bottom of NMR tube in place, water filling void between cuvette walls and NMR tube walls, protein sample in the NMR tube, and rubber stopper to hold top of NMR tube in place.

The NMR tube instead of the PMMA cuvette was chosen to measure transition temperature for three reasons. First, when using a regular PMMA cuvette the volume required for analysis is approximately 2 mL. To prepare this amount of highly concentrated ELP would be expensive and difficult using the current laboratory setup. Instead, using the NMR tube apparatus, as shown above, required only a 250 μl sample size. Second, in the experimental procedure samples were measured from high to low concentration and therefore, it was more conducive and efficient for
diluting and mixing in an NMR tube than in a cuvette. Third, the NMR tube being removable for centrifugation, which was necessary to force the highly viscous protein samples to the bottom of the NMR tube.

During the measurement, two different constant temperature ramps of 0.1 °C/minute and 0.5 °C/minute were used when heating and cooling samples in the instrument. A wavelength of 550 nm was chosen because the highly concentrated protein absorbs too much light at lower wavelengths. To determine this wavelength a spectrum was obtained (Figure 11)\textsuperscript{[13]} over a temperature range from 25 to 60 °C.

![Figure 11. Absorbance with respect to wavelength for highly concentrated ELP. (Reprinted with Creative Commons License \textsuperscript{[47]} from reference [13]. Copyright OhioLINK Electronic Theses & Dissertations Center).](image)

Absorbance was measured as a function of solution temperature and ELP concentration. The purpose of this experiment was to determine the transition temperature of (GVGVP)\textsubscript{40}-Foldon as a function of ELP concentration. As the temperature was increased phase separation occurred resulting in solution turbidity and an increase in absorbance at 550 nm.
2.7 Microscope Measurements

To explore the phase transitioning process and phase behavior of ELPs, especially in highly concentrated ELP solutions, an optical microscope was used to observe the sample during its phase transition. An Olympus BX51W1 Microscope combined with a Hamamatsu Digital Camera C10600 was used (Figure 12 A), allowing the phase separation to be viewed on a computer monitor, making it more convenient to observe and record the data. Additionally, the sample data was recorded as a video, which provided a continuous data stream during the transition. A mechanical correction collar on the objective lens allowed for the correction of image distortion due to the cover glass thickness. A regular cover glass was used in this experiment, with a thickness of 0.17 mm, therefore the mechanical correction collar was set to 0.17 (The red circle in Figure 12 B).
Figure 12. (A): An Olympus BX51W1 Microscope combined with Hamamatsu Digital Camera C10600 were used to observe the phase separation process. (B): setting of the objective lens.

To observe the phase transitioning process of ELPs, it is necessary to heat samples above their transition temperature. However, there was no heating device or temperature control interfaced with the microscope. Therefore, we designed a heating sample holder (Figure 13).
Figure 13. A heating sample holder used in the microscope measurements. (a): hollow metal block with a hole in the middle. (b): resistive heating elements as the heating device. (c): printed suitable sized plastic plate. (d): an ordinary glass microscope slide was pasted on the bottom of the metal block. (e): a small hole was drilled on the surface of metal block near the middle hole.

The microscope sample heater was constructed from a 2x5x1/8 inch piece of aluminum with a 1/4 inch diameter hole drilled into its center. Two, 24 volt DC, Minco HK21747 resistive heating elements were attached to the bottom of the aluminum sample heater, and connected to a 12 volt DC power supply that was controlled by a PID type temperature controlled relay. Two pieces of ABS plastic plate were produced with a 3D printer, and inserted into the metal block from opposite sides, ensuring that the surface of the heating elements were in intimate contact with the aluminum sample heater to provide more efficient heat transfer. A glass microscope slide was attached to the bottom of the sample heater to allow the device to fit onto the microscope stage. To measure the sample temperature during heating, a small hole
was drilled on the surface of the metal block near the middle hole to place a
thermocouple, which would monitor temperature as a function of time.

