APPLICATION OF ELASTIN-LIKE POLYPEPTIDES

IN VIVO AND *IN VITRO* APPLICATION OF ELASTIN-LIKE POLYPEPTIDES

By

XIN GE, B. Eng., M. Eng.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

Doctor of Philosophy (2008) McMaster University

(Chemical Engineering) Hamilton, Ontario

TITLE: Inverse Phase Transition of Elastin-Like Polypeptide: Characteristics and Applications

AUTHOR: Xin Ge

B. Eng. (Tsinghua University, Beijing, China)

M. Eng. (Tsinghua University, Beijing, China)

SUPERVISOR: Professor Carlos Filipe

NUMBER OF PAGES: xii, 136

Abstract

Elastin-like polypeptides (ELP) are artificially designed protein biopolymers that can be produced by living organisms. These proteins have the unique ability to undergo reversible inverse phase transition, in response to changes in temperature and/or addition of chaotropic salts. Below the transition temperature (T_t), ELP is soluble in water. Increasing the temperature above T_t, ELP coacervates into an aqeous ELP-rich phase. In this thesis, this unique feature of ELP was used in for recombinant protein purification and for the formation of aqueous multiple-phase systems.

For protein purification, ELP was fused with an intein and a model protein (thioredoxin), to demonstrate a simple and inexpensive approach for recombinant protein purification. The ELP tags replace the chromatographic media and the intein replaces the use of the protease in conventional methods. Using ELP tags was found to be consistent with large -scale recombinant protein production/purification by purifying an ELP tagged protein using a stirred cell equipped with a microfiltration membrane. When the temperature and/or salt concentration is increased for mixtures containing free ELP and ELP tagged proteins, simultaneous phase transition takes place. This served as the basis for the development of a method suitable for selectively recovering molecules from complex mixtures with high specificity, full reversibility, and virtually unlimited affinity.

The second parts of this thesis focus on the ability of ELP to form aqueous two-phase systems (ATPS) *in vitro* and most importantly, *in vivo*- with the formation of

aqueous microcompartments in living cells. These compartments exclude the protein making machinery of the cell, acting as depots for newly expressed protein. It is also shown (*in vitro*) that ELP based droplets exclude proteases, protecting proteins from degradation. These observations are important for high-level production of recombinant proteins. Also described, is the formation of protein based aqueous multiphasic systems, with tunable morphologies.

Acknowledgements

I would like to express my strongest appreciation to my advisor, Dr. Carlos Filipe. Thank you for your countless support, encouragement and help given to me during my studies at McMaster. He guided me not only on how to conduct the research, but also on how to identify the importance of the research that we were working on. He allowed me a lot freedom and always encouraged me to develop new and independent research ideas. In addition to our professional relationship, he helped me on a personal level. Specifically, I really appreciate him for his help while I was in the hospital. He also helped me to find extra financial aid, which was important for my young family. Overall, from the bottom of my heart, I feel lucky to have him being part of my life. He will act as my role model for my professional career as well in terms of personality.

I would also like to thank my PhD committee members, Dr. Raja Gosh at Chemical Engineering Department, and Dr. Daniel Yang at Biochemistry Department. They constitute true examples of how brilliant and enjoyable scientists can be. Thank you for continuously motivating me.

Most importantly, I have to thank my wife Lining Ren. She decided to join me here in Canada, and made it possible for me to pursue my dream and to be my very best in future. You are my soul mate and you give me unconditional support, during good times and during bad times. Thank you for your love and friendship.

I also had a lot of help from many researchers at McMaster, which made it possible for me to finish my experiments. Thanks go to Victor Pau and Michael Yuchi for countless discussions and helps. I would also like to thank Dharmesh Kanani for his help with the AKTA equipment, and Dr. Todd Hoare for his help with light scatting measurement. Thanks to Lina Liu for ^{125}I labeling, and radio-counting experiments and Dr. Tony Colin for training me and helping me with confocal microscope imaging. Thanks go to Dr. Razvan Nutiu and Dr. Yingfu Li for the initial work on aptamer and NIPPAM. I would also like to thank Dr. Rebort Pelton and Dr. Harald Stöver for their insightful comments on ATPS formation and nanocolloid fabrication. Finally, I would like to acknowledge Sherry Xie, Kun Zhang, Jessie Xu and Han Liu for their help in maintaining the lab.

I would like to thank Nilesh Patel for his friendship and for the good time we spent together in lab. There are also many friends both in and out McMaster that made life in Hamilton memorable.

I thank Dr. Ashutosh Chilkoti, from the Department of Biomedical Engineering, Duke University, for the donation of the plasmids encoding ELP sequences.

I would like to thank the China Scholarship Council and the Ontario Ministry of Training, for providing me scholarships. Finally, I would like to thank NSERC for providing research funding to Dr. Filipe, which allowed me to purchase equipments, reagents and supplies for research.

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Chapter 1

Introduction

Natural polymers, such as proteins, are most well known for their ability to perform functions that make cells work, including reaction catalysis, duplication of genetic material, transport of molecules across the cell membrane and various other functions essential for life. Artificially designed polypeptides are macromolecules that enable bridging the gap between natural based polymers and their synthetic counterparts. A parallel between the world of synthetic chemistry and the world of protein polymers can be found by looking at how some polymers (synthetic and proteins) respond to temperature changes.

Certain polymers, such as Poly(N-isoprpylacrylamide) (PNIPAAm) respond in a peculiar way to changes in temperature. Below a certain temperature, the lower critical solution temperature or LCST (32 °C), the polymer is soluble. As the temperature is increased above the LCST, the polymer switches from a fully soluble hydrophilic random coil to a hydrophobic globule, resulting in the formation of insoluble particles (Pelton and Chibante, 1986).

A similar phenomenon is found in the natural protein tropoelastin: it has the ability to self-aggregate, or more specifically, it is able to coacervate (Vrhovski and Weiss, 1998). Coacervation consists of a phase separation and it occurs as a result of an

increase in temperature above a critical level, which leads to the separation of the tropoelastin from the solvent as a second phase (Miao et al., 2003). Tropoelastin molecules can covalently cross-link with each other leading to the formation of elastin, which is prevalent in the human body to provide elasticity.

Sequencing of the protein tropoelastin revealed that it contains repeating domain structures with alternating hydrophobic and cross-linking regions (Vrhovski and Weiss, 1998). The cross-linking domains contain numerous lysine residues that lead to the formation of covalent intermolecular networks, which stabilize the polymeric matrix. The hydrophobic domains of tropoelastin are rich in nonpolar amino acids (mainly glycine, valine, proline, alaine, leucine, etc.), present as tandem repeats (Miao et al., 2003). There is substantial evidence that the hydrophobic domains are necessary for self-aggregation (Urry et al., 1992b; Morelli et al., 1993; Reiersen et al., 1998).

A group of biopolymers, designed by drawing inspiration from the sequences found in the hydrophobic domains of tropoelastin, has been developed and these are referred to as Elastin-Like Polypeptides (ELPs). ELPs are composed of repeats of the five-amino-acid units, Val-Pro-Gly-Xaa-Gly, where Xaa can be any amino acid expect proline (Urry 1988, 1992a, 1997a). ELP undergoes a sharp inverse phase transition in response to temperature (similar to PNIPAAm). Below the phase transition temperature (T_t), a solution containing ELP is clear and homogeneous but it becomes turbid upon increasing the temperature above T_t. This process is reversible.

Three mechanisms are believed to be responsible for the ability of ELP to undergo phase transition: hydrophobic hydration, conformational changes of ELP, and intermolecular hydrophobic interactions.

Hydrophobic hydration is a phenomenon that occurs when a non-polar molecule is present in an aqueous solution and water molecules shell the hydrophobic regions of the solute. Urry et al. (1997b) measured the number of hydrophobic hydration water molecules (N_{hh}) associated with ELP and found that a large number of interacting water molecules are involved. It was estimated that on a *per* pentamer basis, $N_{hh} \sim 80$ for $(GVGVP)_n$ and $N_{hh} \sim 240$ for $(GVGIP)_n$. By raising the temperature above T_t, the values for N_{hh} decrease to values close to zero, indicating that the hydration water becomes bulk water.

The secondary structures of various ELPs have been probed using a variety of spectral measurements, including Raman spectroscopy (Prescott et al., 1987), circular dichroism (CD) (Arad et al., 1990), solution nuclear magnetic resonance (NMR) (Urry et al., 1989), X-ray diffraction (Cook et al., 1980), solid-state NMR (Hong et al, 2003), and infrared spectroscopy (FTIR) (Serrano et al., 2007). These studies, together with the results of molecular dynamics simulations (Li et al., 2001), provide consistent evidence that ELP experiences significant conformational changes during phase transition. Below T_t, ELP molecules have no defined secondary structures (random coil), while above T_t, ELP molecules fold and form a repeated ß-turn structure termed ß-spiral (Urry, 1992a, 1997a).

Based on these findings, a well-accepted interpretation of the phase transition behavior of ELP has been established. At $T<T_t$, the hydrophobic hydration shield makes ELP fully extended and soluble in aqueous solution. At $T>T_t$, ELP looses its hydration water and simultaneously folds into a ß-spiral structure, in which the apolar side chains are facing outwards. As a result, the interactions between hydrophobic ELP molecules trigger coacervation. The process of coacervation has been described as two step process: "hydrophobic folding and assembly transition" (Urry, 1997a). Yamaoka et al. (2003) proposed that these two steps are highly integrated explaining the reason why the T_t of ELP is dependent on its concentration.

The process of coacervation can also be analyzed from a thermodynamic point of view. Luan et al. (1990) measured the energy change (ΔH) of this process using differential scanning calorimetry (DSC) and found that this is an endothermic process ($\Delta H>0$). By raising temperature, ELPs fold into a more ordered structure and assemble together, thus the entropy associated with the ELP molecules is decreased ($\Delta S_{ELP}<0$), a process that seems to go against the second law of thermodynamics. However, when the entire system is considered, accounting not only for the ELP but for the water molecules as well, it is clear that the second law of thermodynamics is indeed obeyed. The number of water molecules involved in hydrophobic hydration is so large that when they become bulk water, the overall entropy of the system increases ($\Delta S_{sys}>0$). When the temperature rises above a certain point, the coacervation process is thermodynamically favorable and it will happen spontaneously ($\Delta G=\Delta H-T\Delta S<0$).

Early studies using ELPs (mainly before 1990) relied on biopolymers synthesized through synthetic organic chemistry approaches. Advances in recombinant DNA technology have been exploited to produce protein-based biopolymers more efficiently and precisely (Cappello et al., 1990; Krejchi et al., 1994). McPherson et al. (1992; 1996) and Guda et al. (1995) were the first to report successful expression of ELP in *Escherichia coli*. Meyer and Chilkoti (2002) made further developments on ELP expression, by generating libraries of ELP with different lengths and amino-acid compositions.

Protein purification. Meyer and Chilkoti (1999) observed that when ELP was fused with a recombinant protein, the ELP tag maintained the ability to undergo reversible phase transition. This finding lead to the development of a simple method for protein purification, termed inverse transition cycling (ITC). The basis of ITC is to selectively induce coacervation of ELP tagged proteins by applying an environmental stimulus (such as a temperature increase or salt addition), followed by isolation of the coacervates (ELP fusion proteins) from other proteins present in the cell lysate by centrifugation. Since phase transition is reversible, lowering temperature or decreasing ionic strength of the buffer can resolubilize these isolated coacervates. This process is usually repeated several times to achieve a high degree of purity of the ELP tagged protein. Since ITC was first demonstrated (Meyer and Chilkoti, 1999), this technique has been successfully applied to purify several different recombinant proteins, with high purity and high recovery (Stiborova et al., 2002; Shimazu et al., 2003; Trabbic-Carlson et al., 2004a; Banki et al., 2005).

Biomedical applications. Due to its biocompatible nature, ELPs have been used in a variety of biomedical applications. Meyer et al. (2001a) developed a thermally responsive drug delivery method for solid tumor treatment. Betre et al. (2002) applied ELP coacervates as scaffolds to culture mammalian cells and found that the cells maintained proper morphology and cellular activity.

Bioremediation. Kostal et al. (2001) produced ELPs fused to hexahistidine clusters, which bind Cd^{2+}, for removing heavy metals from dilute waste streams using thermally triggered phase transition. A similar approach has been developed for the removal of organophosphorus compounds, by producing ELP fused to organic phosphate hydrolase (OPH) and using phase transition to immobilize the fusion enzyme on hydrophobic surfaces (Shimazu et al., 2003).

Surface immobilization and bioanalytical applications. Frey et al. (2003) demonstrated that an ELP fusion protein could be reversibly immobilized onto hydrophobic surfaces. This finding used to reversibly capture / release proteins at the nanoscale (Hyun et al., 2004), and more generally for bioanalytical assays, *e.g.* highly sensitive immunoassay (Kim et al., 2005; Gao et al., 2005); scaffolds for protein microarrays (Nath and Chilkoti, 2003; Zhang et al., 2005; Jenikova et al., 2007); ELP adsorbed on functional gold nanoparticles for sensing (Nath et al., 2001).

Molecular machinery. The reversible conformational changes of ELP and the associated mechanical contraction / extension associated with phase transition enable the use of ELPs as molecular machines. Megeed et al. (2006) produced single-chain antibodies (scFv) with controlled binding / release of a target by placing an ELP-based

peptide linker between the V_L and V_H domains of scFv. Jung et al. (2006) have integrated single ELP loops within the cavity of an ion channel, and successfully constructed a protein based open / close switch that responds to environmental stimuli.

This thesis focuses on applying ELP in two different areas: (1) further development of novel methods for recombinant protein purification (Chapter 2, 3, 4); (2) *in vitro* and *in vivo* generation of protein-based aqueous two-/multi- phase systems (Chapter 5, 6).

Protein purification

Self-cleavable stimulus-responsive tag

Large-scale protein purification usually requires chromatographic separation of the final product, which can be expensive and time-consuming. The fact that elastin-like polypeptides (ELPs) retain their inverse phase transition behavior when fused to other proteins, enables purifying recombinant proteins without the need of chromatography (Meyer and Chilkoti, 1999). However, there was a difficulty associated with previous reports on ELP based separation of recombinant proteins (Stiborova et al., 2002; Shimazu et al., 2003; Trabbic-Carlson et al., 2004a)- a protease was required to cleave the target protein from the ELP tag. Three problems are associated with the use of a protease: (1) it adds additional cost to the purification process; (2) it adds an additional purification step to remove protease; and (3) due to their limited specificity, proteases can attack the target protein.

Inteins (initially called protein introns) are internal polypeptide sequences that are posttranslationally excised from a protein precursor by a self-catalyzed protein-splicing reaction (Perler et al., 1994). Investigations of the splicing mechanism (Chong et al., 1996) and the determination of the crystal structure of inteins (Duan et al., 1997) showed that mutating specific catalytic residues resulted in the disruption of the tightly coupled splicing process, leading to the cleavage of the peptide bond at the N- or C- terminal splicing junction (Chong et al., 1997).

For recombinant protein purification, inteins have been used as self-cleaving elements that can replace protease recognition sites. New England Biolabs developed and commercialized the IMPACT system (Intein Mediated Purification with an Affinity Chitin-binding Tag; Chong et al., 1998; Southworth et al., 1999), which makes use of inteins for recombinant protein purification. Furthermore, Wood et al. (1999) used directed evolution to engineer an intein with pH-sensitive C-terminal cleavage activity for protein purification. Commercially available intein based systems still require the use of affinity columns for isolation of the tagged proteins, which adds to the total cost of protein purification.

The objective of the research in Chapter 2 was to establish a protein purification method, which provides a general solution to the problems associated with the use of both chromatography and proteases. We hypothesized that ELPs and inteins could be used in a compatible manner and be part of a single system- their functions could be activated sequentially, in an independent and controlled manner: use the ELP tag to purify the fusion protein and then activate the intein to cleave the ELP tag from the target

protein. In Chapter 2, it is shown that this system works well. The attractive features of the technique developed in this study are its simplicity and low cost associated with the purification. This technique can be used at the lab-scale but it also has high potential for scaling-up and production of recombinant proteins at the industrial level.

A scalable membrane based process

Using intein-ELP tags provides a simple, rapid and economical method of protein purification, however, to allow its application at the industrial level, it is necessary to further demonstrate scalability. Additionally, in lab-scale studies, especially in proteomics, researchers increasingly find the need to handle a large number of protein samples at the same time. In Chapter 3 of this thesis, it is shown that the previously studied self-cleaving ELP system (Chapter 2) is suitable for large-scale recombinant protein production, and it also has the potential to treat a large number of samples in a parallel manner.

It was observed that when phase transition takes place, ELP forms coacervates with diameters ranging from hundreds of nanometers to a few micrometers (Meyer et al., 2001b, Meyer and Chilkoti, 2002). Ghosh (2004) found that a membrane-based technique could be applied to separate ammonium sulfate induced reversible precipitations of human immunoglobulin G. We hypothesized that a similar microfiltration process could be established for purification of ELP tagged proteins, in a process in which these microscopic coacervates of ELP fusions could be easily retained by a microfiltration membrane, while other contaminating proteins would pass through the membrane.

The major advantages of this membrane based separation process are: (1) microfiltration membranes are inexpensive; (2) these membranes can be used at high fluxes to achieve high productivity; (3) membrane based processes are suitable for application in large scale production; (4) the proposed process is amenable for parallel operation, *e.g.* placing a microfiltration membrane sheet on a 96-well plate for multiple sample operation.

The results in Chapter 3 show that the microfiltration/ELP based purification method is amenable to massive parallelization in high-throughput studies and it is a very simple method for recombinant protein purification in industrial applications.

Simultaneous phase transition between ELP and ELP tagged molecules

ELP based protein purification, i.e. inverse transition cycling (ITC), has been demonstrated in the past with proteins that were expressed to high levels (Meyer and Chilkoti, 1999; Trabbic-Calson et al., 2004a; Banki et al., 2005). Poor expression levels are expected for toxic proteins (Studier, 1991; Cinquin et al., 2001), complex multidomain proteins (Turner et al., 2004; Martins et al., 2003), or in some cases of heterelogous protein expression (Georgiou and Valax, 1996). In proteomics, massive numbers of proteins are expressed in parallel, and optimizing expression conditions for each one of them is not feasible.

Affinity based purification systems are commonly used to capture molecules that are present at low concentrations in a complex mixture. There are, however, two main drawbacks associated with affinity based systems: (1) the higher the affinity level

between the target and the ligand, the more difficult it is to release the target molecule from the ligand; (2) affinity systems are associated with the use of a surface onto which the ligand is coupled. If a large surface area is used (such as that associated with chromatographic resins), there is high potential for nonspecific and irreversible binding of either contaminating molecules or the target.

The objective of Chapter 4 was to develop a highly efficient capture method that can be used when it is necessary to purify proteins present at very low concentrations. This method also to address the two problems associated with affinity based systems, since the new method is based on a process that is fully reversible and exposes the sample to an absolute minimum surface area.

In preliminary studies we found that for very low concentrations of ELP tagged proteins, ITC in its original format did not allow recovery of the proteins. This result is consistent with the fact that that phase transition is a concentration dependent phenomenon (Meyer and Chilkoti, 2004). We hypothesized that if an ELP tagged molecule is present in a solution at a very low concentration, adding an excess amount of free ELP to the sample and inducing phase transition should result in hybrid coacervation, via the interaction of the ELP moieties of the two molecules. The work presented in Chapter 4 is a quantitative study of simultaneous phase transition using free ELP as a coacervation additive. There we demonstrate: (1) the efficiency of the method by capturing an ELP tagged recombinant protein present at different concentrations (ranging from μM to as low as pM); (2) the specificity of the method by spiking crude cell lysate with the ELP tagged protein to a final concentration of 1 nM, a concentration

that is approximately equal to the presence of a single molecule of the recombinant protein per *E. coli* cell.

The results of this research are particularly useful in high-throughput proteomic studies, where small amounts of poorly expressed proteins need to be recovered for analysis, *e.g.* by ultra sensitive mass spectrometry (Mann et al., 2001). In a more general context, the concept presented in this study provides a method that is highly efficient, specific, and fully reversible and it should have potentially wide ranging applications in many diverse areas.

Aqueous two-/multi- phase system

There is strong evidence that hydrophobic dehydration, conformational change and hydrophobic interaction, all work together and result in the coacervation of ELP. "Phase transition" is usually used to describe the phenomena in which ELP separates out of an aqueous solution and forms an additional phase. However, a fundamental question has not been clearly answered yet: what is the nature of the phase of the ELP coacervates? A solid phase (as the polymer analogy, PNIPAAm), liquid phase, "coacervate phase" (Urry et al., 1985; Meyer and Chilkoti, 2004), "hydrophobic-to-hydrophilic phase transition" (Nath and Chilkoti, 2001; Hyun et al., 2004), "soluble-insoluble phase transition" (Kostal et al., 2001)? The essence of the ELP coacervate is also surrounded by uncertainty in the literature, which include descriptions such as *particle* (Meyer et al., 2001b; Zhang et al., 2003; Rodríguez-Cabelloet al., 2005), *aggregate* (Urry, 1997a; Shimazu et al., 2003; Yamaoka et al., 2003; Meyer and Chilkoti,

2004), *droplet* (Kaibara et al., 2000; Flamia et al., 2004; Zhang et al., 2006), *glue* (Urry, 1997a), *gel* (Serrano et al., 2007), *crystalline* (Urry, 1992a), *insoluble precipitate* (Banki et al., 2005), etc. The second half of this thesis (Chapter 4, 5) aims to address this conceptual blur. After clarifying the nature of this ELP coacervates, results are presented on some applications that originated from having a clear and unambiguous description of the physical nature of the ELP coacervates.

Clarification of the nature of the coacervates formed upon phase transition with ELP is critical to direct bioengineering applications with ELPs. For example, we tried to use confocal microscopy and a laser to develop an *in vivo* labeling technique at the single cell scale. It was based on the assumption that above T_t, ELPs form insoluble particles (solid phase). In preliminary studies, we found that phase transition took place inside of *E. coli* expressing high levels of an ELP - green fluorescent protein (GFP) fusion protein. We used a laser to bleach regions in the cytoplasm of the cell that contained the ELP-GFP intracellular coacervates and obtained a result that clearly demonstrated that our initial assumption of solid phase coacervates was incorrect. It was found that when the coacervates were partially bleached, fluorescence was rapidly recovered during Fluorescence Recovery After Photobleaching (FRAP) experiments. FRAP is a technique used to study the mobility of proteins (Lippincott-Schwartz et al., 2003). This finding revealed that the coacervates were liquid and that the protein in the coacervate was mobile and not in the format of insoluble particles.

ATPS in vivo

Urry et al. (1985) found that ELP coacervates have high water contents, i.e. 63% water and 37% polypeptide by weight. Kaibara et al. (2000) used optical microscopy to observe the coacervation of α-elastin, and Flamia et al (2004) used AFM to study ELP. All these results suggest the formation of droplets. Furthermore, Zhang et al. (2003; 2006) used dark field microscopy to study the kinetics of coacervation in an on-chip linear temperature gradient, and proposed the "one component aqueous two phase system (ATPS)". Based on these results, together with our findings of the mobility of the assembled ELP, we hypothesized that coacervation of ELP leads to the formation of ATPS both *in vitro* and *in vivo*. Furthermore, the fact that ELP tags can be used for recombinant protein purification suggest that ELP coacervates exclude other molecules present in the cell, therefore we also hypothesized that ATPS could lead to the formation of functional micro-compartments inside living organisms, separating protein synthesis from protein storage in the cytoplasm of a cell.

As a clean alternative for traditional water-oil two-phase system, ATPS (aqueous two-phase system) are composed of two water-based immiscible phases. Examples of this phenomenon usually involve two polymers, such as dextran and PEG (Piculell and Lindman, 1992) or a single polymer plus a high concentration of the appropriate salt (Ananthapadmanabhan and Goddard, 1987).

The objective of the study reported in Chapter 5 is to provide clear evidence that ELP forms ATPS both *in vitro* and *in vivo*. Results of this study are important to provide theoretical guidance for applications of ELP in recombinant protein expression.

Additionally, in the interior of living cells, there are differences in local composition that have profound implication for cell function (Ovadi and Saks, 2004; Campanella et al., 2005). Up to now, there was no an experimental model system available to study the phenomenon of microcompartmentalization in living cells (Pielak, 2005; Long et al., 2005). Such a system is reported in Chapter 5, where functional microcompartments were created in living microorganisms, which could also have a significant impact on efforts currently being made to generate "synthetic cells" (Koonin 2000; Long et al., 2005).

Protein-based aqueous multiple-phase system

Changing external conditions and/or the composition of ELP molecules can modify the phase transition temperature of ELP. The external conditions effecting T_t include salt type and concentration (Urry, 1992a; Yamaoka et al., 2003), pH (Urry, 1992a), pressure (Urry, 1992a; Yamaoka et al., 2003) and ELP concentration (Yamaoka et al., 2003; Meyer and Chilkoti, 2004). There are two ways of changing T_t based on the composition of ELP: fusing a partner protein to ELP (Trabbic-Carlson et al., 2004b), and changing the length (Meyer and Chilkoti, 2002; 2004) and amino acid sequence of ELP, especially at the guest residue site (Urry et al., 1991; Meyer and Chilkoti, 2002). Because different T_t are associated with different species of ELP molecules, we propose that aqueous multi-phase systems (AMPS) could be formed by mixtures of different ELPs.

Dynamic light scattering studies revealed that the size of ELP droplets in emulsions range from ~200 nm to a few micrometers (Meyer et al., 2001a; Meyer and Chilkoti, 2002; Figure 3.2B in the thesis). The size and size distribution of elastin-based

droplets can be manipulated by changing protein concentration and temperature (Kaibara et al., 2000; Meyer and Chilkoti, 2002). The ELP droplets spontaneously form a separate ATPS (Chapter 5). The kinetics of this process were recently studied and it was found that adding surfactant such as SDS makes the emulsion stable for several hours (Zhang et al., 2003). These findings allow one to generate, in a controllable manner, protein-based nanocolloids that have reversible stimulus-responsive behavior. Thus, we also proposed that the formation of AMPS could enable the production of protein based anisotropic colloids composed of ELP species.

Controlling the material distribution in ultra-small scales is the key challenge in nanotechnology. Synthesis of multi-compartment nanoparticles has allowed the micro-fabrication of biphasic and triphasic Janus droplets by simultaneous microfluidic jetting of parallel polymer solutions in an electric field (Roh et al., 2005; 2006) or through sheath flowing (Nisisako and Torii, 2007). To date, all the fabricated anisotropic nanoparticles are composed of synthetic polymers, not proteins. Due to the advantages of recombinant biopolymers, such as the exact chain-length and biocompatibility, these protein-based materials have recently gained more and more interest for biotechnology applications (Sarikaya 2003, Banta 2007). In Chapter 6 of the thesis, the formation of polypeptide based aqueous multi-phase systems and anisotropic nanocolloids is demonstrated for the first time.

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Chapter 2

Self-Cleavable Stimulus Responsive Tags for Protein Purification without Chromatography

This chapter is based on Ge, X. *et al.* (2005). *Journal of the American Chemical Society* **127**(32): 11228-11229.

2.1 Abstract

A simple method to purify recombinant proteins is described by fusing a target protein with an intein and an elastin-like polypeptide that only requires NaCl, dithiothreitol, and a syringe filter to isolate the target protein from *Escherichia coli* lysate. This tripartite fusion system enables rapid isolation of the target protein without the need for affinity chromatography for purification or proteases for cleavage of the target protein from the fusion. The elastin-like polypeptide tag imparts reversible phase transition behavior to the tripartite fusion so that the fusion protein can be selectively aggregated in cell lysate by the addition of NaCl. The aggregates are isolated by microfiltration and resolubilized by reversal of the phase transition in low ionic strength buffer. After resolubilizing the fusion protein, the intein is activated to cleave the target protein from the elastin-intein tag, and the target protein is then isolated from the elastin-intein fusion by an additional phase transition cycle.

2.2 Introduction

The need for simple methods to purify recombinant proteins is increasingly urgent and is driven by two technological imperatives. At the laboratory level, the advent of proteomics and high-throughput structural biology has created an urgent demand for simple methods to express and purify recombinant proteins. Although the use of affinity tags in combination with chromatographic separation has gained widespread use for purification of recombinant proteins, affinity tags have several problems for lab-scale purification of recombinant proteins: (1) they require chromatographic separation to achieve high purity, which limits their application in a high-throughput setting, and (2) when implemented in batch mode, the level of purity is typically insufficient for structural biology. At an industrial scale, an increasing number of protein and peptide therapeutics are in the development pipeline, and the emphasis on reducing their cost of production provides a strong impetus for the development of economical and large-scale protein purification methods. Chromatographic separation of proteins is, in general, difficult to scale up, and in the case of affinity chromatography, the cost of the resin is prohibitive at a large scale. Another significant problem with the use of most affinity tags is that a protease is usually required to cleave the target protein from the affinity tag. Three problems are associated with the use of a protease: (1) it adds additional cost to the purification process, (2) it adds an additional step to the purification process, because the protease needs to be separated from the target protein, and (3) due to their limited specificity, proteases can attack the target protein. The system described in this communication provides a general solution to these problems.

The first solution to these problems exploits the finding by Chilkoti and co-workers that elastin-like polypeptides (ELPs) retain their inverse phase transition behavior when fused to other proteins (Meyer and Chilkoti 1999). They showed that the phase transition behavior of ELP fusion proteins could be triggered by gentle heating of *Escherichia coli* lysate or by the addition of NaCl to aggregate the protein without nonspecific salting out or irreversible precipitation of other proteins. The aggregates were then recovered by centrifugation, isolated, and redissolved by reversing the phase transition to yield purified, and in all cases tested thus far, functionally active fusion proteins (Meyer and Chilkoti, 1999; Trabbic-Carlson et al., 2004). In this study, we have combined this purification method, inverse transition cycling, with self-cleaving inteins for protease-independent cleavage of the target protein to further expand the utility of this technology.

2.3 Material and Methods

2.3.1 Plasmid Construction and Protein Expression

The gene encoding for Mxe GyrA mini intein (Telenti et al., 1997) was amplified from template pTXB1 (New England Biolabs) through PCR using *Pfu* polymerase (Fermentas) and 5'-phosphorylated primers, Ph-CATGCGTATGTGCATCACG GGAGAT and Ph-AGGCCTGAGTTCAGACCGGTGA. The vector containing the Trx-ELP fusion structure (based on pET-32b, Novagen) (Meyer and Chilkoti, 1999) was digested with *BalI* (Promega) to generate blunt-end fragments between the thioredoxin and the ELP coding regions, and treated with calf intestinal phosphatase (Fermentas). The

DNA fragments of the PCR product and the linearized vector were purified through agarose gel electrophoresis, and then linked together with T4 DNA ligase (Fermentas). The intein and ELP were linked by the same ten amino acids as in the pTXB1 vector (New England Biolabs), which separate the intein from the chitin binding domain in that system, followed by the amino acids that correspond to a His tag and a thrombin cleavage site, from the pET32a vector (Novagen). The ligation mixture was transformed into *E. coli* JM109, and recombinants contaning the insert were identified by restriction endoincuelase digestion and agarose gel electrophoresis to obtain the plasmid pTME (Trx-Mex-ELP). The sequence of the inserted Mxe intein was confirmed by automated nucleic acid sequencing (Mobix facility, McMaster Univ). pTME was then transformed into *E. coli* expression BLR(DE3) competent cells (Novagen), and cultured in LB media supplemented with 100 μg/ml of ampicillin. Induction of fusion protein expression was initiated when the OD_{600} reached 0.6, by adding IPTG to a final concentration of 0.3 mM. The induction was carried out at a temperature of 20 °C overnight to minimize *in vivo* self-cleavage of the intein (Telenti et al., 1997). Cells were harvested by centrifugation and sonicated (VirTis Corp.). Nucleic acids were removed from the crude extract using polyethyleneimine precipitation, followed by centrifugation.

2.3.2 Purification and Cleavage

The Trx-Mex-ELP chimeric protein was separated from crude *E coli* lysate by using a 0.2 μm filter. Briefly, 5 M NaCl was added into cell lysate to a final salt concentration of 1.67 M and maintained at room temperature for 5 min. The visibly

turbid suspension was then passed though a 0.2 μm microfiltration membrane (Supor, Pall Corp.). After washing with 1.67 M NaCl, water was added to resolubilize the aggregates of Trx-Mxe-ELP, leading to elution of soluble the Trx-Mxe-ELP fusion from the membrane. After elution of the Trx-Mex-ELP fusion, dithiothreitol (DTT) was added to a final concentration of 20 mM and incubated overnight at room temperature, following another cycle of inverse transition cycling and microfiltration to separate the cleaved Trx from the Mex-ELP tag.

2.3.3 Desalting and Protein Assay

The free Trx obtained at the end of the purification scheme was passed through a 3 kDa MW cutoff ultrafiltration membrane (Pall Corp) to remove DTT and concentrate the protein,. The samples obtained at each step of the purification were collected and analyzed by SDS-PAGE (10-20% PROTEAN 3; Bio-Rad). The concentration of the purified Trx was determined using a calculated[7] extinction coefficient of 13,940 $M^{-1}cm^{-1}$. Trx activity was monitored by the insulin-disulfide reduction assay (Holmgren, 1979). The completeness of DTT removal from the purified Trx was monitored by insulin precipitation assay.

2.4 Results and Discussion

To demonstrate proof-of-principle of this methodology, thioredoxin (Trx) was fused at its C-terminus with a mini-intein from *Mycobacterium xenopi* GyrA gene (Mxe) (Telenti et al., 1997), and an ELP tag (Meyer and Chilkoti, 1999) to create the tripartite

fusion Trx-Mxe-ELP (Figure 2.1A). We chose the mini-intein from the *M. xenopi* GyrA gene that has an Asn -> Ala mutation at the C-terminus of the intein for N-terminal cleavage (Evans et al., 1998). The amino acids Met-Arg-Met were added before the Mxe intein sequence to control the cleavage reaction (Evans et al., 1999). The ELP used in the fusion consists of 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio of 5:2:3, respectively, as reported previously (Meyer and Chilkoti, 1999; 2002).

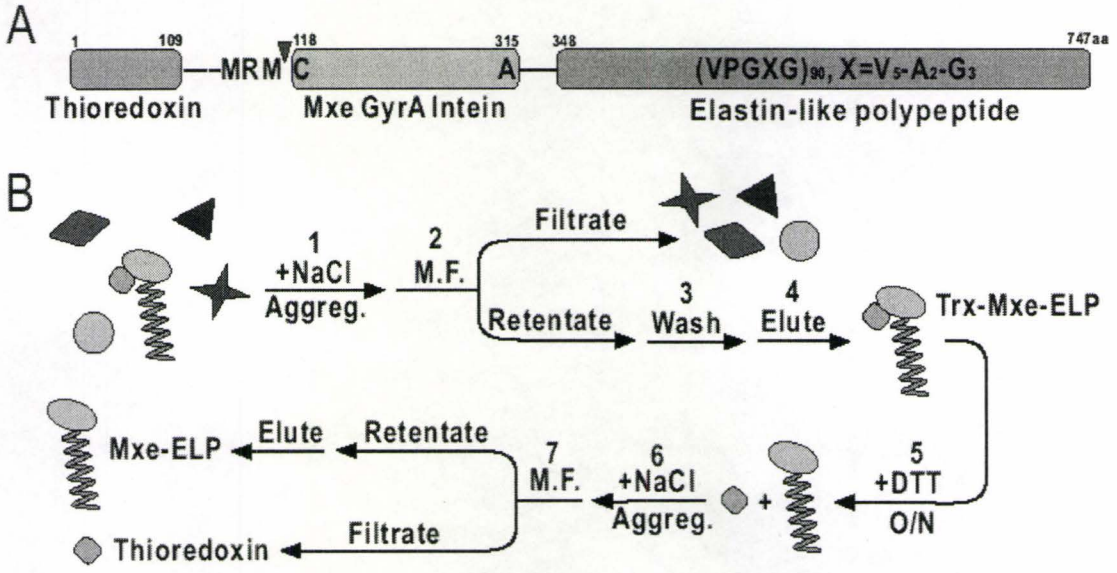

Figure 2.1 Scheme of tripartite self-cleaving environmentally responsive purification tag. (A) Triple fusion structure. Thioredoxin as the target protein was fused with Mxe GyrA mini intein and elastin-like-polypeptide. A peptide (MRM) was inserted before the N-terminal end of the intein sequence to control the cleavage activity. The C-terminal end of Mxe was modified (N->A) to block C-terminal cleavage or splicing. The arrow indicates the site of self-cleavage, which is induced by DTT. Numbers denote the amino acid sequence length. (B) Purification scheme; M.F (microfiltration membrane), DTT (dithiothreitol), O/N (overnight incubation).

This protease-free and chromatography-free protein separation process is remarkably simple, as shown schematically in Figure 2.1B. NaCl is added to crude cell lysate at room temperature to trigger the ELP phase transition, which results in the aggregation of the ELP fusion protein to form micrometer-sized particles (Meyer et al., 2001; Meyer and Chilkoti, 2002). The mixture is then filtered through a 0.2 μm membrane, resulting in the retention of the ELP fusion aggregates and elution of other *E. coli* proteins and macromolecules. Next, the aggregates are resolubilized and recovered by passing water through the membrane, which reverses the phase transition and results in dissolution of the aggregated fusion protein. We chose microfiltration, as opposed to centrifugation (Meyer and Chilkoti, 1999), to recover the aggregated fusion protein to simplify the purification process to the level associated with DNA "minipreps". Dithiothreitol (DTT, 20 mM) is then added to self-cleave the Mxe GyrA intein, releasing Trx from the Mxe-ELP tag. Another round of inverse transition cycling is performed to isolate thioredoxin from the Mxe-ELP fusion. The purified thioredoxin is then desalted and concentrated by using an ultrafiltration centrifugation filter with a MW cutoff of 3 kDa (Pall Corp).