Samples were made at different concentrations of (GVGVP)$_{40}$-foldon then
applied to the microscope slide, care taken to reduce the number of air bubbles
trapped between the cover glass and glass slide. For the measurement, the glass slide
was placed on the apparatus discussed previously (Figure 13), and the thermocouple
was placed between the glass slide and metal heat sink. Then the sample,
thermometer, and metal device were held together using tape (Figure 14). Finally, the
microscope slide heating device with sample was placed onto the microscope and data
was recorded while slowly changing the sample temperature.

Figure 14. Microscope slide heating device used to observe phase transition
behavior.
CHAPTER III

RESULTS AND DISCUSSION

3.1 UV-Visible Spectrophotometry Phase Diagram Analysis

The transition temperature of an ELP solution is defined as the point where ELPs associate and aggregate leading to a cloudy solution which is detectable by UV-Visible spectrometer. As a clear protein solution becomes turbid, there is a transition happening from a single phase to a two phase system. To better understand this phase transition behavior, a concentration – temperature phase diagram was constructed.

In previous studies \cite{51}, a sudden increase in absorbance was observed which quickly reached a maximum value, so the transition temperature was defined at the mid-point temperature between the onset of the turbidity and the maximum absorbance of the curve, equation 3.

\[
\frac{\text{Absorbance}_{\text{onset}} + \text{Absorbance}_{\text{max}}}{2} = \text{Absorbancer}_t
\]

Equation 3
This method works well for samples where the curve of absorbance vs. temperature exhibits a nearly vertical slope. However, this method cannot be applied to all curves. In some instances, the absorbance does not sharply increase with temperature. In these cases, a different method is necessary to determine the transition temperature. In this research, three different methods have been used to calculate the ELP transition temperature from absorbance vs. temperature data.

The (GVGVP)$_{40}$-foldon sample was analyzed by starting at high concentration 423 mg/ml, then was diluted a total of 17 times, resulting in 18 different ELP concentrations. Sample temperature was increased at two different ramp rates: 0.1 °C/min and 0.5 °C/min. For example, sample absorbance curves for (GVGVP)$_{40}$-foldon at concentration of 387 mg/ml were measured at two different ramp rates (Figure 15). The separation of the two curves is approximately 1 °C. Therefore, a 0.1 °C/min ramp rate was used to analyze all ELP concentrations greater than 20 mg/ml. However, when the concentration was lower than 20 mg/ml, it was found that both ramp rates resulted in similar results, with a deviation no greater than 0.3 °C. Since the lower ramp rate resulted in a greater amount of time required for analysis, 12 hours compared to the faster ramp rate of 2.5 hours, and provided no significant difference in data. In order to save time, the faster ramp rate was used to measure samples when their concentration was less than 20 mg/ml.
Figure 15. Sample absorbance curves for (GVGVP)$_{40}$-foldon at concentration of 387.15 mg/ml at 0.1 °C/min ramp rate (orange) and 0.5 °C/min ramp rate (blue).

Depending on whether there is a significant deviation in the absorbance prior to rapid increase in absorbance from the baseline, the 18 concentrations can be divided into four different behaviors (Figure 16). The complete data sets are presented in the Appendix.
Absorbance vs Temperature (℃)

Graph A:
- 423.61 mg/ml
- 387.15 mg/ml
- 361.16 mg/ml

Graph B:
- 328.73 mg/ml
- 264.07 mg/ml
- 243.53 mg/ml
- 208.28 mg/ml
- 189.32 mg/ml
Figure 16. Absorbance versus temperature for (GVGVP)$_{40}$-foldon. (A): concentrations of samples are from 361 mg/ml to 423 mg/ml. (B): concentrations of samples are from 189 mg/ml to 328 mg/ml. (C): concentrations of samples are from 27 mg/ml to 173 mg/ml. (D): concentrations of samples are from 1 mg/ml to 20 mg/ml.