SDS-PAGE analysis for each step of the purification process is shown in Figure 2.2A. The cell lysate from nontransformed cells (lane 1) and transformed cells expressing Trx-Mxe-ELP after induction with IPTG (lane 2) were obtained by ultrasonic cell disruption followed by centrifugation and precipitation of nucleic acids with polyethylenimine. Addition of NaCl to a final concentration of 1.67 M NaCl triggered the ELP phase transition, and the fusion proteins aggregated to form micrometer-sized

particles that resulted in a visibly turbid solution (Meyer et al., 2001; Meyer and Chilkoti, 2002). After filtration, other proteins passed through the membrane (lane 3), but the aggregated Trx-Mxe-ELP fusion was retained on the microfiltration membrane, as seen from the absence of the band corresponding to the fusion protein in the SDS-PAGE of the filtrate. When the high salt buffer was switched to water, the resolubilized ELP fusion was eluted out from the membrane as soluble protein (lane 4). The two bands in lane 4 are due to partial in vivo cleavage of the Trx-Mxe-ELP, resulting in a fraction of ELP-intein fusions that do not contain Trx. After incubation with 20 mM DTT overnight, Trx was released from the Mxe-ELP tag (lane 5). A second round of inverse transition cycling resulted in the separation of the Trx (lane 6) from the ELP fusion tag (lane 7). A quantity of 50 mg/L of the Trx-Mxe-ELP fusion protein was obtained, as determined by UV-vis (using a calculated extinction coefficient (Gill and von Hippel, 1989) for Trx equal to 13 940 M^{-1} cm^{-1}). The first round of inverse transition cycling provided ~90% purity; we note that greater levels of purity (≥95%) can be easily obtained by simply repeating the cycling procedure several times, as shown previously by Meyer and Chilkoti (1999). Taking into account that 25% of the total amount of the fusion produced was cleaved in vivo, the total expression level of the Trx-Mxe-ELP fusion protein was estimated to be ~70 mg/L. When Trx-ELP was expressed without the intein, the expression level was found to be 80 mg/L, consistent with a previous study (Trabbic-Carlson et al., 2004), indicating that there is only a small decrease in the yield by incorporating the intein in the construct.

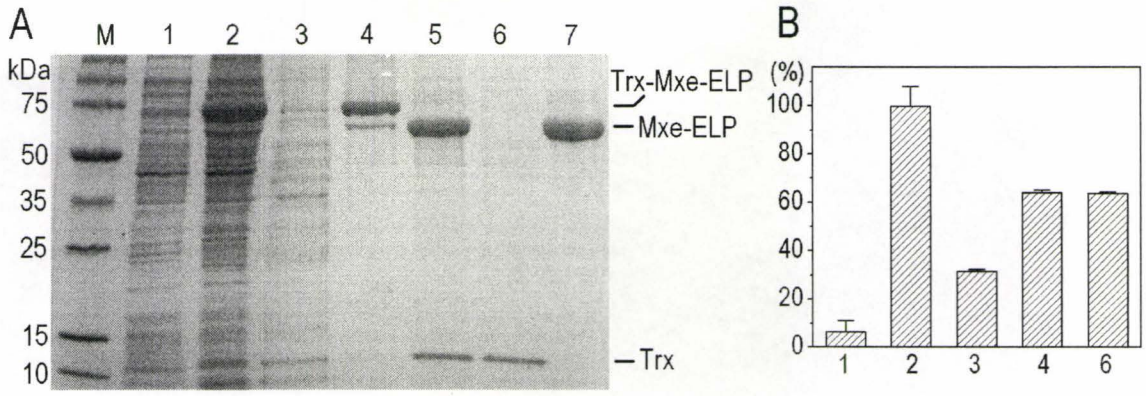

Figure 2.2 Purification by the self-cleaving stimulus responsive tag. (A) SDS-PAGE of each step of the purification and self-cleavage process. Lane 1, crude extract of untransformed cultured cells; lane 2, crude extract of cultured cells expressing the tripartite fusion Trx-Mxe-ELP; lane 3, *E. coli* proteins in the filtrate from a 0.2μm microfiltration membrane; lane 4, Trx-Mxe-ELP fusion (72.6 kDa) eluted with water from the membrane; lane 5, overnight cleavage with 20mM DTT; lane 6, thioredoxin (12.6 kDa) filtered through a 0.2 μm membrane; lane 7, eluted Mxe-ELP tag (60.0 kDa); lane M, molecular mass marker proteins. (B) Thioredoxin activity assay for each stage of purification (lane 1-4, 6 as in part A) by insulin reduction assay. Values were normalized to the activity in the cell lysate.

Figure 2.2B shows the corresponding Trx activity at each purification step, which was determined by the insulin reduction assay (Holmgren, 1979; 1984). The ~25% activity of Trx (normalized to the activity level in cell lysate as being 100%) observed in the filtrate (lane 3) is due to the endogeneous Trx encoded by the *E. coli* genome (not transformed host, lane 1) and due to premature in vivo release of Trx from the fusion protein during culture, which accounts for the majority of the Trx activity in the filtrate, as also seen in lanes 1 and 3 of Figure 2.2A. After filtration, the activities in the filtrate (lane 3) and of the eluted Trx-Mxe-ELP fusion (lane 4) account for 100% of the activity observed in the cell lysate, showing that activity was not lost during purification. The activity of Trx after cleavage and a second round of inverse transition cycling (lane 6)

was experimentally indistinguishable from the activity of the Trx-Mxe-ELP fusion that was eluted from the microfiltration membrane in the first round of purification. In a control experiment with Trx-ELP (without the intein), we observed that capture and recovery of the fusion protein through the microfiltration membrane was greater than 95% (Figure 3.7 in Chapter 3), indicating that this simple purification method allows remarkable recovery of the target protein.

2.5 Conclusions

In summary, we have demonstrated that it is possible to purify an ELP fusion protein by a simple microfiltration procedure and that the target protein can be cleaved from the ELP tag using an intein, thus obviating the need for expensive proteases and their removal. The primary limitation of this methodology, the premature *in vivo* cleavage of the fusion protein, can be solved, we believe, by the use of ligand-activated inteins (Buskirk et al., 2004; Skretas and Wood, 2005). The attractive features of this approach for lab-scale purification are its extraordinary technical simplicity and low cost: the only reagents and equipment required to purify milligram levels of protein at the lab bench are NaCl and DTT, a 0.2-μm membrane filter, and a syringe. This methodology could also be easily scaled up for kilogram-level purification of peptide and protein therapeutics (associated experiments and results are in Chapter 3). Furthermore, because this procedure eliminates the need for proteases for isolation of the target protein, it is attractive for structural biology, where contaminating levels of the proteins interfere with structure determination, and for industrial applications, where the cost of the protease can

represent a significant cost of the final product. The simplicity of the self-cleaving inverse transition cycling procedure demonstrated here is reminiscent of DNA minipreps and should liberate biologists from the tedium and complexity of protein purification by making it eminently feasible to simultaneously purify multiple proteins on the laboratory bench without recourse to expensive reagents and equipment.

2.6 Reference

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Chapter 3

Purification of an Elastin-Like Fusion Protein by Microfiltration

This chapter is based on Ge, X. *et al.* (2006). *Biotechnology and Bioengineering* **95**(3): 424-432.

3.1 Abstract

This article describes a simple and potentially scalable microfiltration method for purification of recombinant proteins. This method is based on the fact that when an elastin-like polypeptide (ELP) is fused to a target protein, the inverse phase transition behavior of the ELP tag is imparted to the fusion protein. Triggering the phase transition of a solution of the ELP fusion protein by an increase in temperature, or isothermally by an increase in salt concentration, results in the formation of micron-sized aggregates of the ELP fusion protein. In this article, it is shown that these aggregates are efficiently retained by a microfiltration membrane, while contaminating *E. coli* proteins passed through the membrane upon washing. Upon reversing the phase transition by flow of Milli-Q water, soluble, pure, and functionally active protein is eluted from the membrane. Proof-of principle of this approach was demonstrated by purifying a fusion of thioredoxin with ELP (Trx-ELP) with greater than 95% recovery of protein and with greater than 95% purity (as estimated from SDS-PAGE gels). The simplicity of this method is

demonstrated for laboratory scale purification by purifying Trx-ELP from cell lysate using a syringe and a disposable microfiltration cartridge. The potential scalability of this purification as an automated, continuous industrial-scale process is also demonstrated using a continuous stirred cell equipped with a microfiltration membrane.

3.2 Introduction

The development of simple, rapid, and scalable methods for protein purification are becoming increasingly necessary for bench scale studies as well as for protein production at an industrial scale. Affinity chromatography of proteins with engineered affinity tags has attained widespread use in the purification of recombinant protein (Nilsson et al., 1997). However, affinity chromatography suffers from major limitations for two emerging applications of proteins. First, it has become increasingly evident that a major bottleneck in high-throughput studies of protein function in proteomics is the time and expense in purifying large numbers of proteins at the milligram scale. Affinity chromatography is not ideal for massively parallel protein purification for high-throughput studies. The main reasons for this are the cost associated with multiple affinity columns and the difficulties in implementing chromatography in a parallel manner especially if gradient elution is required. Second, recombinant proteins are increasingly important as therapeutics, with many protein and peptide drugs currently in the pre-clinical pipeline worldwide. The limitations of affinity chromatography are even more severe for the kg-ton industrial scale purification of proteins, as the cost of the resins to bind the tagged proteins is high, adding significantly to the cost of purification.

In the case of metal affinity chromatography, the potential toxicity of the metal chelates that may leach from the column, prohibits their use for purification of protein therapeutics.

An orthogonal approach to recombinant protein purification that eliminates all chromatographic separation steps, first demonstrated by Meyer and Chilkoti (1999), has the potential to address these limitations of chromatographic separations. These authors fused a target protein to an elastinlike polypeptide (ELP) at the gene level. ELPs are biopolymers composed of repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa can be any amino acid except proline (Urry et al., 1985; Urry 1992). ELPs have a unique and technologically useful property; they undergo an inverse phase transition within a very narrow temperature range in aqueous solution (Urry 1988; 1992, 1997). At a temperature below the inverse transition temperature (T_t), ELPs are soluble but become insoluble if the solution temperature exceeds T_t. This behavior is completely reversible, in that the insoluble ELPs will redissolve in water or buffer if the solution temperature is lowered belowthe T_t of the ELP. Chilkoti and coworkers showed that this feature of ELPs is retained when it is fused to a variety of proteins (Meyer and Chilkoti, 1999; Trabbic-Carlson et al., 2004b) which led to the development of a very simple method – inverse transition cycling (ITC) – for protein purification, by performing centrifugation of the cell lysate at a temperature higher than T_t. This results in the recovery of a pellet mainly composed of the ELP fusion protein and resolubilization of the pellet at a temperature below T_t yields the soluble purified protein (Meyer and Chilkoti, 1999). To date, ITC has been used to successfully purify a number of different proteins (Meyer and Chilkoti,

1999; Shimazu et al., 2003; Trabbic-Carlson et al., 2004a, 2004b), and it has been demonstrated that the activity of every target protein is preserved with ITC.

When the inverse phase transition is triggered, the aggregates of the ELP fusion protein have a radius in excess of one micron (Meyer et al., 2001; Meyer and Chilkoti, 2002). We hypothesized that these microscopic aggregates could be easily retained using a simple microfiltration membrane. A similar observation has been done by Ghosh (2004), but using ammonium sulfate induced reversible precipitation of Human immunoglobulin G. Confirmation of this hypothesis would allow substitution of the centrifugation step by a microfiltration step. This would result in a method that is more amenable to massive parallelization in high-throughput studies, as well as a significantly easier method for recombinant protein purification in industrial applications. In this article, we show that microfiltration works remarkably well to purify a thioredoxin-ELP (Trx-ELP) fusion protein by ITC. The use and efficacy of microfiltration is demonstrated for bench scale purification, consisting of a syringe coupled to a microfiltration cartridge, as well as in a continuously operated stirred cell, indicating that this approach is potentially scalable to gram-kilogram levels for industrial scale purification of proteins.

3.3 Material and Methods

3.3.1 ELP Fusion Protein Production

The plasmid used in this study was as described by Meyer and Chilkoti (1999). In summary, it is derived from the pET-32b expression vector (Novagen, Madison, WI), which encodes a $(His)_6$ tag and a thrombin recognition site downstream of a thioredoxin

gene, that had been previously modified to include a unique *Sfi*I site located 3' to the thrombin cleavage site for insertion of an ELP gene (Meyer and Chilkoti, 1999). The ELP gene encodes for 90 repeats of the pentapeptideVal-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio of 5:2:3, which we denote as ELP [$V_5A_2G_3$-90]. The ELP gene was ligated into the *Sfi*I digested, modified pET-32b vector to encode the fusion protein thioredoxin-ELP [$V_5A_2G_3$-90] (Meyer et al., 2001), herein referred to as Trx-ELP. The vector encoding for the fusion protein was transformed into *E. coli* strain BLR (DE3) (Novagen) through standard protocols.

The transformed cells were cultivated and induced according to standard protocols. The culture media was discarded and the pellet was resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.2 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH7.4), in 4% of the initial volume of the culture medium. The resuspended cells were lysed in an ice-water bath using ultrasonic disruption (VirTis Comp.) using discontinuous ultrasonic pulses for 2 min, at the maximum power at which no substantial amount of foam was generated. The lysate was centrifuged at 16,000 g, 4 °C for 10 min to remove insoluble particles. Nucleic acids were precipitated by adding polyethyleneimine (MW = 600, 0.5% final concentration), followed by centrifugation at 16,000 g 4 °C for 10 min, to obtain the soluble cell lysate. This sample was then filtered through a 0.2 μM membrane. This constituted the initial samples for all the studies described in this article for protein purification, herein referred to as pre-treated cell lysate. Typical expression levels for the fusion protein were 40 mg/L of culture media.

For studies to characterize phase transition behavior and measurement of particle

size after aggregation, Trx-ELP was purified by ITC (Meyer et al, 2001) with minor modifications. To cell free extracts, 5 M NaCl was added resulting in a final NaCl concentration of 1.5 M. The sample was then heated to 50 °C for 10 min in a water bath, and centrifuged at 16,000 g at 35-40 °C for 10 min. The supernatant was discarded and the pellet was resuspended in cold PBS buffer on ice for 5 min to resolubilize the ELP fusion protein. Any remaining insoluble particles were removed by an additional centrifugation step at 16,000 g, 4°C for 10 min. SDS–PAGE, using Coomassie Blue stain, was used to verify the purity of the Trx-ELP obtained by ITC. The apparent molecular weight was slightly higher than the expected size (50 kDa), a phenomenon that has been previously reported with other elastin-based proteins (Shimazu et al., 2003).

3.3.2 Determination of Trx-ELP Fusion Protein Concentration, Enzymatic Activity, and Inverse Phase Transition Behavior

The concentration of purified Trx-ELP samples was determined spectrophotometrically, using a molar extinction coefficient of 19,630/M/cm, which was calculated based on the amino-acid composition of the fusion protein (Gill and von Hippel, 1989). The specific enzymatic activity of Trx was measured using the insulin reduction colorimetric assay (Holmgren, 1979; 1984). The reaction was initiated by adding thioredoxin reductase (Sigma Aldrich, St. Louis, MO), and the consumption of NADPH (Sigma Aldrich) was monitored by measuring the decrease in absorbance at 340 nm. The decrease of OD_{340} at 1 or 2 min intervals was used to calculate the enzymatic activity.

Temperature-dependent aggregation of the Trx-ELP fusion protein was characterized on a Cary 100 Ultraviolet-Visible spectrophotometer equipped with a Peltier multicell temperature controller (Varian Instruments, Palo Alto, CA), by measuring the turbidity at 350 nm as a function of solution temperature. The temperature was increased from 15 to 50 °C at a constant rate of 1 °C/min. The experiments were performed with Trx-ELP at a concentration of 40 μM in PBS or a concentration of 20 μM in PBS + 2.5 M NaCl.

The kinetic data for the formation of ELP aggregates was obtained by monitoring the increase of absorbance at 350 nm as a function of time, at a constant temperature of 25 °C. A solution of Trx-ELP in PBS (1 ml 40 μM) was added to a 1 cm path length cuvette. At time zero, 1ml of 5MNaCl was added and the turbidity increase at 350 nm was recorded as a function of time.

The particle size distribution of the soluble and aggregated Trx-ELP fusion protein was determined by dynamic light scattering (DLS) at different temperatures. A Lexel 95 ion laser operating at a wavelength of 514 nm was used as the light source, with the detector at an angle of 90° relative to the incident light beam. The laser power was set to 200 mW and 100 mW for DLS of soluble and aggregated Trx-ELP fusion, respectively. The correlation data was analyzed using a BI-9000AT digital autocorrelator, version 6.1 (Brookhaven Instruments Corp., Holtsville, NY). The CONTIN statistical method was used to generate the particle size distributions. Light scattering data was collected within a 1 min interval, and five repeat measurements were done for each sample.

3.3.3 Trx-ELP Purification by Microfiltration

To trigger the aggregation of the Trx-ELP fusion protein, pretreated cell lysate was mixed with an equal volume of 5.0 M NaCl at room temperature. After 5min had elapsed, to ensure complete aggregation, the purification of the aggregated Trx-ELP fusion protein by microfiltration was demonstrated using three different schemes, representing three different scales of operation (Figure 3.1):

Scheme A. A syringe was used to load a sample for purification, which was coupled with a 0.2 μm Supor (Pall Corp., East Hills, NY) disposable microfiltration cartridge (Figure 3.1A), with a diameter of 25 mm. The membrane material was hydrophilic polyethersulfone to minimize nonspecific protein adsorption. Five milliliters of the pre-treated cell lysate with aggregated Trx-ELP was transferred to a syringe and passed through the filter. To wash the aggregates deposited onto the filter, 5 ml of 2.5 M NaCl was injected through the filter to remove proteins other than Trx-ELP, while still maintaining the Trx-ELP in its aggregated state. To elute the aggregated Trx-ELP, 3 ml of Milli-Q water was passed through the filter, causing resolublilization of the retained aggregates. Samples were taken at each stage of the purification for analysis by SDS–PAGE and enzyme activity assay.

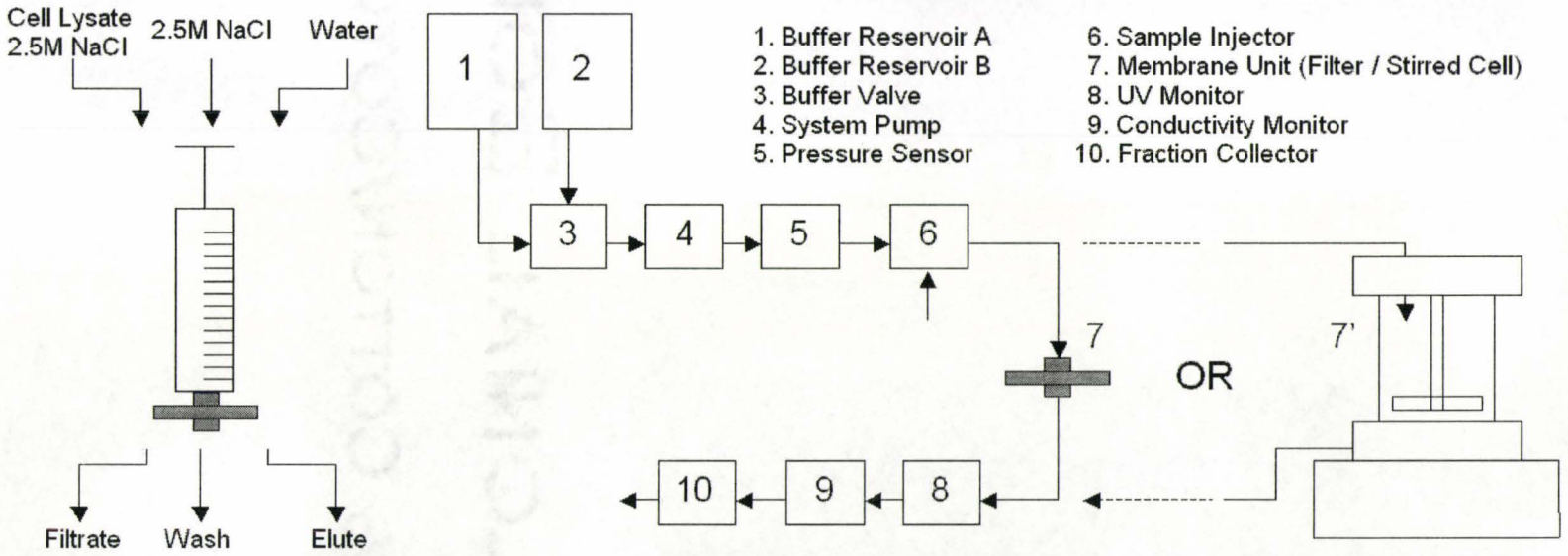

Figure 3.1 Diagrams of microfiltration purification processes. A: Syringe with a microfilter. B: AKTA system with a microfilter. C: AKTA system with a stirred cell module.

Scheme B. A 0.2 μm Supor disposable microfiltratio cartridge (Pall Corp.) was connected to an AKTA Prime system (Amersham Biosciences, Piscataway, NJ), as shown in Figure 3.1B. This system allows continuous monitoring of the absorbance at 280 nm (AU_{280}) and conductivity of the permeate stream, as well as the overall pressure in the system. The flow through the system was kept constant at 10 ml/min. Prior to sample injection, the system was operated by continuously passing 2.5 M NaCl, until all readings stabilized. The sample, consisting of pre-treated cell lysate with pre-aggregated Trx-ELP, was loaded to the system through a 5 ml sample injection loop. During this period, a rapid increase of the AU_{280} was observed due to the washout of contaminating proteins present in the lysate. After the AU_{280} reached a value of zero, resolubilization of the Trx-ELP aggregates retained by the membrane was achieved by switching the buffer to Milli-Q water. The elution of Trx-ELP from the membrane could be observed by an increase in the AU_{280}. Samples were collected at each of the different stages of the purification process for analysis using SDS–PAGE.

Scheme C. A stirred cell module, was connected to the ÄKTAprime™ system (Amersham Biosciences) (Figure 3.1C), as described by Ghosh (2004). In large scale operations, shear stress is used in the vicinity of membranes to minimize cake formation, membrane fouling, and concentration polarization, as all of these factors result in a premature decrease in membrane permeability and hence productivity. A stirred cell unit was used to evaluate the effect of shear stress on the retention of the Trx-ELP aggregates. The unit had a 25 mm diameter disc membrane (Millipore Express disc, pore size of 0.22 μm) at the bottom of the cell and a working volume of 10 ml. A Superloop (Amersham

Biosciences), capable of holding sample volumes up to 50 ml was connected to the AKTA Prime system. The system was operated at a constant flow rate of 1 ml/min. The steps for purification of the Trx-ELP protein from pre-treated cell lysate were similar to those in Scheme B. The system was initially operated with 2.5 M NaCl, and 25 ml of pre-treated cell lysate with preaggregated Trx-ELP was loaded to the Superloop and injected into the stirred cell module. The aggregates retained by the membrane were continuously washed with 2.5 M NaCl. After an initial large peak of AU_{280}, associated with elution of non-aggregated proteins and after return of the baseline to zero, the buffer was switched to Milli-Q water to trigger resolubilization of the aggregated Trx-ELP. During this elution stage, permeate samples were collected for analysis by SDS–PAGE.

3.3.4 Determination of Percent Recovery

To determine the mass recovery associated with microfiltration (defined as the ratio of the mass of target protein recovered after microfiltration, to the mass of target protein before the microfiltration step), the experimental procedure described in Scheme B was used. A mixture containing 2.5 ml of purified 40 μM Trx-ELP solution and 2.5 ml of 5 M NaCl was injected into the system was operating at a flowrate of 10 ml/min. The absorbance at 280 nm was continuously measured to determine the mass of Trx-ELP eluted after switching buffers from 2.5 M NaCl (wash buffer) to Milli-Q water (elution buffer) by integrating the area under theAU_{280} curve. This experiment was done in duplicate. To determine the total mass of protein injected into the system, the absorbance at 280 nm was similarly measured from a 5 ml sample of pure 20 μM Trx-ELP (in PBS),

which was loaded into the sample loop now lacking a purification filter. The ratio of the integrated areas calculated with and without a purification filter is a direct measure of the percent mass recovery of the microfiltration system for the Trx-ELP fusion protein. To determine the activity percent recovery associated with microfiltration (defined as the ratio of the activity of the target protein recovered after microfiltration, to the activity of the target protein before the microfiltration step), a sample containing 40 μM Trx-ELP in PBS was added to an equal volume of 5 M NaCl, and protein retention and elution was done using Scheme A. After elution with Milli-Q water, the elution fraction containing the Trx-ELP was diluted to a final concentration of 40 μM. The activity of Trx before and after the filtration step was measured using the insulin reduction assay (Holmgren 1984).

3.4 Results and Discussion

3.4.1 Characterization of the Phase Transition Behavior of Trx-ELP

The phase transition behavior of Trx-ELP was evaluated by following the progress of absorbance at 350 nm as a function of temperature. The samples consisted of 40 μM Trx-ELP; this concentration was chosen because it is typical of the concentration of the expressed protein in the soluble cell lysate. The phase transition behavior of Trx-ELP as a function of solution temperature is shown in Figure 3.2A, for different concentrations of NaCl. Increasing the temperature of the Trx-ELP solution above a critical point, results in a sharp increase in the turbidity in a ~2 °C temperature range due to the formation of Trx-ELP aggregates. The phase transition temperature (T_t) is herein defined as the temperature at which 50% of the maximum absorbance is recorded.

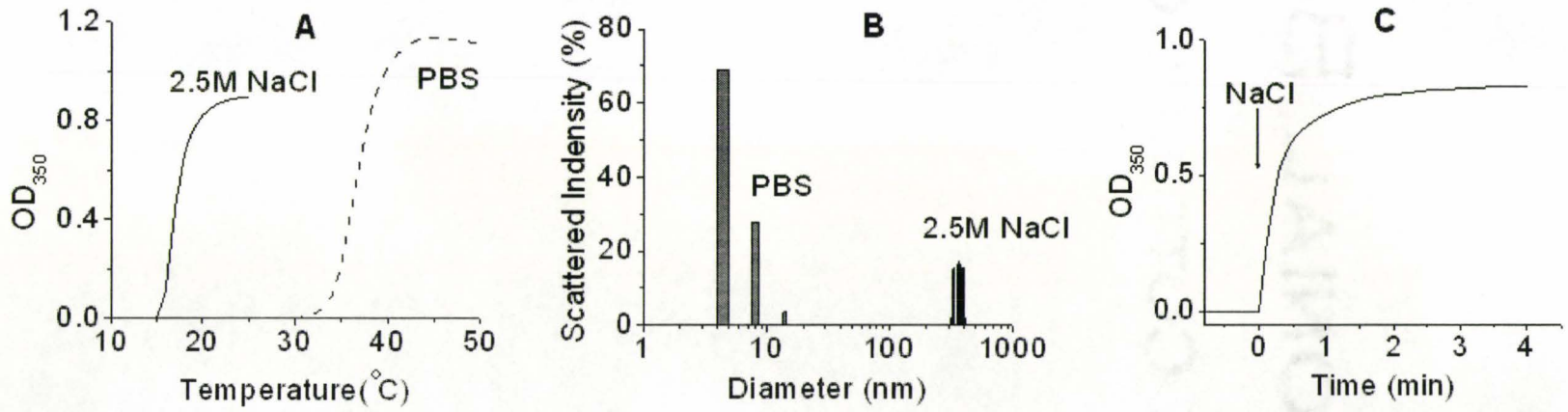

Figure 3.2 Characterization of phase transition of Trx-ELP fusion protein. A: Thermal behavior of the fusion protein at different salt concentrations (labeled in Figure) as a function of solution temperature. B: Particle size distribution of soluble and aggregated ELP. C: Kinetics of aggregation of Trx-ELP fusion upon isothermally triggering the phase transition by the addition of an equal volume of 5 M NaCl to 40 μM Trx-ELP in PBS.

Figure 3.2A shows that Trx-ELP exhibits a T_t of 60 °C in PBS, 38 °C in 1.0 M NaCl and 18 °C in 2.5 M NaCl (all salt concentrations are in addition to PBS). These data show that the inverse phase transition of Trx-ELP can be isothermally triggered at room temperature upon the addition of 2.5 M NaCl to a solution of the fusion protein in PBS.

DLS was used to measure the particle size distribution of a sample of 40 μM Trx-ELP in PBS and a sample of 20 μM Trx-ELP in 2.5 M NaCl, at 25 °C (Figure 3.2B). When PBS was used, Trx-ELP is soluble, with molecules having a diameter in the range of 2-10 nm (average ~7.5 nm). When the NaCl concentration was increased to 2.5 M, the diameter of the particles ranged from 310 to 410 nm, with an average diameter of 357 nm, consistent with aggregation of the fusion protein.

The inverse phase transition and consequent aggregation of Trx-ELP also occurs rapidly. Figure 3.2C shows the progress of the absorbance of a sample consisting of 40 mM Trx-ELP at 25 °C, to which an equal volume of 5 M NaCl was added. One minute after the salt was added, the absorbance reached ~90% of its maximum value. The turbidity continued to increase, due to additional aggregation, reaching a maximum at ~3 min. This data shows that inverse phase transition and aggregation are rapid processes.

These observations indicate that the change in particle size could be used as the basis of a simple method for purification of ELP-tagged fusion proteins from a complex mixture of proteins, such as found in cell lysate, using a microfiltration membrane. To achieve this, the phase transition of a solution of Trx-ELP is triggered at room temperature by the addition of NaCl leading to the aggregation of the ELP-tagged fusion protein. This makes the effective "size" of the ELP-tagged protein much larger than other

proteins present in the mixture. From the size range observed in this and other studies (Meyer et al., 2001; Meyer and Chilkoti, 2002), a microfiltration membrane, which is intrinsically capable of processing material at high fluxes, can be used to retain the ELP-based aggregates. This can be done while allowing non-tagged proteins to be eluted out through the membrane by washing with a buffer than maintains the ELP-tagged protein in an aggregated state. Resolubilization of the ELP-tagged protein can be achieved by changing the environmental conditions (decrease in temperature and/or salt concentration), allowing recovery of the tagged protein in a concentrated manner. In the remainder of this article, we show that this approach is not only feasible, but also easily scalable and amenable to applications that require the simultaneous purification of many different proteins.

3.4.2 Feasibility of Microfiltration Purification: Scheme A

A scouting study was performed to test the ability of a microfiltration membrane to retain aggregated Trx-ELP and to determine if it is possible to resolubilize the aggregates so that the fusion protein can be eluted out of the membrane. This was done using Scheme A, as described in Materials and Methods Section. Figure 3.3 shows the SDS-PAGE gel analysis for the samples collected during each step of the procedure. No fusion protein was detected when the sample was loaded and washed with 2.5 M NaCl, demonstrating that the aggregates were totally retained on the membrane. When Milli-Q water was injected through the filter, the Trx-ELP aggregates were resolubilized and eluted out of the filter, with the majority of the fusion protein eluting out in the first

elution fraction (1 ml), and with very little protein eluting in the second 1 ml fraction and none thereafter. These observations show that it is indeed possible to retain the aggregates using the membrane, and to resolubilize them and elute them in a very small elution volume.

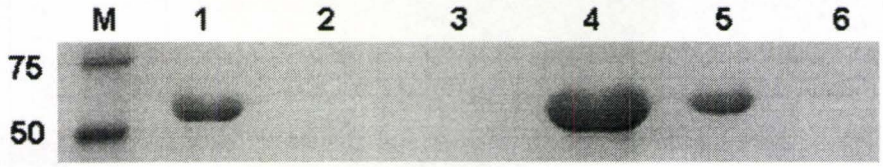

Figure 3.3 Demonstration of the ability of a microfiltration membrane to totally retain Trx-ELP fusion aggregates formed at room temperature in PBS+2.5 M NaCl and elution of soluble ELP upon reversing the phase transition by buffer change to water. Lane 1, initial Trx-ELP sample; lane 2, filtrate obtained by NaCl triggered aggregation of the fusion protein; lane 3, washing of aggregated proteins with 2.5 M NaCl; lanes 4-6, three elution fractions obtained by dissolving the aggregated Trx-ELP fusion and flushing the Trx-ELP fusion protein with water; M: Protein MW Marker (50 and 75 kDa shown only).

3.4.3 Purification of ELP Fusion Protein From Pre-Treated Cell Lysate: Scheme B

Trx-ELP was purified from pre-treated cell lysate using Scheme B. The results are presented in Figure 3.4A. For reference, lane 1 in the gel corresponds to the expression profile of the non-transformed *E. coli* host and lane 2 corresponds to the expression profile of the transformed cells. After the inverse phase transition was induced in the pre-treated cell lysate by adding NaCl to a final concentration of 2.5 M, the sample was filtered. The eluted volume from this step (lane 3), contains all native *E. coli* proteins, whereas the low intensity of the band corresponding to Trx-ELP indicates that the aggregates were largely retained on the filtration membrane. A washing step with 2.5 M NaCl (lane 4) revealed that no loosely adsorbed contaminating proteins were retained on the membrane and that purity is certainly higher than 95%. In the final step of

purification, the Trx-ELP aggregates were dissolved by passing Milli-Q water through the filter. Lane 5 shows that the fusion protein was obtained in a highly pure and concentrated form in the eluted volume.

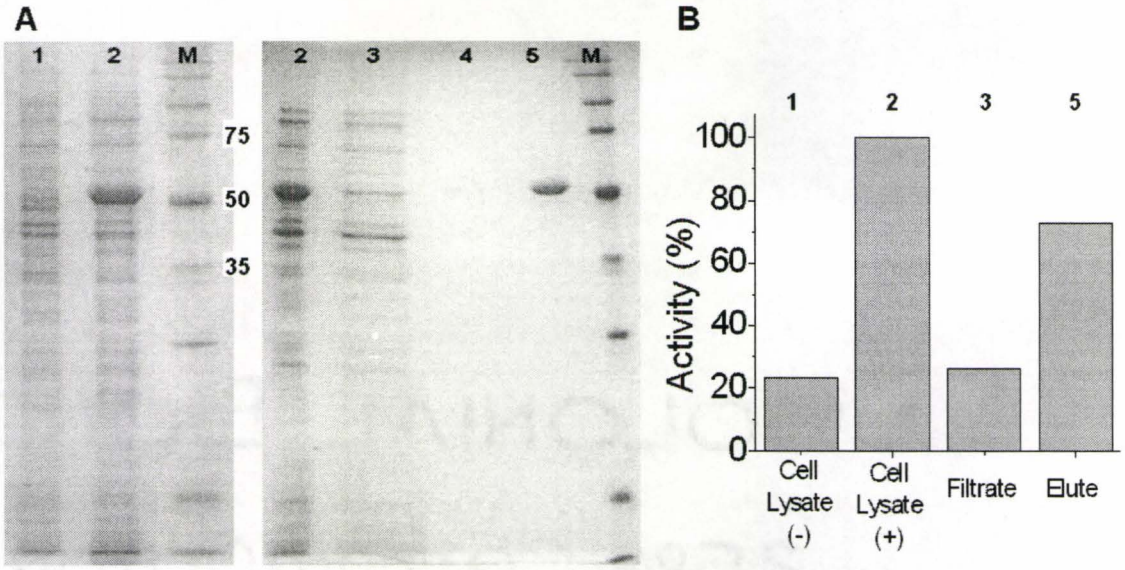

Figure 3.4 Purification of the Trx-ELP fusion protein purification directly from cell lysate using a 0.2 μm filter coupled with a syringe. A: SDS–PAGE analysis of each stage of purification. Lane 1, cell lysate without transformation; lane 2, cell lysate with transformation and induction with IPTG; lane 3, filtrate using 2.5 M NaCl; lane 4, washing of aggregates with 2.5 M NaCl; lane 5, elution of fusion protein with water. M: Protein MW markers (in kDa). B: Thioredoxin activity assay of samples.

In Figure 3.4 (B), the total activity of Trx measured in the cell lysate was normalized to 100%, and 76% of the initial activity was recovered in the final elution step (corresponding to lane 5 in Figure 3.4A), with the remainder, 23% of activity present in the filtrate (corresponding to lane 3 in Figure 3.4A). When the activity was measured in cell lysate from non-transformed *E. coli*, the activity corresponded to 22% of the activity of cell lysate from transformed cells. This baseline Trx activity of ~22% corresponds to the expression of the endogeneous, genomic copy of Trx in *E. coli*, that is obviously not

tagged with ELP, and hence does not aggregate upon addition of NaCl, and is eluted in the filtrate. Together, these results clearly demonstrate that close to 100% of Trx-ELP present in the soluble cell lysate is retained on the membrane upon the phase transition triggered aggregation of Trx-ELP, and is completely recovered from the membrane upon dissolution of the aggregates in the final low salt elution step.

It is important to demonstrate that indeed aggregates were formed and retained by the membrane through sieving. Solublilization and elution of Trx-ELP, should result in a noticeable drop in the transmembrane pressure (TMP). For this purpose, an AKTA Prime system was used, which allows continuous monitoring of system pressure, and it was connected to a 0.2 μm disposable cartridge filter. The flow rate was kept at 10 ml/min. Figure 3.5 shows the results of a typical run. The sample loaded into the 5ml loop consisted of pre-treated cell lysate and 5M NaCl, mixed in equal volumes. As the sample was injected into the system, a large increase in absorbance was observed, due to elution of non-tagged and non-aggregated protein. A large increase in the pressure is also observed during this initial phase, due to the high concentration of total protein present in the sample and due to the formation of a Trx-ELP "cake" on the membrane. Continuously running 2.5 M NaCl through the system washed the aggregates retained in the membrane and stabilization of the pressure. Switching the buffer to Milli-Q water (sharp decrease in conductivity), resulted in immediate decrease of the TMP which was accompanied by a sharp peak of the AU_{280}, indicating elution of Trx-ELP, indicating that the aggregates had been retained by the membrane and were rapidly resolubilized. The SDS–PAGE gel of samples taken during the purification process clearly indicates that

ELP fusion protein purification from cell lysate was achieved since the product resulted in a single band in the gel.

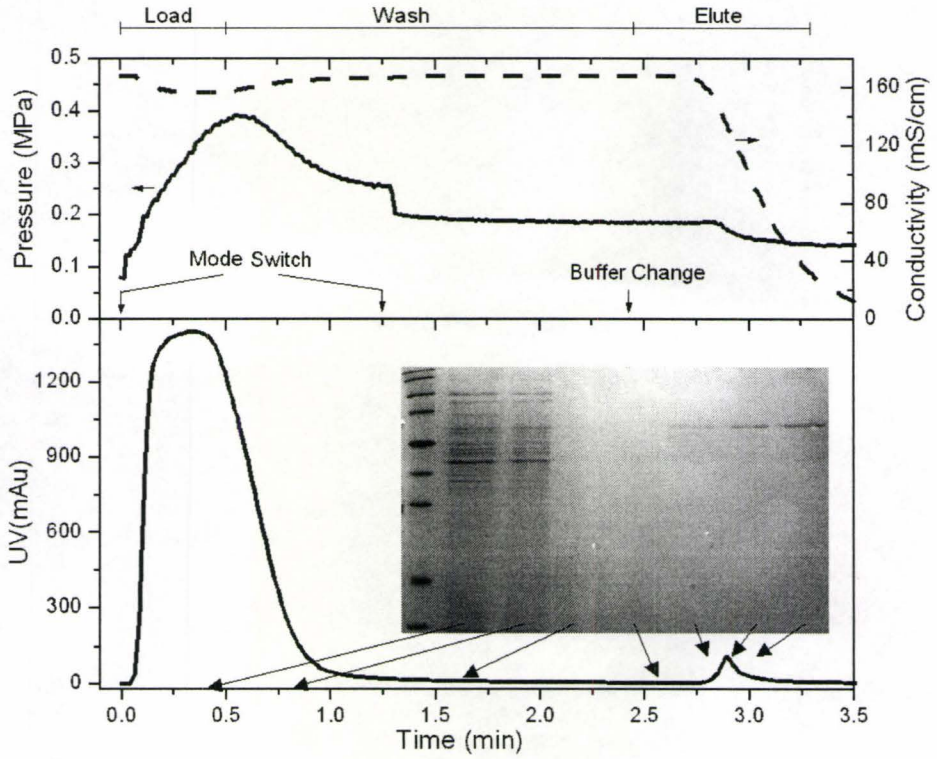

Figure 3.5 Purification of the Trx-ELP fusion protein from cell lysate, using a 0.2 μm microfiltration disposable cartridge. The system was operated at a flow rate of 10 ml/min.