It can be seen that in the two concentration ranges (Figure 16 B and D) the absorbance rose very rapidly and suddenly without a slow increase from the baseline. However, in the other two concentration ranges (Figure 16 A and C), the absorbance
gradually increased from the baseline starting between 1 to 3 °C prior to a rapid increase in absorbance. I refer to this slow rising process as a toe region, and it will be discussed in detail later. Due to the diversity of the curves the mid-point method (Eqn 3) which defined the transition temperature as the midpoint of the absorbance curve could not be applied to all of the curves successfully. Therefore, three different methods of calculation were used to determine the transition temperature of the ELP solutions, the tangent line method, the toe region method, and the slope method.

3.1.1 Tangent Line Method

The first method used to determine the transition temperature from absorbance vs. temperature data is called the tangent line method. This method calculates the transition temperature as the temperature at which the tangent line, occurring at maximum slope, crosses the temperature axis. To systematically determine the transition temperature in this manner, a tangent line was calculated for every three point. The intersection of these tangent lines with the x axis was plotted with respect to temperature. The transition temperature was defined as the temperature at which the maximum x intercept occurred (Figure 17 A and B).
Figure 17. Tangent line method for determining transition temperature. (A): the intersection of the tangent lines (solid red) with the baseline (solid black), the sample of (GVGVP)$_{40}$-foldon at 387.15 mg/ml at 0.1 °C/min ramp rate. (B): those intersections were plotted with respect to temperature, and the maximum intercept was the transition temperature, 37.2 °C for this sample.

For the two types of curves shown in Figure 18, in graph A, the absorbance rises slowly until approximately 37 °C, while in graph B the absorbance rises quickly from
the onset of transition, 30 °C. Since the tangent line method determines the transition temperature from the steepest slope, in graph A there is approximately a 3 °C difference between the onset of transition and the calculated transition temperature. In graph B, since the absorbance rises quickly from the onset of transition very little difference in calculated transition temperature, using this method, and the onset of transition is observed. This method was applied to all the curves (Figure 19). After introducing the others methods, these phase diagrams will be discussed in greater detail.
Figure 18. Sample absorbance curves for (GVGVP)$_{40}$-foldon with different concentrations. (A): concentration of 387.15 mg/ml at 0.1 °C/min ramp rate. (B): concentration of 189.32 mg/ml at 0.1 °C/min ramp rate.
3.1.2 Toe Region Method

As described earlier, the absorbance vs. temperature curves for some samples showed a toe region at the onset of absorbance from the baseline. The toe region is defined as the region of slow increase in absorbance with temperature that occurs before a more rapid increase in absorbance. In Figure 20, the red brackets represent the range in which the toe behavior was observed, and the red circle indicates the region where the initial deviation in the absorbance curve from the baseline occurred. All absorbance vs. temperature data curves were analyzed using this method, then based on this data a second phase diagram was created (Figure 21).
Figure 20. Toe region for (GVGVP)$_{40}$-foldon at 423.61 mg/ml at 0.1 °C/min ramp rate. The red brackets represented the range of the toe behavior is observed. The red circle indicated the onset of the absorbance from the baseline (zero).

Figure 21. Phase diagram created using the toe region method of analysis for (GVGVP)$_{40}$-foldon.
3.1.3 Slope Method

Typically, the determination of the onset of the toe region is a subjective method. To reduce the subjectivity, we developed a quantitative process to identify it, called the slope method. Similar to the tangent line method, this method uses the slope of the curve. In this method, the slope was calculated for each set of neighboring data points, then plotted with respect to temperature. For example, the concentration of (GVGVP)_{40}-foldon for the sample curve in Figure 22 was 387 mg/ml, and there existed a first point that had significant deviation from x-axis. This means that the absorbance had a spontaneous change compared to the data before. Therefore, this point was determined as the onset of the toe region. In this method, the transition temperature was determined as the temperature corresponding to the obvious initial deviation of the point from the x-axis. This method was used to analyze all of the data and a phase diagram was prepared (Figure 23).
Figure 22. Slope method for (GVGVP)$_{40}$-foldon sample at 387.15 mg/ml at 0.1 °C/min ramp rate. The obvious initial deviation observed was represented by the red circle.