3.4.4 Purification of ELP Fusion-Protein Using a Stirred Cell Amenable to Industrial Scales: Scheme C

These two previous schemes of operation are representative of what one would encounter in a bench scale application. In large-scale systems, membrane-based systems require the use of shear stress to minimize concentration polarization and cake formation on the membrane surface. To evaluate the effect of shear on the retention of Trx-ELP aggregates we used a stirred cell unit. In this experiment, 25 ml of pre-treated cell lysate,

with pre-aggregated Trx-ELP fusion (by addition of NaCl, to a final concentration of 2.5 M), were injected into the stirred cell using the AKTA Prime system.

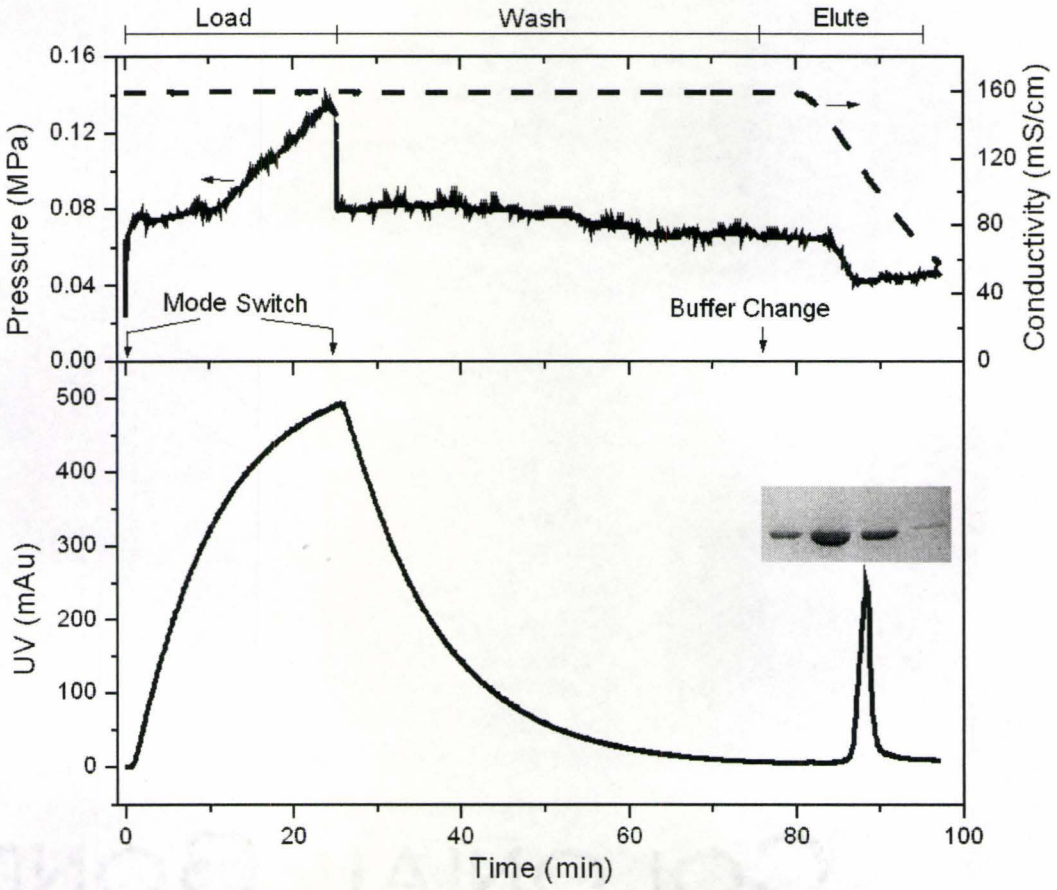

Figure 3.6 Purification of the Trx-ELP fusion protein from cell lysate, using a stirred cell module, to evaluate the effect of the use of stirring on the ability to retain the fusion protein aggregates. The system was operated at a flowrate of 1 ml/min, a 0.22 μm was used, and 25 ml of crude cell lysatewas injected to a 10 ml stirred cell.

The result of this experiment is shown in Figure 3.6, where AU_{280}, conductivity and system pressure profiles are presented. During the load and wash phases of purification, the AU_{280} curve shows a typical response associated with a completely mixed tank, with a first order decay process associated with the elution of non-tagged and non-aggregated proteins through the membrane. When the buffer was switched to Milli-Q

water, the aggregates dissolved and eluted out of the membrane, and SDS-PAGE analysis indicates that Trx-ELP was recovered with a high degree of purity. The fact that Trx-ELP was recovered using the stirred cell also indicates that mixing did not result in significant disturbance of the aggregates, which would lead to leakage of the Trx-ELP during the load and washing phases.

3.4.5 Determination of Mass and Activity Percent Recovery

As presented in Figure 3.3, the mass recovery rate of Trx-ELP using microfiltration is nearly 100%. To further confirm that high levels of mass recovery where obtained by this procedure, without loss of activity of Trx, two experiments were performed. In the first experiment, the injected sample consisted of a mixture containing 2.5 ml of pure 40 μM Trx-ELP solution with 2.5 ml of 5 M NaCl, and the sample was continuously washed with 2.5 M NaCl. The aggregates retained by the membrane were resolubilized and were eluted by switching the running buffer to Milli-Q water. For the test without the membrane, a 5 ml sample of pure 20 μM Trx-ELP was loaded into the sample loop and injected directly to the UV detector using Milli-Q water as the running buffer. Integration of the area under the AU_{280} curve for both tests directly allows calculation of the mass recovery.

In the second experiment, 40 μM Trx-ELP was added to an equal volume of 5 M NaCl, and protein retention and elution was done using Scheme A. After elution with Milli-Q water, the elution fraction containing the Trx-ELP was diluted to a final concentration of 40 μM. The activity of Trx before and after the filtration step was

measured using the insulin reduction assay (Holmgren, 1979; 1984). This test was done to determine if the aggregation/filtration/elution steps result in any decrease of the enzyme activity. The results from these two tests are shown in Figure 3.7. A mass recovery rate very close to 100 % was achieved without any loss of enzyme activity, which indicates that the method proposed is not only simple, but it also allows for very high levels of protein recovery, both in terms of mass and activity.

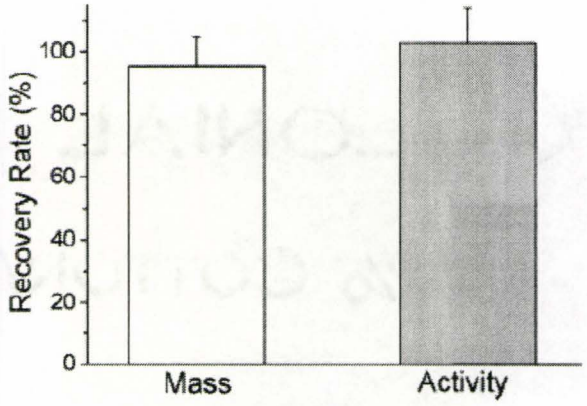

Figure 3.7 Mass and activity recovery of Trx-ELP using microfiltration based ITC. The mass recovery was determined using UV measurements at 280 nm on the sample after and before microfiltration-based ITC. Activity was measured for these same samples using the insulin reduction colorimetric assay.

One of the main benefits of microfiltration-based ITC is that the entire purification process is done *in situ* and in a continuous and time efficient manner, which is not possible with the use of centrifugation. Centrifugation-based ITC requires multiple rounds of a multi-step process in which the inverse phase transition is triggered in the cell lysate, the lysate is centrifuged to isolate the aggregated ELP fusion protein, the ELP fusion protein is mechanically resuspended, and contaminating aggregated biomolecules are removed from the soluble fusion protein by another centrifugation step. This entire

process may need to be repeated several times to achieve the desired fusion protein purity. In contrast, microfiltration-based ITC is performed entirely in a single vessel where high ionic strength buffer (in this case 2.5 M NaCl) is used not only to efficiently retain the Trx-ELP in its aggregated form on the membrane, but also to allow contaminating proteins to be continuously and efficiently washed out of the aggregated fusion protein. This process achieves a high level of purity in the final Trx-ELP product (for all the gels we obtained, only the Trx-ELP band was observed for the product), which is eluted from the membrane by simply lowering the ionic strength of the buffer. The entire purification process takes only a few minutes to complete, with very high recovery rates, and with no appreciable loss in activity.

In its simplest form, microfiltration-based ITC can be performed using a simple syringe and a disposable microfiltration membrane. As such, it is easy to envision how a 96-well plate could be retrofitted with a microfiltration membrane sheet and be operated under vacuum for highthroughput protein purification. Furthermore, microfiltration-microfiltration-based purification is significantly more amenable to scale-up than centrifugation. We used a dead-end filtration system for proof of principle, and show that the method proposed in this article has good potential for large-scale applications, where shear stress is used. However, it shoul be pointed that a more detailed study is needed to assess the processing capacity of a unit area of membrane. We envision that a tangential cross-flow microfiltration module would be more suitable for retention of the aggregated fusion protein, which would allow minimizing the pressure at which the system is operated. It should be noted that in tangential crossflow systems, shear stress will be

higher than in our stirred cell and as such studies using these systems is needed to fully assess the feasibility of the approach described in this article. Continuous mode microfiltration-based ITC has the potential to allow for large volumes of lysate to be efficiently and economically processed to produce industrial sized quantities (gram to low kg) of protein.

3.5 Conclusions

In this article, we demonstrate the feasibility of purifying Trx-ELP by exploiting the reversible inverse phase transition of the ELP tag combinedwith retention of the aggregates on a microfiltration membrane. We demonstrate the simplicity of this method for bench scale purification of milligram quantities of ELP fusion protein, by purifying Trx-ELP from cell lysate with 100 % recovery using a syringe microfilter in a scheme that is reminiscent of plasmid DNA mini-preps. The entire purification process can be completed in a matter of few minutes. This technique should be of value to researchers that need to purify small quantities of many different proteins, and it is ideally suited to high-throughput applications where massive parallelization is required. Furthermore, we demonstrate the potential scalability of this process, which is critical for industrial scale applications, by successfully purifying Trx-ELP from cell lysate using a continuous filtration system with a stirred test cell, to include the presence of shear stress in the system. The continuous mode of operation, the in situ wash step, and the high permeability associated with microfiltration membranes should allow large volumes of cell lysate to be processed, with potential production of recombinant proteins in the gram

to low kilogram range.

3.6 Reference

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Chapter 4

Simultaneous Phase Transition of ELP Tagged Molecules and Free ELP: An Efficient and Reversible Capture System

This chapter is based on Ge, X. and C. D. M. Filipe (2006). *Biomacromolecules* **7**(9): 2475-2478.

4.1 Abstract

In this chapter, we demonstrate proof-of-principle for a method that allows selective recovery of molecules present at very low concentrations in complex mixtures. The method makes use of an elastin-like polypeptide (ELP) as a coaggregant for the capture of an ELP tagged recombinant protein present at concentrations as low as 10 pM, with a recovery higher than 90%. This coaggregation process was found to be independent of the concentration, at least up to 10 pM concentration of the ELP tagged protein. The coaggregation process is highly specific as was demonstrated by spiking crude cell lysate with the ELP tagged recombinant protein to a final concentration of 1 nM and recovering more than 80% of it to a high level of purity. The method should be particularly useful for high-throughput proteomic studies, where small amounts of poorly expressed proteins could be recovered for analysis by mass spectrometry. In a more general context, the concept presented in this paper provides a method that is highly

efficient, specific, and fully reversible, which should render it useful in areas other than recombinant protein purification.

4.2 Introduction

Researchers working in a variety of fields often need to capture, in an efficient and highly specific manner, molecules that are present at very low concentrations in a very complex matrix. The common approach is to use affinity based systems, with the streptavidin-biotin (SA-B) interaction being the prime example of this approach due to its unmatched level of affinity (Green 1963; 1990). There are, however, two main drawbacks associated with affinity based systems: the higher the affinity level between the target (e.g., B) and the ligand (e.g., SA), the more difficult it is to release the target molecule from the ligand; affinity systems are associated with the use of a surface (beads, resins, etc.) onto which the ligand is coupled. If a large surface area is used (such as that associated with polymeric beads), there is high potential for nonspecific and irreversible binding of either contaminating molecules or the target. The objective of this work is to provide proof-of-principle for a method that has high capture efficiency, has full reversibility, and that exposes the sample to an absolute minimum surface area.

A growing number of reports have been made on the use of elastin-like polypeptides (ELPs) tags as fusion partners to recombinant proteins, which allows purification of the recombinant proteins without the use of chromatography. ELPs consist of repeats of the pentapeptide sequence Val-Pro-Gly-Xaa-Gly (VPGXG), where Xaa is any amino acid except proline (Urry 1988). ELPs are able to undergo a reversible inverse

phase transition (RIPT), within a very narrow temperature range (~2 °C) (Urry 1988; 1992; 1997). Below a critical transition temperature (T_t), ELPs are soluble, whereas above this temperature, they become insoluble. Meyer and Chilkoti (1999) observed that when ELP is fused to a recombinant protein, the ELP tag maintains the ability to undergo RIPT. This observation lead to the development of a very simple method for purification of recombinant proteins: inverse transition cycling (ITC) (Meyer and Chilkoti, 1999).

The basis for ITC is to selectively aggregate ELP tagged proteins using environmental stimuli (either increasing the temperature, adding NaCl, or a combination of both), so that the aggregates can be separated from other proteins present in the mixture, either by centrifugation (Meyer and Chilkoti, 1999) or by microfiltration (Chapter 2, 3). These aggregates can be resolubilized by decreasing the temperature of the mixture and/or decreasing the ionic strength of the buffer in which the protein is present. This approach has been demonstrated with a variety of target proteins (Shimazu et al., 2003; Trabbic-Calson et al., 2004b; Banki et al., 2005), and in all cases, the target fusion protein was obtained with purity levels higher than 95%. This suggests minimal nonspecific binding of contaminant molecules to ELP based aggregates. This approach has also been shown to yield purified proteins in quantities that are similar with those obtained using oligohistidine tags (Trabbic-Carlson et al., 2004a).

ITC has been demonstrated in the past with proteins that were expressed to high levels (Meyer and Chilkoti, 1999; Trabbic-Calson et al., 2004a; Banki et al., 2005), but poor expression levels are expected for toxic proteins, complex multidomain proteins, or in some cases of heterelogous protein expression. Later in this paper, we describe how for

very low concentrations of ELP tagged proteins, ITC in its original format does not allow recovery of the proteins. In proteomics, massive numbers of proteins are expressed in parallel, and optimizing expression conditions for each one is not feasible. Mass spectrometry for protein studies requires protein concentrations as little as 0.1-1 pM (Mann et al., 2001). We hypothesized that if an ELP tagged molecule is present in a solution at a very low concentration (even in the pM range) adding an excess amount of free ELP to the sample and inducing RIPT would form hybrid aggregates via the interaction of the ELP moieties of the two molecules.

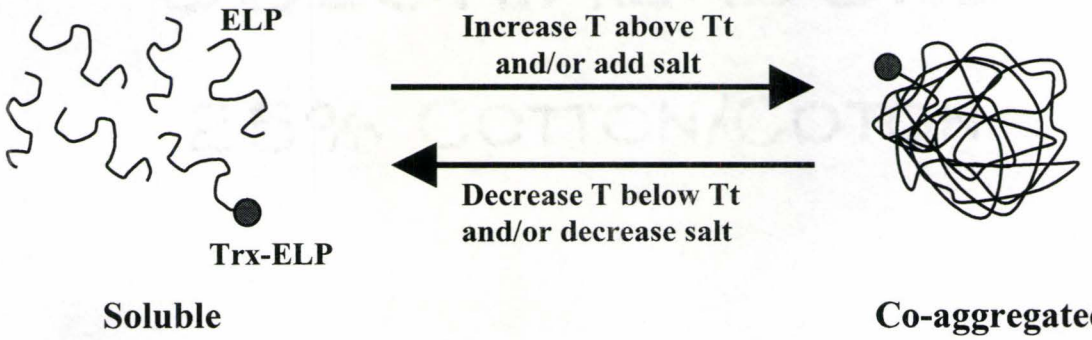

Figure 4.1 Scheme of simultaneous phase transition of ELP fusion (Trx-ELP) and free ELP

Coaggregation of free ELP and ELP tagged proteins has been reported for the purification of a fusion of a single-chain Fv of an anti-atrazine antibody to ELP (Kim et al., 2003), and as a component of a competitive phase-separation immunoassay (Kim et al., 2005), but no quantitative data was presented on the efficiency of coaggregation. In the work presented here, it is quantitatively shown that this coaggregation process is

highly specific and efficient even when using very low concentrations of ELP tagged molecules. Especially noteworthy, this interaction is fully reversible.

4.3 Material and Methods

4.3.1 Plasmids and Host Cells

To test the method described in this paper, we used a fusion of thioredoxin (Trx) and ELP (herein referred to as Trx-ELP) as a model protein. The plasmid that encodes for Trx-ELP used in this study was pTrx-ELP90, and its assembly is described elsewhere (Meyer and Chilkoti, 1999). It is based on the pET-32b expression vector (Novagen, Madison, WI) and the fusion protein consists of thioredoxin, followed by a $(His)_6$ tag, a thrombin cleavage site and the ELP tag. The ELP tag in this plasmid is denoted as ELP $[V_5A_2G_3\text{-}90]$, which comprises 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio of 5:2:3. To obtain a plasmid that encodes for the ELP tag only, the plasmid that encodes the fusion protein was digested with *Nde*I (Fermentas), and the large fragment was separated by agarose gel electrophoresis and purified with DNA extract kits (Qiagen). The purified DNA fragment was then self-circulated under the catalysis of T4 DNA ligase (Fermentas) to generate the plasmid that encodes for the expression of the ELP without thioredoxin. This plasmid is called pELP90. The two vectors used in this study were transformed into *E. coli* strain BLR (DE3) competent cells (Novagen), using the $CaCl_2$ transformation method.

4.3.2 Protein Expression and Purification

The transformed cells were cultivated in Luria-Bertani media, supplemented with 100 μg/mL ampicillin. Shaker flasks were inoculated from a single colony and cultured overnight at 37 °C. It has been shown that expression of ELP and ELP-tagged proteins is greatly enhanced when the cells are not induced using isopropyl β-thiogalactopyranoside (IPTG) but rather when the cultivation period is extended (Guda et al., 1995; Trabbic-Carlson et al., 2004a), and this approach was used in this study. An additional culture experiment was done with nontransformed *E. coli* BLR (DE3) strain, to provide cell lysate devoid of ELP or ELP tagged proteins.