Figure 23. Phase diagram created by the slope method for (GVGVP)$_{40}$-foldon.
3.1.4 Analysis of Phase Diagrams

By combining the phase diagrams obtained from the three methods of calculation (Figure 24), it is showed that the transition temperatures of the tangent line method and the toe region method have notable difference within some ranges of concentration. However, in other regions of the phase diagram, the transition temperatures have almost no difference and even overlap. This phenomenon is consistent with the visually apparent differences shown in the absorbance vs. temperature curves and can be divided into four regions depending on whether or not the sample curve has a toe region (Figure 16). When the curve has a toe region, the transition temperatures deviate. In those areas that no toe region is observed, the difference between the calculated transition temperatures is small. It can be seen that the calculations in transition temperature for the toe region method and the slope method are similar. Therefore, if the onset of the toe region needs to be used to determine the transition temperature, the slope method would be a better calculation approach because this method is less subjective.
Figure 24. Phase diagram for (GVGVP)$_{40}$-foldon using three different methods. Blue square: tangent line method. Red circular: toe region method. Green triangle: slope method.

There exists some interesting phenomenon in all of the phase diagrams. Referring to Figure 25 A, at a concentration of 328 mg/ml, the phase diagrams begin to deviate. For the three samples whose concentrations were greater than 328 mg/mL, after the sample was measured it was visually noticed that only a single phase existed (Figure 25 B right). However, for the samples whose concentrations were 328 mg/ml or less, after UV measurement the two phases were clearly observed (Figure 25 B left) with an upper water rich phase and a lower protein rich phase. As would be expected, with the decreasing ELP concentration, the volume of water rich phase increases and the volume of protein rich phase decreases until the protein concentration is too low to see the protein rich phase on the bottom of the NMR tube.
Figure 25. Change in behavior observed with increase in protein concentration. (A): two regions separated by the black dotted line is observed. (B): shown in left is a sample of (GVGVP)$_{40}$-foldon at 189.32 mg/ml after UV measurement at room temperature, and the right is a sample of (GVGVP)$_{40}$-foldon at 387.15 mg/ml at the same conditions.
3.2 Microscope Analysis of Phase Transition Behavior

For a better understanding of the difference in the phase behavior, optical microscopy is used to observe the phase transition process with different concentration samples. According to the data discussed before, four different regions were identified within the (GVGVP)$_{40}$-foldon phase diagram (Figure 24). To investigate the phase transition behavior within each phase region, one sample from each region was selected (Table 4). These samples were observed by microscopy while increasing and decreasing the temperature using the apparatus that was previously introduced (Figure 14).

Table 4 The concentration and property of four different (GVGVP)$_{40}$-foldon samples

<table>
<thead>
<tr>
<th>(GVGVP)$_{40}$-foldon sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>16 mg/ml</td>
<td>92 mg/ml</td>
<td>204 mg/ml</td>
<td>410 mg/ml</td>
</tr>
<tr>
<td>Toe region in absorbance curve</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

51
3.2.1 The (GVGVP)_{40}-foldon sample at 16 mg/ml

The first sample observed had a concentration of 16 mg/ml and is in an area of the phase diagram that does not display a toe region. The phase transitioning process of this sample occurs rapidly upon heating (Figure 26). At 32.7 °C a single phase exists, then at a temperature of only 0.2 °C higher small droplets begin to appear. This rapid transition is consistent with the rapid increase in turbidity observed in the absorbance curve. Therefore, the process of phase separation observed by the microscope, matches the curvature of an absorbance vs. temperature curve without toe region behavior.
Figure 26. The phase separate behavior of a (GVGVP)$_{40}$-foldon sample at 16 mg/ml. No change was observed at 32.7 °C, while many small drops appeared at 32.9 °C. The number of drops did not significantly change at 33 °C. The drops do increase in size as the temperature increases as is observed at 33.7 °C.

As the temperature was increased, droplet aggregation occurred forming several large drops (Figure 27). At this time, there are two different phases observed in the solution. One is the dispersed phase which is the droplets, and the other one is the continuous phase. It is reasonable to assume that the continuous phase is the protein poor phase because the concentration of the sample is 16 mg/ml (1.6% w/v). This corresponds to the volume of the appeared drops. When the temperature was held
at approximately 45°C, for the small drops, no movement or random motion of the drops was observed.