The cells were harvested by centrifugation at 5000 *g*, 4 °C, for 10 min. The pellet was resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.2 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH7.4) in a volume ratio of 1:25 to the initial volume of culture medium. The resuspended cells were disrupted by introducing discontinuous ultrasonic pulses (VirTis Comp.) in an ice-water bath. The lysate was then centrifuged at 16 000 *g*, 4 °C, for 10 min to remove insoluble particles. From these samples, Trx-ELP or ELP was purified using three rounds of ITC (Meyer and Chilkoti, 1999). The ELP protein contained an $(His)_6$ tag and a thrombin cleavage site which were both removed by incubating (overnight) the sample with thrombin (Sigma) followed by another round of ITC to obtain pure ELP. The purity of the proteins was determined using SDS-PAGE followed by staining with Coomassie Brilliant Blue for Trx-ELP or with copper (Bio-Rad) for ELP. The concentrations of purified ELP and ELP-fused proteins were determined by measuring the UV absorbance at 280 nm and by using the extinction coefficients

determined from the amino acid sequence (19,700 M^{-1} cm^{-1} for Trx-ELP and 5690 $M^{-1}cm^{-1}$ for ELP). The molecular weights of Trx-ELP and ELP are 50 kDa and 36 kDa.

4.3.3 Labeling of Trx-ELP with ^{125}I

Trx-ELP was radioiodinated by the iodine monochloride method (MacFarlane, 1958; Helmkamp et al., 1960; Horbett et al., 1981). To a tube containing 50 μL of glycine buffer (2 M glycine, 2 M NaCl, pH 8.8) and 50 μL of iodine monochloride reagent (3.3 mM ICl, 1.8 M NaCl), 5 μL of ^{125}I isotope was added, and the contents of the tube were mixed for 1 min. To this mixture was added 4 mg of Trx-ELP in 600 μL of PBS, supplemented with 40 μL of glycine buffer, and the mixture was allowed to react at room temperature for 2 min. This mixture was subjected to two rounds of microfiltration based ITC (Chapter 2,3) to separate free radioactive iodine from Trx-ELP. This was achieved by adding 500 μL of 5 M NaCl to induce aggregation of the Trx-ELP, filtering the solution through a 0.2 μm Supor (Pall Corp.) disposable microfiltration cartridge, and collecting the filtrate. The aggregates retained in the membrane were washed with 500 μL of 2.5 M NaCl and then resolublized and eluted from the filter with 500 μL of PBS. This process was repeated twice. To ensure that no free radioactive iodine was present, gamma counting was done on the filtrate obtained in the second round of ITC. This filtrate accounted for 0.006% ± 0.0003% of the initial counts in the mixture before thefirst round of ITC; therefore, the amount of the radioactive free iodine left is negligible. The concentration of ^{125}I-labeled Trx-ELP was determined by measuring the

UV absorbance of the sample at 280 nm. Gamma counting was performed using a Wizard 3 1480 Automatic Gamma Counter (Perkin-Elmer Life Sciences).

4.3.4 Capture of Trx-ELP by Coaggregation with Free ELP in PBS

Free ELP and a mixture of labeled and unlabeled Trx-ELP were added to a counting vial containing 500 μL of PBS. To the vial was added an equal volume of 5 M NaCl to induce aggregation at room temperature. For all experiments, the final concentration (in 1 mL) of free ELP was 20 μM and for Trx-ELP, and the concentration ranged from 1 μM to 10 pM. To maximize the accuracy in gamma counting, for total concentrations of Trx-ELP higher than 1 nM, the labeled protein was 1 nM and the rest was un-labeled, and for total Trx-ELP concentration equal to or less than 1 nM, and all of the Trx-ELP was labeled. To separate the Trx-ELP and ELP coaggregates, the samples were centrifuged at 16 000 *g*, for 5 min, at room temperature. The supernatant was transferred to another tube and the pellet was resolubilized with 500 μL of PBS.

Measurement of the radioactivity of the initial mixture (before NaCl addition), the supernatant, and the resolublized pellet allowed the recovery percentage to be calculated. The time limits for counting were set to 1 min for the tests in which the Trx-ELP concentration ranged from 1 μM to 1 nM, 5 min for Trx-ELP at 100 pM, and 10 min for Trx-ELP at 10 pM. To amplify the radioactivity counts at very low Trx-ELP concentrations (10 pM), a sample with a total volume equal to 10 mL (as opposed to 1 mL) was used. To reduce the loss of Trx-ELP due to nonspecific binding onto the surface of pipet tips, the experiments were designed to minimize the number of pipetting steps.

Each experiment (at each concentration) was repeated three times, and the average and standard deviation was calculated from the results. A set of control experiments was conducted similarly but using bovine serum albumin (BSA) at a final concentration of 20 μM instead of free ELP.

4.3.5 Capture of Trx-ELP from Cell Lysate

To 10 mL of cell lysate obtained from an overnight 250 mL culture of the nontransformed host cells were added free ELP and labeled Trx-ELP. To this sample was added 10 mL of 5 M NaCl to induce aggregation (high salt conditions). The final concentrations of ELP and Trx-ELP in the 20 mL samples were 5 μM and 1 nM, respectively. For each round of ITC, two centrifugation steps (16 000 *g* for 5 min at room temperature) were done. After centrifugation using high salt concentrations (high salt spin), the supernatant containing soluble contaminants was removed, and the pellet was resuspended in 10 mL of cold PBS, followed by an additional centrifugation step (low salt spin). The pellet obtained after centrifugation with PBS contained particulate contaminants and the supernatant contained the resolublized free ELP/Trx-ELP mixture, which was transferred to a new tube. The same procedure was used for the second and third rounds of ITC.

Radioactivity levels were measured for the initial sample before the addition of NaCl and for the samples from each purification step in every round of ITC. The radioactivity observed in the resuspended pellet represents the amount of protein that was recovered. The amount of protein lost was determined by adding the radioactivity

obtained for the supernatant produced in the high salt conditions spin and the radioactivity obtained for the pellet from the low salt spin.

After three rounds of ITC, 1 unit thrombin (Sigma) was added to the sample containing Trx-ELP and ELP. After incubation (overnight) at room temperature, one additional round of ITC was preformed to separate the Trx from the ELPs. The supernatant, containing the Trx, was desalted and concentrated by centrifugation using an ultrafiltration membrane filter with a cutoff MW of 3 kDa (Pall Corporation). The purity of the final sample was assessed by 15% SDS-PAGE, and stained with Coomassie Brilliant Blue.

4.4 Results

4.4.1 High Recovery Rate is Independent of Concentration

To determine the capture efficiency of the coaggregation process, ITC purified Trx-ELP was labeled with ^{125}I. To a tube containing a mixture of free ELP and labeled Trx-ELP in PBS (500 μL) was added an equal volume of 5 M NaCl to trigger RIPT at room temperature. For all experiments, the final concentration (after NaCl addition) of free ELP was 20 μM and for Trx-ELP, and the concentration ranged from 1 μM to 10 pM. After centrifugation, the supernatant was transferred to another tube, and the pellet was resolubilized in 500 μL of cold PBS. Gamma counting was done for the initial sample containing free ELP and labeled Trx-ELP (before salt addition), for the supernatant and for the tube with the resolubilized pellet. A set of control experiments, using BSA at a concentration of 20 μM, instead of free ELP, was conducted to clearly

identify the need for addition of free ELP as a coaggregant to recover Trx-ELP when present at very low concentrations.

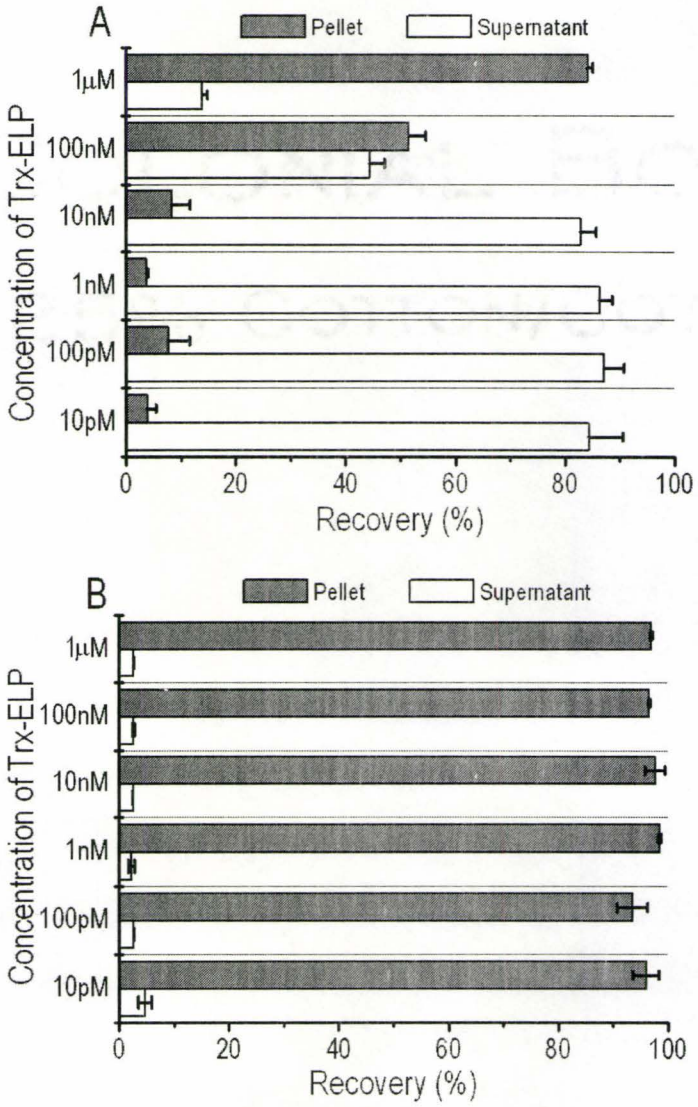

Figure 4.2 Percent of the initially labeled Trx-ELP that is associated with the supernatant (white bars) and resuspended pellet (gray bars) as a function of the Trx-ELP concentration, after one ITC cycle. (A) BSA at a concentration of 20 μM was used as the background for all experiments; (B) free ELP was supplemented to a concentration of 20 μM for all experiments. Error bars represent the gamma counting error and the standard deviation associated with three different experiments.

The results of the control experiments with BSA supplementation are presented in Figure 4.2A. The percent recovery is ~80% for the highest Trx-ELP concentrations (1 μM), an observation consistent with previous reports (Meyer and Chilkoti, 1999). The percent recovery decreases sharply as the concentration of fusion protein decreases, with virtually no recovery in the 1 nM to 10 pM concentration range. As shown in Figure 4.2B (addition of free ELP to a final concentration of 20 μM) coaggregation allows recovery of more than 95% of Trx-ELP when the fusion protein concentration was varied from as much as 1 μM to as little as 10 pM. For all samples, the supernatant contained less than 5% of the total Trx-ELP added to the initial sample. It is remarkable that the capture efficiency of this process is independent of the Trx-ELP concentration, to a level of 10 pM when free ELP is used as the coaggregant.

These results clearly demonstrate that the method could be used for a wide range of concentrations at which a target fusion is present: from high concentrations, where RIPT would allow recovery of the target without ELP supplementation, to extremely low concentrations, where coaggregation is required to recover the target. It is not possible, with the techniques we used (gamma counting), to determine the lowest concentration of Trx-ELP that can be recovered using coaggregation. A different set of experiments were done using a 0.2 μm membrane to capture the coaggregates formed during phase transition, instead of centrifugation. These experiments consistently generated percent recoveries of ~70% of the target protein, these values being lower than those shown in Figure 4.2B. After retaining the aggregates in the membrane, using 2.5 M NaCl, the membrane was washed with cold PBS, and the radioactivity associated with the PBS after

passage through the membrane only accounted for ~1% of the initial amount of labeled protein. This means that ~30% of the initial labeled protein was irreversibly bound to the membrane. By using a membrane, the samples were exposed to a large surface area associated with the filter, which resulted in high levels of nonspecific and irreversible binding to the membrane. This shows the need to reduce the surface area to which the sample is exposed in order to minimize losses.

4.4.2 Coaggregation is Highly Specific

The efficiency of the coaggregation process was evaluated, in terms of percent recovery and purity of the final product, using cell lysate (obtained from nontransformed host cells) as the background matrix in which the capture is done. To 10 mL of cell lysate were added free ELP and labeled Trx-ELP. To induce coaggregation, 10 mL of 5 M NaCl were added to start the first round of ITC. The final concentrations of ELP and Trx-ELP in these 20 mL samples were 5 μM and 1 nM, respectively. Two additional ITC cycles were performed, and the reported percent recovery for all samples was calculated with respect to the radioactivity present in the sample before the first ITC cycle was performed.

After 3 cycles of ITC, more than 80% of the labeled Trx-ELP was recovered (Figure 4.3A). It is important to note that for each ITC cycle both the percentage of recovered and lost Trx-ELP go down. Since recovery of Trx-ELP is less than 100% for each ITC cycle, the total mass of Trx-ELP decreases in consecutive ITC cycles. Because percent recoveries were calculated with respect to the radioactivity of the sample at the

very beginning of the experiment, before any ITC cycle was done, both the percent recovered and percent lost decrease reflecting loss of protein as more ITC cycles are performed.

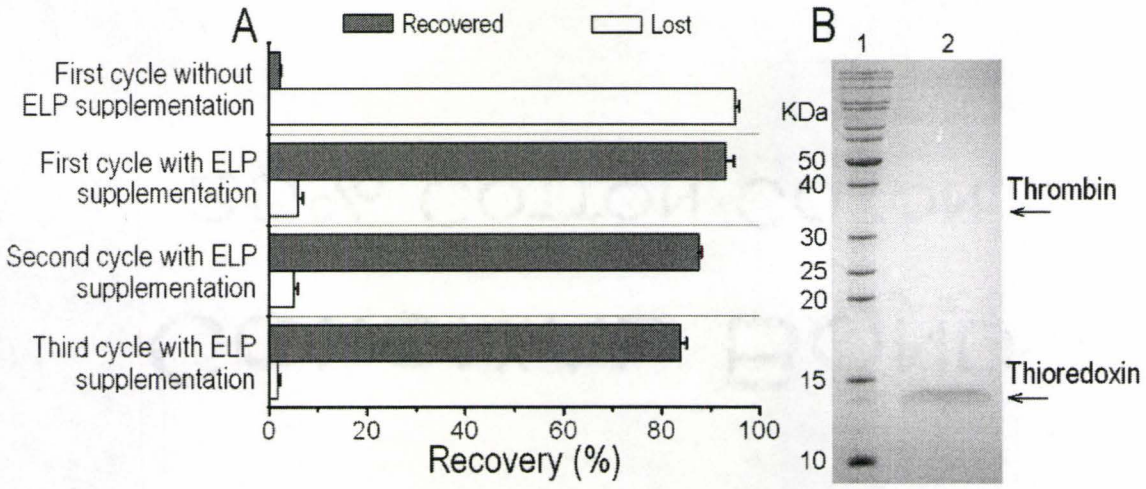

Figure 4.3 Capture of Trx-ELP at a concentration of 1 nM, from cell lysate supplemented with free ELP at a concentration of 5 μM. (A) Percent of the initially labeled Trx-ELP (in the initial sample prior to any ITC cycle was done) that is associated with the resuspended pellet (gray bars) and the summation of the radioactivity (white bars) in the supernatant from the first centrifugation step of ITC (high salt spin) and pellet from the second centrifugation step (low salt spin) of the same ITC cycle. Three ITC cycles were done, and the recovery percentages were calculated based on the initial sample in the experiment as being 100%. As the control, no free ELP was supplemented to the cell lysate containing Trx-ELP at 1 nM. Error bars represent the gamma counting error and the standard deviation associated with four different experiments. (B) SDS-PAGE for the sample obtained after 3 cycles of ITC, overnight cleavage with thrombin, followed by one additional cycle of ITC to separate the Trx from the ELP tag. The only other visible band corresponds to thrombin (37 kDa).

A control experiment was conducted with cell lysate, using Trx-ELP at a final concentration of 1 nM, but without addition of free ELP as a coaggregant. As shown in Figure 4.3A, more than 95% of the labeled Trx-ELP is lost in the supernatant of the first round of ITC, proving that free ELP supplementation is essential to enable recovery of the Trx-ELP at this low concentration.

The SDS-PAGE gel (Figure 4.3B) obtained for this experiment reveals that after three ITC cycles and overnight cleavage with thrombin, Trx was obtained to a high level of purity (thrombin was the only other protein detected in the gel, resulting in a very faint band). Coaggregation results not only in high capture efficiency, but also is a highly specific process. The ultimate limit of this method is still to be determined and alternative detection techniques must be used to determine what this limit is.

4.5 Discussion

Although affinity based systems have a large surface area associated with the matrix onto which the ligand is coupled, the surface area that is required for coaggregation based capture, as described in this paper, is only that associated with the walls of the vial that contains the sample. This should minimize the potential for contamination due to nonspecific binding and loss of target molecules due to irreversible binding. The set of experimental conditions at which RIPT occurs can be manipulated using various combinations of temperature, salt concentration, and total ELP concentration. Also, ELP tags with different lengths and sequences (Meyer and Chilkoti, 2002) have been developed that should allow the use of coaggregation for desired sets of experimental conditions.

One important aspect of ELPs is that single conjugation points, via incorporation of a lysine or a cysteine residue, can be engineered into the ELP sequence, allowing site-specific coupling of molecules with ELP tags. A possible application of this method is conjugating a library of nucleic acids to ELP, and using the coaggregation process as part

of the "in vitro selection" (Tuerk and Gold, 1990; Ellington and Szostak, 1990) scheme to enrich for aptamers. In the initial rounds of selection, the aptamers account for a small percentage of the molecular population being screened. As such, they should be recovered with the maximum level of efficiency possible. We believe that the general concept demonstrated in this paper is applicable to more than capture and purification of proteins present at very low levels and has potentially wide ranging applications in many diverse areas.

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Chapter 5

In Vivo Formation of Protein Based Aqueous Microcompartments

5.1 Abstract

In this chapter, we report the formation of protein based liquid droplets resulting in the formation of microcompartments *in vivo* in *E. coli*. These microcompartments where generated by expressing elastin-like polypeptides (ELP) which have the ability to undergo a reversible phase transition, resulting in the formation of an aqueous two-phase system (ATPS) in the cytoplasm of the cell. We prove that these microcompartments are liquid by expressing a fusion protein consisting of ELP and GFP and by performing FRAP experiments at different stages of cell cultivation. In the initial phases of cell growth, the fusion protein concentration is low and is not large enough to drive the formation of a second aqueous phase. As the intracellular fusion protein concentration increases with longer cultivation time, droplets start forming and as protein expression continues, the droplets coalesce at the cells' poles. Fluorescence recovery after photobleaching (FRAP) experiments with cells at different growth stages, revealed that the protein in these ELP based droplets are aqueous and not solid aggregates, as seen in typical inclusion bodies. Staining of the ribosomes and co-imaging the GPF fusion protein, showed that these compartments exclude the protein making machinery of the cell, acting as depots for newly formed protein. It is also shown, *in vitro*, that ELP based

droplets result in the exclusion of proteases, protecting proteins from degradation. To the best of our knowledge, this is the first report on engineering the formation of an extra aqueous phase in a living organism.

5.2 Introduction

Aqueous two-phase systems (ATPS) can be obtained by the presence of two or more polymers (Piculell and Lindman, 1992), or one polymer and a chaotropic salt (Ananthapadmanabhan and Goddard, 1987). In these systems, two or more distinct aqueous phases are formed with a well defined interface separating them. These systems have been used in a wide array of applications, ranging from methods for extraction of biomolecules (Albertson, 1986), to mimics of cytoplasmic organization (Long et al., 2005, 2008) since ATPS systems share a common feature in biological systems, which is compartmentalization. Keating's group described the use of an ATPS using PEG/dextran pairs and encapsulating the droplets with lipid bilayers to generate vesicles that mimic molecular crowding inside cells (Long et al., 2005). It has been noted that current ATPS systems used as models of biological organization are formed using synthetic polymers and it would be highly desirable to find equivalent systems that would be plausible in cells (Pielak, 2005).

In a series of publications from the mid 1980s, Urry's group reported on the synthesis and unique behavior of a series of synthetic and repetitive peptides (Urry, 1985, 1988). They consist of repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is any amino acid except proline. These peptides are able to undergo a reversible inverse

phase transition resulting in ATPS formation (Zhang et al., 2003, 2006). Phase transition can be triggered using a variety of stimuli, such as temperature and addition of chaotropic salts (Meyer et al., 2001; Urry, 1992, 1997) and is affected by the ELP sequence and chain length (Meyer et al., 2001; Meyer and Chilkoti, 2004).

In this chapter, we show that ELP allow the formation of ATPS *in vivo* in *E. coli*. These results demonstrate that ELP are ideal for bridging synthetic ATPS based systems, currently used to study the importance of molecular crowding in biological systems, by allowing generation of ATPS *in vivo*, thereby allowing for a more plausible framework for these type of studies. The results also suggest that ATPS formation *in vivo* can be a valuable tool for high-level expression of proteins that are easily degraded by proteolysis.

5.3 Material and Methods

5.3.1 Plasmids

The elastin-like polypeptide used in this work was ELP [$V_5A_2G_3$-90], which comprises 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio of 5:2:3. Herein this protein will be referred to as ELP. The green fluorescence protein (GFP) mutant used in this study was GFPgcn4, which has enhanced sensitivity for microanalysis at 488 nm excitation (Ito et al., 1999). Standard molecular biology techniques were used to generate the plasmid encoded for the production of GFP fused to the C-terminus of ELP (ELP-GFP). The starting plasmid, pTrx-ELP (for the production of thioredoxin fused to the N-terminus of ELP) (Meyer and Chilkoti, 1999), was digested by *Bsu*RI, and the 1.3kb ELP fragment was gel recovered and inserted into

*Sma*I site of pUC19 (Invitrogen) to obtain pUC19-ELP. The ELP sequence with *Eco*RI and *Hind*III sticky ends was cut out of pUC19-ELP by double digestion with these two restriction enzymes and sub-cloned into the corresponding sites of pET28b (Novagen) resulting in pET28b-ELP. The GFPuv4 gene was PCR amplified from pCA24N (Kitagawa et al., 2005) as the template and using 5'-gtacctgcaggggccgcagtaaaggagaagaa-3' and 5'-catggcatggatgaactatacaaataagcttgcgcc-3' as the primers. The PCR product was digested with *Pst*I and *Hind*III, and inserted into the corresponding sites on pET28b-ELP to finally obtain the ELP-GFP expression plasmid, pELP-GFP. The plasmid pELP, which codes for expressing ELP alone, was also constrcuted as described in Chapter 4. The restriction and modification enzymes were purchased from Fermentas (Burlington, Ontario). DNA fragments were separated by agarose gel electrophoresis and purified with DNA extraction kits (Qiagen). All the plasmids were analyzed by restriction mapping and confirmed by DNA sequencing (Mobix, McMaster facility).

5.3.2 Protein Expression and Purification

The plasmids coding for ELP-GFP, Trx-ELP, and ELP were transformed into *E. coli* strain BLR (DE3) competent cells (Novagen), which is used for expressing recombinant proteins with tandem repeats, because of its deficiency in homologous recombination. For hyper-expression of ELP-GFP, the non-induction method was used (Guda et al., 1995; Trabbic-Carlson et al., 2004; Chow et al., 2006). Transformed BLR (DE3) were cultured for 36 hr at 37 °C in Terrific Broth supplemented with 2 mM proline. The cells were harvested by centrifugation and the pelleted cells were

resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.2 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH7.4) in a volume ratio of 1:25 with respect to the initial volume of culture medium. The cells were lysed by applying discontinuous ultrasonic pulses (VirTis) in a ice-water bath. ELP-GFP were purified using inverse transition cycling (ITC) (Meyer and Chilkoti, 1999). The purity of the proteins was determined with SDS-PAGE. The concentration of purified ELP-GFP was measured by its absorbance at 280 nm using a spectrophotometer (Cary 100), with the extinction coefficient of 62730 M^{-1}, calculated from amino acid sequence.

5.3.3 Sample Preparation For Imaging

For *in vitro* studies, a sample containing 100 μM of purified ELP-GFP in 1.5 M NaCl was prepared, which resulted in phase transition of the ELP-GFP fusion protein. This turbid solution was transferred to a microscope slide and fluorescence microscopy was used to collect and stack images in the Z-direction. This solution (100 μM ELP-GFP in 1.5 M of NaCl) was also centrifuged to enhance droplet coalescence. The ELP-GFP rich phase was collected and spread onto a microscope slide and imaged. For *in vivo* studies, Terrific Broth supplemented with proline was used to culture *E. coli* BLR (DE3) transformed with pELP-GFP. Fresh overnight-grown cultures were inoculated (1% of the volume of fresh media) and cultured at 37 °C, 200 rpm. Cells were sampled at various cultivation times, and imaging was completed no more than 1 hour after sampling. Fluoresce recovery after photobleaching (FRAP) were done *in vivo* using ELP-GFP expressing *E. coli* cells grown for 24 hours. To prevent cell movement during the FRAP

experiments and for three dimensional Z-stack imaging, the cells were immobilized with 10% polyacrylamide gel, which was prepared as follows: 500 μl of the cell culture was harvested by centrifugation and the pelleted cells were resuspended in 330 μl H_2O and then adding 165 μl of 30%/0.8% acrylamide/bi-acrylamide (EMD), 3 μl 10% ammonium persulfate (EMD) and 0.3 μl TEMED (EMD). After mixing, 5 μl of the mixture was immediately transferred onto microscope slides (VWR) and covered with #1 cover glass (VWR). As soon as polymerization took place, the edges of the cover glass were sealed with nail polish (MAC Cosmetics) to prevent drying. Imaging of cell membranes and nucleic acids distribution *E. coli*, were done using lipophilic styryl dye FM 4-64 (Molecular Probes) and SYTO 62 (Molecular Probes), respectively. *E. coli* cells were harvested after 24 hours culture by centrifugation and resuspended in PBS (for FM) or 150mM NaCl (for SYTO). The final concentration for both dyes was 1 μM, and the cells were stained for 20 minutes before imaging.

5.3.4 Microscopy and Imaging System

All microscopic images were taken using a Leica TCS SP5 confocal microscope (Leica Microsystem) equipped with an Acousto-Optical Beam Splitter and a 63× 1.3NA glycerol objective lens. The microscope was operated with the Leica Application Suite Advanced Fluorescence software (LAS AF confocal). The laser source for excitation was Argon ion (488 nm) for imaging the green fluorescence protein, DPSS (561 nm) for imaging the FM 4-64 red fluorescence dye, and HeNe (633 nm) for imaging the SYTO 62 red fluorescence dye. Emission wavelength ranges were adjusted according to the

spectrum of the fluorophores FM 4-64 and SYTO 62, as recommended by Molecular Probes. For 3D imaging, a minimum of 30 Z-stacked images were collected and processed with the Imaris V5.0 (Bitplane) software to create 3D sectional images. Quantitative data for FRAP experiments were obtained using the FRAP wizard program (Leica). Relative fluorescence intensity was determined by N_t/N_0, where N_0 is the average fluorescence intensity of a given area before photobleaching and N_t is the average fluorescence intensity for that same area after a certain period of time had elapsed after photobleaching.

5.3.5 Phase Transition

The phase transition behavior of ELP at different concentrations (500 μM, 200 μM, 100 μM, and 50 μM and in PBS), was monitored using a Cary 100 Ultraviolet-Visible spectrophotometer equipped with a multicell temperature controller (Varian Instruments). The absorbance of the samples at 350 nm (OD_{350}) was monitored as a function of temperature. The temperature was increased at a constant rate of 0.5 °C/min. The phase transition temperature (T_t) was defined as the temperature at which 50% of the maximum OD_{350} of the sample was obtained.

5.4 Results and Discussion

5.4.1 In vitro *Formation of Aqueous Two-Phase System*

In our studies, we used an ELP consisting of 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly (Xaa being Val, Ala, and Gly in the ratio of 5:2:3), fused to the mutant of the green fluorescent protein (GFP gcn4) with enhanced fluorescence when excited at 488 nm (Ito et al., 1999). The first step was to unequivocally demonstrate that this fusion protein results in the formation of ATPS *in vitro*. Figure 5.1A(i) shows a sample containing ELP-GFP, to which NaCl was added (ELP-GPF final concentration of 100 μM for ELP-GFP and 1.5 M for NaCl), immediately causing the solution to become turbid (Figure 5.1A(ii)). Fluorescence imaging of this sample revealed the presence of fluorescent spheres with a diameter of about 5 μm (Figure 5.1B), with the entire volume of these spheres comprising a single homogenous and continuous phase (sections of spheres shown in Figure 5.1B). To enhance droplet coalescence, centrifugation was applied to the coacervated mixture and this resulted in the formation of total coalescence of the ELP-GPF droplets into a single large droplet (Figure 5.1A(iii)), which is optical transparent. This large droplet was sampled and it was observed under the microscope (Figure 5.1C). This droplet consisted of a single and continuous ELP-GFP rich phase with uniform fluorescence intensity. It was also observed that upon agitation, the giant droplet was destroyed and the solution became turbid, which indicates reversibility of coalescence (data not shown). The key features of aqueous two-phase systems are: both phases are composed mainly of water, and each of the phases is relatively rich in one of the solute components (Ananthapadmanabhan, 1987). This means that the solute rich

phase is fluid. As shown in Figure 5.1A(iv), the shape of the ELP-GFP rich phase changes 5 minutes after centrifugation due to gravity, indicating that the isolated ELP-GFP rich phase is indeed a fluid and not a solid precipitate.

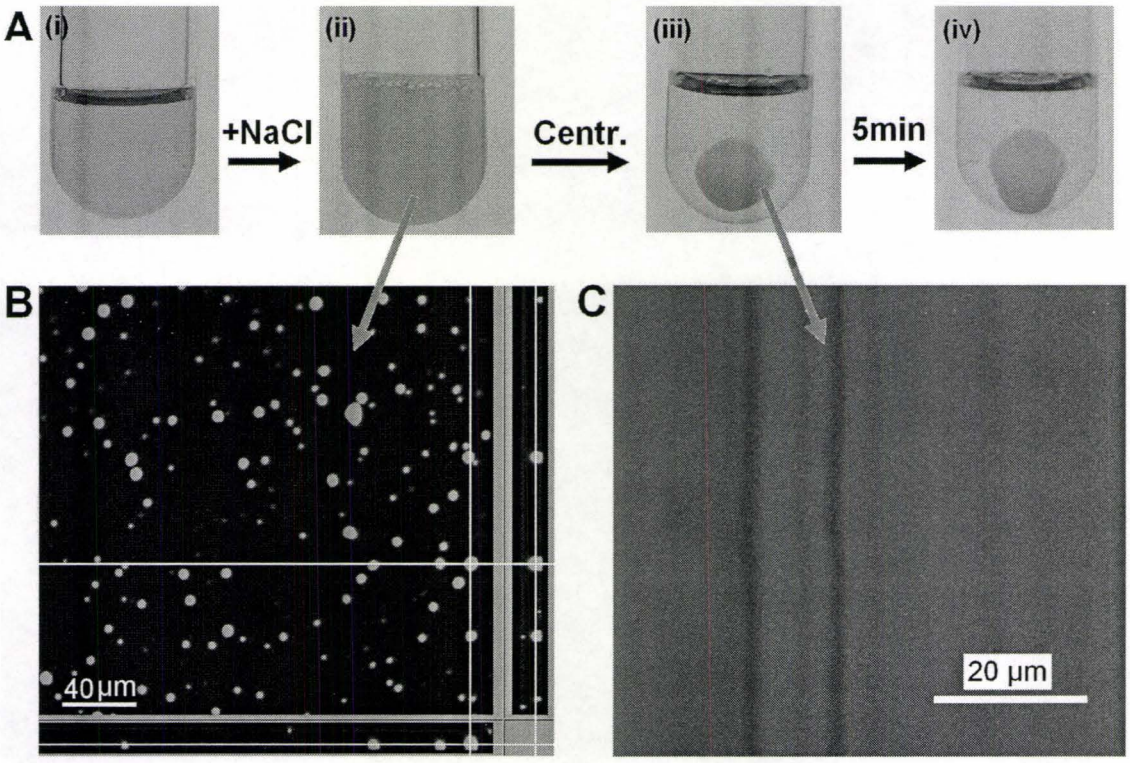

Figure 5.1 Formation of ELP-GFP/NaCl aqueous two-phase system *in vitro*. (A) The process of formation and partitioning of the two phases: (i) ELP-GFP in PBS solution; (ii) formation of the aqueous two-phase turbid solution by addition of NaCl; (iii) droplet coalescence by centrifugation; (iv) gravity acts on ELP-GFP giant droplet, causing it to move (5 minutes) without the droplet breaking. (B) 3D fluorescent microscope imaging of the turbid solution in Figure 5.1A(ii). (C) Imaging of separated ELP-GFP rich phase from Figure 5.1A(iii) reveals a single and homogenous phase.

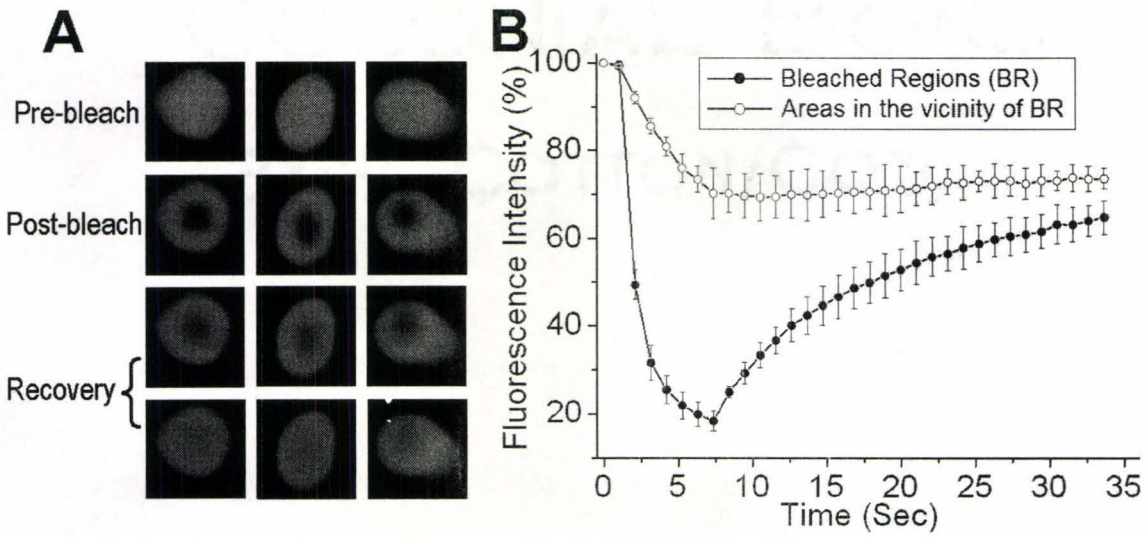

Figure 5.2 *In vitro* fluorescence recovery after photobleaching (FRAP) analysis of ELP-GFP rich phase droplets: (A) Fluorescent imaging done at different stages of FRAP experiments; (B) Quantitative FRAP analysis of bleached regions and areas in the vicinity of the bleached regions.

FRAP experiments were conducted to further confirm protein mobility, and the existence of an aqueous phase, in the ELP-GFP rich phase. The results of these experiments and the quantitative analysis are shown in Figure 5.2. After photobleaching, the fluorescence of the bleached regions recovered rapidly, with more than 60% of the initial fluorescence being recovered. The areas surrounding the bleached regions were also monitored for fluorescence intensity. It was found that during bleaching, the fluorescence intensity in these area decreases, due to the mobility of ELP-GFP in the entire droplet. The data shown in Figure 5.1 and Figure 5.2 unequivocally shows that the ELP-GFP fusion protein allows the formation of aqueous two-phase systems (Ananthapadmanabhan, 1987) *in vitro*.

5.4.2 ATPS Formation In vivo

The formation of ATPS is dependent on the concentration of the solute (Yamaoka et al., 2003; Meyer and Chilkoti, 2004). Results from phase transition temperature measurements (Figure 5.3) show that for ELP concentrations higher than 200 μM, ATPS formation occurs under physiological conditions (in PBS and 37 °C).

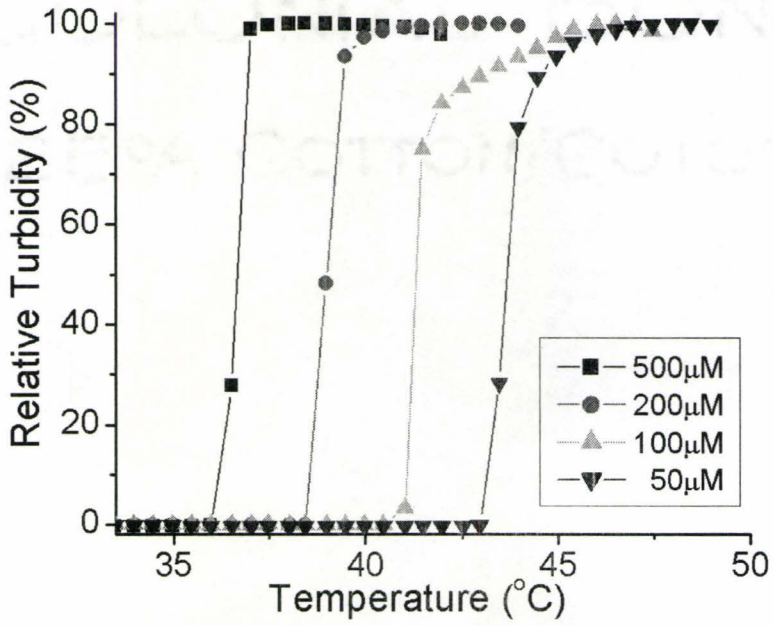

Figure 5.3 Phase transition of elastin-like-polypeptide (ELP) solutions with different ELP concentrations. Under physiologically relevant conditions (in PBS), the phase transition temperatures with deferent concentration of ELP are: 43.5 °C for 50 μM; 39.0 °C for 100 μM; 41.0 °C for 200 μM; and 36.5 °C for 500 μM ELP.

Extremely high expression levels have been reported for proteins fused to ELP – as much as 1.6 g purified ELP fusion protein can be obtained per liter of culture (Chow et al., 2006). Considering that the entire mass of ELP fusion protein is contained in the small volume associated with the cytoplasm of *E. coli*, the concentration of the ELP

fusion proteins inside the cell must be at levels well above those required for the formation of ATPS. Figure 5.4 shows the process by which ATPS are formed *in vivo*, by imaging cells at different points of culturing. Three distinct stages could be observed:

1. For fairly short cultivation times, the amount of ELP-GPF produced is still low, resulting in an intracellular concentration that is not sufficient to drive the formation of ATPS. Figure 5.4A and 5.4B (shown sections in the xyz directions) reveal that after 10 hours of cell growth, the ELP-GFP is uniformly distributed within the cytoplasm of the cells. The fluorescence intensity of the cells is fairly low, which suggests that the ELP-GPF concentration in the cells is not high enough to allow formation of ATPS. The arrows point to the poles of some cells where ATPS starts to form, as protein is accumulated.
2. When cells were cultured for 20 hours, small droplets started to appear, clearly showing the formation of ATPS *in vivo* as the intracellular concentration of ELP-GFP continues to increase. Sections of the cells were imaged and it can be seen that these droplets are isolated from each other.