Figure 27. Microscope was used to measure the (GVGVP)$_{40}$-foldon sample at 16 mg/ml at 34.7 °C.

Based on the phase diagram (Figure 24), it can be determined that the transition temperature of the 16 mg/ml solution should be 30.3 °C (the phase diagram plotted by the tangent line method). However, when observed by microscope, the temperature that the solution starts changing is approximately 33 °C, and that is higher than the transition temperature shown on the phase diagram. This is attributed to the difference between measured and actual temperature of the sample in the microscope, with the three possible causes.

First, there is a separation between the temperature probe and the observed area of the sample. Although the temperature probe is placed close to the center of the glass slide there is still some space between them. If the temperature probe had been placed closer to the center of the glass slide it would have interfered with the
optical path of the microscope and would have added a significant slant to the glass microscope slide, making focusing of the microscope difficult. This can be observed in Figure 26, where not all of the image on the screen was in focus when the sample was measured. Second, it could be caused by the difference in thermal conductivity between the aluminum heat sink and the glass microscope slide. One side of the temperature probe is close to the glass microscope slide while the other side is close to the metal block. However, the sample does not touch the metal block. Therefore, the temperature around the thermocouple could be a little different than the temperature of the actual sample. Also, the heater is placed on the bottom of the metal block and based on the limitations of the microscope, the metal block does not have a container to insulate it from the ambient environment. Third, the temperature discrepancy can be attributed to the temperature controller. The temperature changes fast as the sample is heated, and the rate of temperature increasing cannot be easily controlled. Also, the temperature cannot be maintained at a constant value for an extended period of time. Irregardless of the cause, the temperature measured by the thermometer appears to be consistently around 2.5 to 3 °C higher than the transition temperature shown in the phase diagram. This temperature shift also appears in other samples that will be discussed later.

When heating is stopped, the sample is allowed to cool down at an uncontrolled rate. Shown in Figure 28, as the temperature decreases, the edges of the drops begin mixing with solution, and the drops become smaller until they disappear into the solution. With the temperature decreasing, those drops and the continuous water rich phase starts mixing together. It was observed that during cooling the droplets began to move across the observing area due to the slight slant of the microscope slide. This
sudden movement was interesting and can be explained by the lower viscosity of the droplets as water diffuses into their structure. Since the water concentration increases in the drops the protein concentration decreases, and these drops become light in color while the border between the two phases begins to fade. Finally, all of the drops disappear into solution and the whole sample becomes a uniform solution the same as before it was heated.

Figure 28. The temperature decreasing process of a (GVGVP)$_{40}$-foldon sample at 16 mg/ml.
3.2.2 The (GVGVP)$_{40}$-foldon sample at 92 mg/ml

The absorbance curve of the second sample (92 mg/ml) has a toe region. From the time when drops start appearing until the number of drops does not increase, there is a temperature range of approximately 2 °C (Figure 29). This phenomenon is different than the sample discussed previously. This corresponds well to an absorbance curve with a toe region, where within the range of the toe region the absorbance increases gradually. When the temperature is kept at a high level, these drops aggregate together and become larger, similarly to the lower concentration sample. In this solution, we believe the dispersed phase is the protein rich phase, and the continuous phase is water rich phase. With the higher protein concentration (10%), the volume of dispersed phase is larger than in the first sample whose concentration is 16 mg/ml.
Figure 29. The temperature increasing process of a (GVGVP)$_{40}$-foldon sample at 92 mg/ml.

**3.2.3 The (GVGVP)$_{40}$-foldon sample at 204 mg/ml**

The third sample is a higher concentration solution (204 mg/ml), whose absorbance curve does not exhibit a toe region. The drops appear quickly, over a short temperature range, and the amount of drops do not increase after they appear, which is similar to the 16 mg/ml sample (Figure 30). It can be seen clearly in this sample, the number of drops is much greater than the previous two samples, and the total volume of drops is larger too. It was also observed that the drops are not all in the same layer. Some drops are in contact with the top surface of the glass slide, while others adhere
to the bottom surface of the cover slide. Drops located on the same surface coalesce into large drops. However, drops that are located on different surfaces are not observed to coalesce. In addition, at the upper right corner of the observation area (Figure 30), there are two large isolated drops. Before heating the sample, the volume of those two drops was small and during cooling the drops did not dissolve into solution. During sample heating the volume of two drops noticeable increase, however, around these two drops, there exists a region where small droplets do not appear (Figure 30). It seems that a depletion zone is formed around these two drops.

Figure 30. The temperature increasing process of a (GVGVP)$_{40}$-foldon sample at 204 mg/ml.

These two drops appear to be the protein rich phase, because during the first of measurement these drops were not observed and only appeared after cycling the
temperature between low and high several times. It is probable that these drops were generated from inadequate mixing of the solution between temperature cycles. In the first temperature cycle, as temperature increased a protein rich phase separated from solution and coalesced into large droplets. As the temperature decreased these large drops dissolved but did not completely mix with the bulk solution. When the temperature was increased again, the regions which had not mixed with solution act as nucleation sites for aggregation of the protein rich phase. This phenomenon is similar to when samples are measured by UV-Visible spectrophotometry (Figure 25 B) in our NMR tube apparatus (Figure 10). When the sample concentration is 328 mg/ml or lower, after measuring by UV-Visible spectrophotometer, the solution in the NMR tube has two clearly distinct layers, and it is hard to mix these two individual layers together again.

Moreover, observing different area, the drops form not only on the top surface of the glass slide, but also on the bottom surface of the cover slide. Referring in Figure 31, when the temperature decreases slowly, those large drops will move slowly. During the movement, those drops combine with all small drops which touch those large drops, whether the small drops are on the top or bottom. Therefore, it is believed that this kind of large drops and the small drops both are protein rich phase, and the continuous phase is water rich phase.
Figure 31. The temperature decreasing process of a (GVGVP)$_{40}$-foldon sample at 204 mg/ml.

Small drops located on different layers are separated by a water rich continuous phase. For two small drops from different layers to coalesce they must overcome a huge resistance. Therefore, in the short observation time recorded in these experiment this event was not observed. This sample under the process of decreasing temperature is similar with other samples, the dispersed phase disappears in the solution.
3.2.4 The (GVGVP)₄₀-foldon sample at 410 mg/ml

The final sample observed by microscopy is a high concentration solution (410 mg/ml), and its absorbance curve has a toe region. Samples of such high concentration have high viscosity, making them difficult to load on the glass slide. For that reason, some air bubbles were present during the measurement of this sample. As measurement temperature was increased small drops began to appear slowly and after a 4 °C temperature increase, from the onset of phase separation, the amount of drops forming no longer significantly increased (Figure 32). This observation is similar to the sample whose concentration is 94 mg/ml, however, for this higher concentration sample, the temperature range over which phase separation is observed is larger.
Figure 32. The temperature increasing process of a \((\text{GVGVP})_{40}\)-foldon sample at 410 mg/ml.

While the sample is cooled, the process is observed to be similar to the previous cases. However, the viscosity of high concentration protein solutions is high, which results in the slow movement for small drops. In this sample, the content of protein is high, up to 40% in solution, and the volume of the dispersed phase is smaller than previous samples. This suggests that the continuous phase is the protein rich phase and the dispersed phase is the water rich phase. It is also observed that only a few
drops adhere to the bottom surface of the cover slide while most drops are held in solution. This observation can be explained that due to the sample’s high viscosity the drops were not able to rise to the surface of the solution. This phenomenon is consistent with the UV-Visible spectrophotometer measurements. After highly concentrated protein solutions were measured by UV-Visible spectrophotometer, a water phase layer on the top of the solution is not observed (Figure 25 B). We propose that this is the result of an inversion of the continuous phase.

### 3.3 Phase diagram analysis

By combining the phase diagram with the microscope images, it is observed that the samples whose absorbance curve have no toe region show from microscopy that phase separation occurs rapidly when the temperature is at the transition point. In the microscope images, this is evident from a dispersed phase rapidly appearing from the continuous phase and in the absorbance curve, absorbance reaches the maximum value in a short time. However, the samples whose absorbance curves have a toe region show from UV-Visible spectroscopy that the droplets appear over a wide temperature range as observed by microscopy.

Moreover, according to the observation from microscope, when small drops appear, the solution starts becoming two phases. Therefore, in considering the phase diagram curves proposed by the three different methods, the phase diagram created by toe region method is better than the other two, since it is more closely represents the actual phase diagram curve. When heating the sample of concentration at 204 mg/ml, it was shown (Figure 30) that a large number of drops appear in the
temperature range from 32.4 °C to 32.6 °C, indicating that phase separation occurred through spinodal decomposition. However, for the two large drops around the upper left corner, it observed that those drops getting bigger from 31 °C to 32.4 °C, which means the equilibrium (binodal) transition was below 32.4 °C. Therefore, for those no toe region areas in the phase diagram curve (Figure 33 B), we propose those parts of curve represent the spinodal curve. When heating the sample of concentration at 410 mg/ml (Figure 32), it was mainly observed that a few small drops appeared, then those drops growth and getting bigger, which is behavior consistent with nucleation and growth phenomenon. So for those having toe region areas in the phase diagram curve (Figure 33 B), we propose those parts of curve more closely represent the binodal curve.
Figure 33. (A) a simple example about the binodal and spinodal curves. The lowest black point is the lower critical solution temperature. (B) Phase diagram created by the toe region method for (GVGVP)$_{40}$-foldon, which divided into four different areas through black dash line.
Previous studies on aqueous poly(N-isopropylacrylamide) (PNIPAM) solutions show many similarities with those of ELPs solution, such as they are amphiphilic molecules, they both have LCST phase separation behavior in the solution, and they both can form hydrogen bonds with surrounding water molecules $^{[52,53]}$. Therefore, for the ELPs phase separation behavior in aqueous solution, it is considered that: when the temperature is lower than the transition temperature, because the hydrogen bonds formed between the protein molecule and neighboring water molecule, result in the ELP molecules to be in a stretched structure. When the temperature is above the LCST, water molecules associated with hydrophobic hydration are released from the ELP surface resulting in aggregation of the hydrophobic parts in the molecules. This aggregation results in a conformational change of the ELP molecule, changing from a stretch structure to a folded structure $^{[54]}$. 
CHAPTER IV

CONCLUSION

In this study, the elastin-like polypeptides (GVGVP)_{40}-foldon were expressed and purified, and it was made into the 18 samples with different concentrations, the highest concentration is 423 mg/ml. In the processing and analysis of data measured by UV-Visible spectrophotometer, several approaches were used, then several phase diagrams were constructed. In those phase diagrams, it was shown that the dependence of protein concentration on the transition temperature from single phase to a two phase system.

We used the microscope to observe the phase transition process of four (GVGVP)_{40}-foldon samples with different concentrations, it was found that they had different transition behavior. At high concentration, the water rich phase is the dispersed phase, and the protein rich phase is continuous phases; however, when concentration below 410 mg/ml, the protein rich phase is the dispersed phase, and the water rich phase is the continuous phase.
There are some deficiencies in this study, such as the poor temperature control system for microscope, and only using the UV-Visible spectrophotometer to measure the transition temperature. It is suggested that further work to be done using the other measured techniques such as NMR or calorimetry to collect the data about the transition temperature of (GVGVP)$_{40}$-foldon protein. In order to clearly understand the phase transition process, especially in the region on the phase diagrams between the tangent line method and the toe region method, a more accurate and appropriate phase diagram may be constructed. In the future research, using a fluorescent material to label the (GVGVP)$_{40}$-foldon protein molecules, which would possibly allow the definitive identification of the phase using fluorescence microscopy.


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APPENDIX