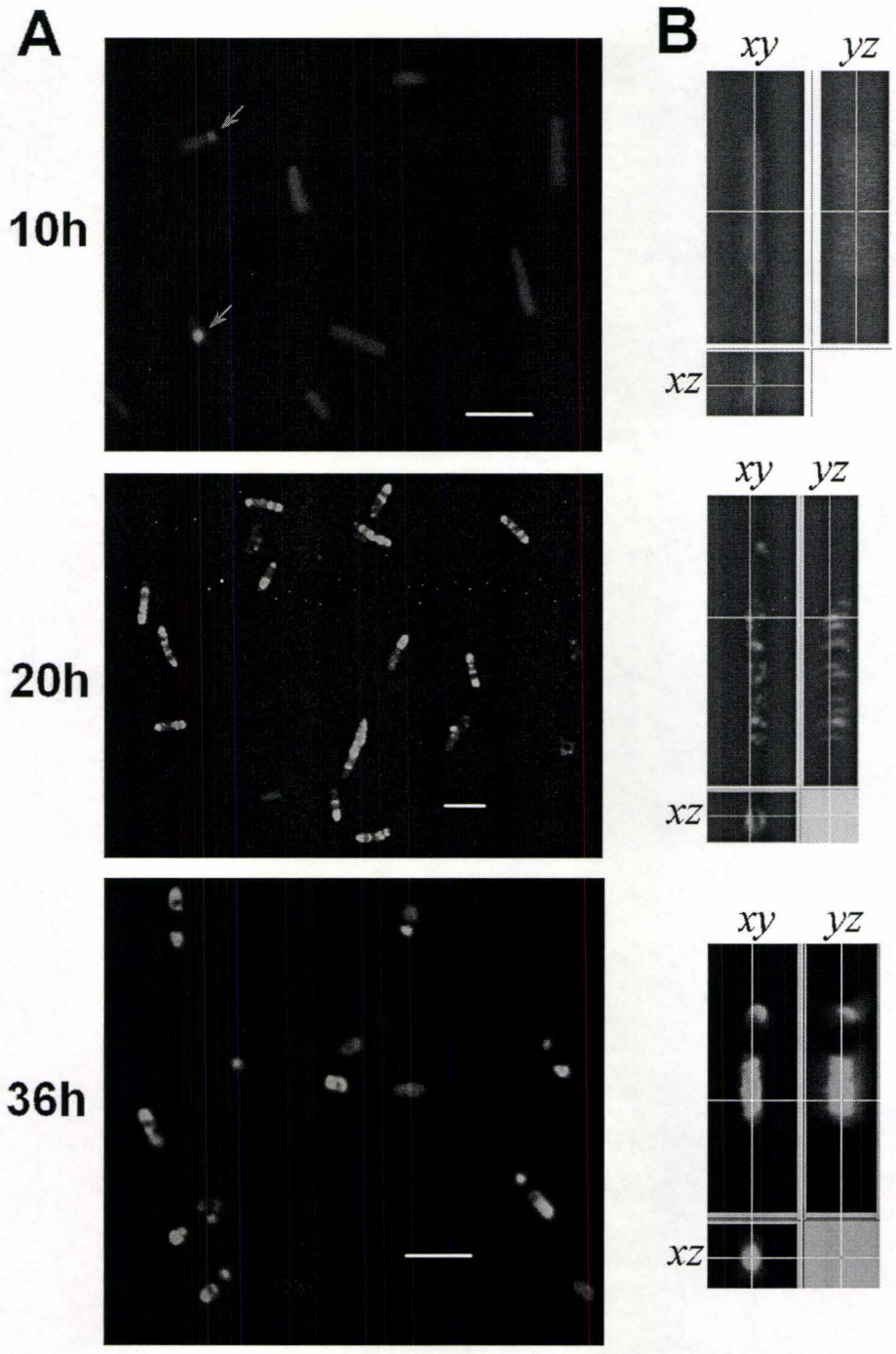

Figure 5.4 Increasing the ELP-GFP concentration by extending cultivation time leads to the formation of ELP-GFP rich ATPS compartments inside *E. coli*: (A) Fluorescent imaging for different cultivation times – 10 hours (top), 20 hours (middle), and 36 hours (bottom); (B) 3D-section fluorescence images of individual cells at various cultivation times, for the same samples as shown in panel A. Bars represent 4 μm.

3. After 36 hours of cultivation, the total amount of ELP-GFP present inside the cells was high (as shown by high levels of fluorescent intensity of the cells, Figure 5.4A). Due to the high intracellular concentration of the fusion protein, the small droplets merge resulting in a smaller number of very large droplets. From Figure 5.4A, the formation of two different compartments can be seen in a single cell: an ELP-GFP rich phase (highly fluorescent) and a ELP-GFP poor phase (dark regions of the cytoplasm). Sections of these cells (Figure 5.4B) reveals that the ELP-GFP rich phase consists of a single homogeneous phase that extends to the entire height of the cytoplasm.

In vivo ATPS formation occurs virtually in the same manner as the ATPS formation observed *in vitro* (shown in Figure 5.1), the main difference was that, *in vitro,* centrifugation was used to coalesce the droplets and *in vivo*, particles increased in size due to both coalescence (diffusion of the particles) and increases in the ELP-GPF concentration, due to continuous protein expression.

We wanted to probe the nature of the intracellular droplets shown in Figure 5.4 to clearly demonstrate that these are not typical inclusion bodies, since it has been reported that enzymatic and fluorescent activity can be found in inclusion bodies (Garcia-Fruitos et. al., 2005). Imaging the ELP-GFP rich compartments with transmitted light (DIC) and fluorescence imaging, showed that these compartments were optically clear without any opacity (Figure 5.5), which means that they are liquid in nature and not typical solid inclusion bodies.

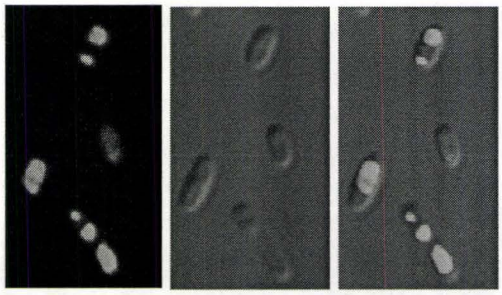

Figure 5.5 The observed intracellular compartments are not usual inclusion bodies. Fluorescent image (left), transmitted light image (DIC) (middle), and superimposing the DIC and fluorescent images (right).

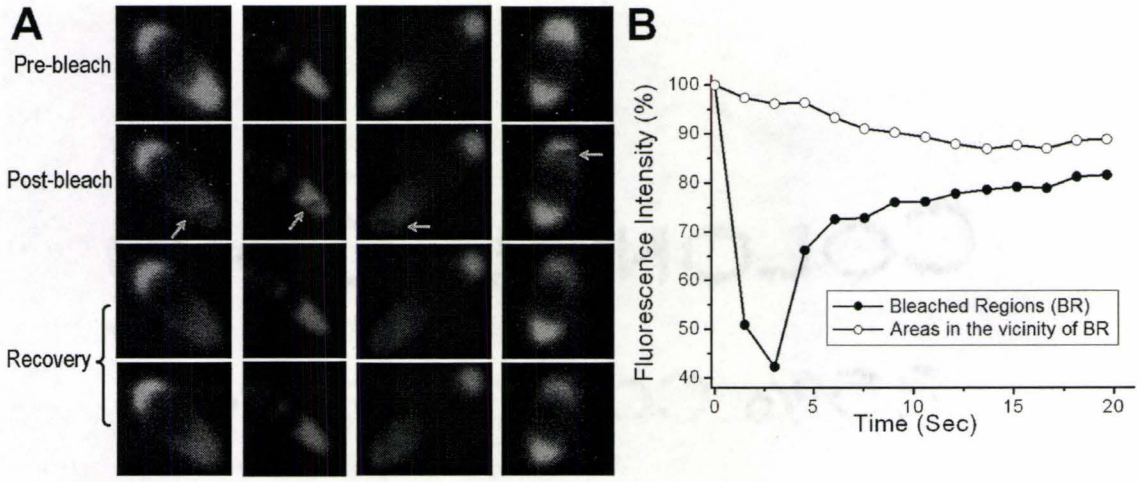

Figure 5.6 FRAP analysis of ELP-GFP rich phase compartments *in vivo*: (A) Fluorescent images of *E. coli* cells (24 hr cultivation) during FRAP experiments: prebleach (top row), immediately after bleaching (the 2nd row), and recovery (bottom two rows). The bleached regions in each cell are indicated with arrows; (B) Quantitative data for a FRAP experiment.

To further confirm that these compartments were liquid, FRAP was used to analyze cells cultivated for 24 hours (Figure 5.6A), and the results of these experiments were quantified (Figure 5.6B). Fluorescence was quickly recovered in the bleached areas. Recovery was achieved at the expense of the ELP-GFP in that particular compartment (cells typically had two compartments, located at each one of the poles)

indicating that ELP-GFP is highly mobile within one ATPS compartment and not in between compartments, since they are separated by the cytoplasm. Recovery rates were different for different cells, due to different ratios of the volume of the bleached region to the total volume of the compartment containing the bleached region. The immobilization of *E. coli* cells in 10% polyacrylamide gel totally prevents cell movement during imaging. The polyacrylamide gel is transparent to optical light and to the laser beam and no detectable deflection observed. This technique should be very useful for accurate FRAP measurements and 3D Z-stack imaging of highly mobile organisms.

5.4.3 Functionalized Compartments

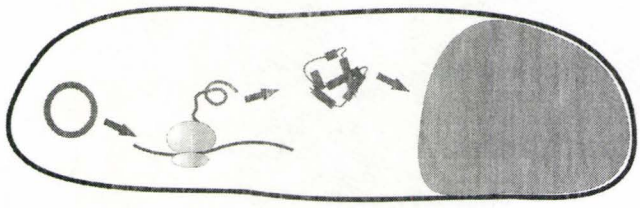

Figure 5.7 Scheme of functional intracellular partitioning: intracellular compartments for recombinant protein synthesis and recombinant protein storage.

The formation of ATPS compartments *in vivo* suggests that functional compartmentalization of cells maybe possible. Figure 5.7 shows that separate compartments contribute to two different functions: protein synthesis and protein storage. Transcription and translation take place in the cytoplasm, and the synthesized ELP containing proteins are accumulated in the second phase, which acts as a separate compartment to store protein. If the storage compartment is formed as an ATPS, one expects that these compartments should be located at the cell poles, to minimize the

interfacial surface between the two phases, so that the free energy is minimized. To locate the ELP-GFP storage compartments in living cells, a lipophilic styryl dye FM 4-64 was used to stain cell membrane and define the boundary of the cell, showing that the ELP-GFP rich compartment is located at the cells' poles (Figure 5.8).

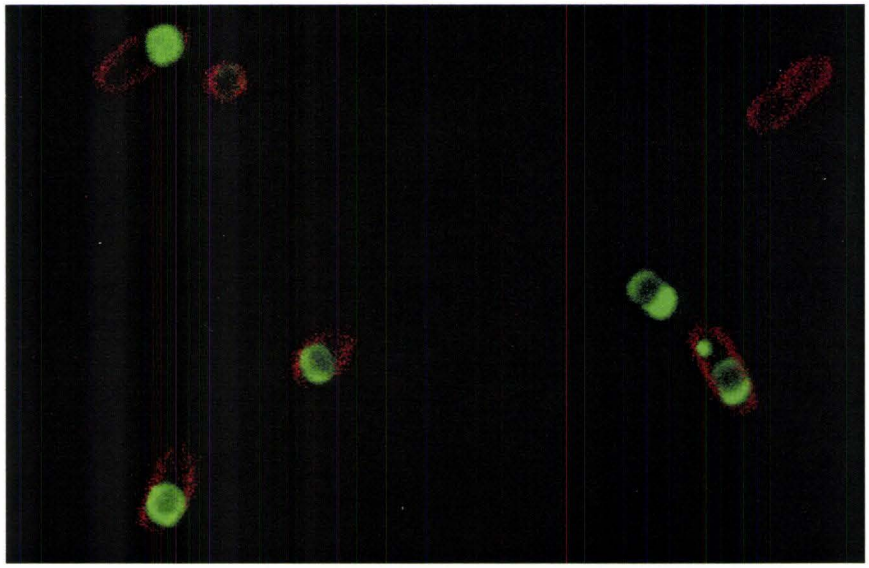

Figure 5.8 Accumulation of ELP-GFP as compartments at the poles of *E. coli* cells (24 hr cultivation). The cellular membrane was stained with a red fluorescent lipophilic styryl dye (FM 4-64).

SYTO 62 is a red fluorescent dye whose fluorescence is enhanced upon binding to nucleic acids. It is widely used to stain DNA, RNA, and to visualize ribosome in living cell (Knowles et al., 1996). Cells expressing ELP-GFP were cultured for 24 hours, stained with SYTO 62 and imaged (Figure 5.9). Enlarged images of selected (boxed) cells are shown in the right panel of Figure 5.9. The green and red fluorescence do not overlap.

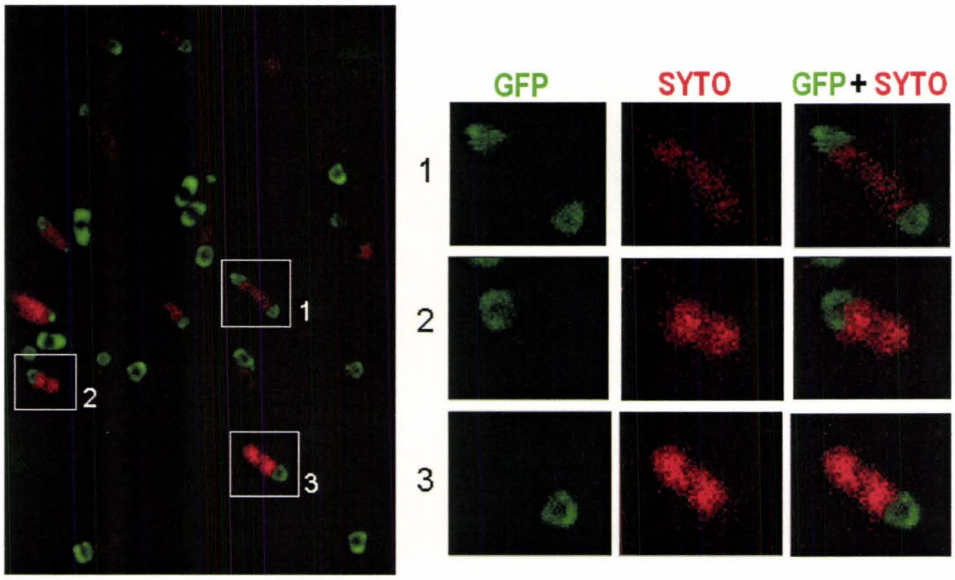

Figure 5.9 Nucleic acids and ELP-GFP are physically separated inside *E. coli* cells (24 hr cultivation). Nucleic acids were stained by SYTO62. Featured cells are enlarged and shown in the right panel.

A control experiment was done *in vitro* to demonstrate that the SYTO dye can permeate through ELP-GFP droplets (Figure 5.10). Taken together, these results show that exclusion of the SYTO stain from the ELP-GFP compartments found *in vivo* is not due to immiscibility of the dye in ELP-GFP rich compartments, but because nucleic acids and ribosomes were excluded from these compartments. It is indeed possible to create functional partitioning of the cell into areas for protein synthesis and areas for protein storage. It is also clear that the formation of ATPS compartments *in vivo* does not interfere with protein expression. As gene transcription and ribosome directed translation continuously take place in the cell, ELP-GFP based droplets start forming at the expense of the ELP-GFP being produced. This results in low concentrations of the ELP fusion

proteins in the cytoplasm of the cell, minimizing protein crowding in the cell, which is of potential value for the expression of aggregation prone proteins.

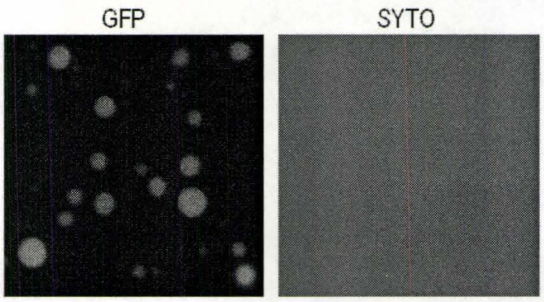

Figure 5.10 The observation that SYTO dye can permeate through ELP-GFP droplets. 50 μM ELP-GFP with addition of 1.5 M NaCl forms ATPS at room temperature. The cloudy mixture was added with SYTO (to the concentration of 1mM), and incubated 20 minutes before florescence images were taken.

5.4.4 ATPS and Proteolytic Protection

The ability of ELP tagged proteins to undergo reversible inverse phase transition has been used to purify a variety of recombinant proteins (Trabbic-Carlson et al., 2004; Banki et al., 2005) from complex mixtures, such as cell lysate, using Inverse Transition Cycling – ITC (Meyer and Chilkoti, 1999). The key feature of this approach is that ELP based droplets contain ELP tagged proteins, but exclude other proteins present in the cell lysate. If this was not the case, ITC would not work a protein purification method. We hypothesized that ATPS formation would also exclude proteolytic enzymes and formation of ELP base droplets may protect ELP fused proteins from proteolytic attack. This could partially explain the very high expression levels that have been reported when expression proteins fused to ELP (Chow et al., 2006; Guda et al., 1995; Scheller et. al.,

2006; and Patel et. al., 2007), since we show that ATPS formation takes place inside the cells. To test this hypothesis, thioredoxin-ELP fusion protein (Trx-ELP) with a thrombin cleavable linker in between the ELP tag and Trx, was expressed and purified as described elsewhere (Meyer and Chilkoti, 1999; Meyer et. al., 2001). This fusion protein was used as the substrate to determine the proteolytic activity of thrombin in the presence and absence of ATPS formation.

Samples consisting of 250 μM Trx-ELP in 1M NaCl were maintained at 20 °C (below T_t) and 30 °C (above T_t). Thrombin (Sigma) was added to these samples in a ratio of 2 U per mg of Trx-ELP fusion and the samples were incubated for 2 hours. The results are shown in Figure 5.11. As compared to the initial amount of Trx-ELP (lane 1), more than 95% of Trx-ELP was cleaved by thrombin when the sample was incubated at a temperature below T_t (lane 3); on the other hand, more than 95% of Trx-ELP remained uncleaved when the sample was incubated at a temperature above T_t (lane 4). These results demonstrate that Trx-ELP, without ATPS formation, is readily accessible and cleaved by thrombin, but is protected from thrombin when ATPS is formed. By excluding the thrombin from the Trx-ELP droplets, the cleavage reaction can only happen at the interface of the two phases, therefore, dramatically reducing the rate of reaction. It should be noted that the NaCl concentration was found to have no effect on the thrombin cleavage activity – no detectable difference of cleavage efficiency was found between samples at low and high NaCl concentrations, without ATPS formation (lane 2 and lane 3).

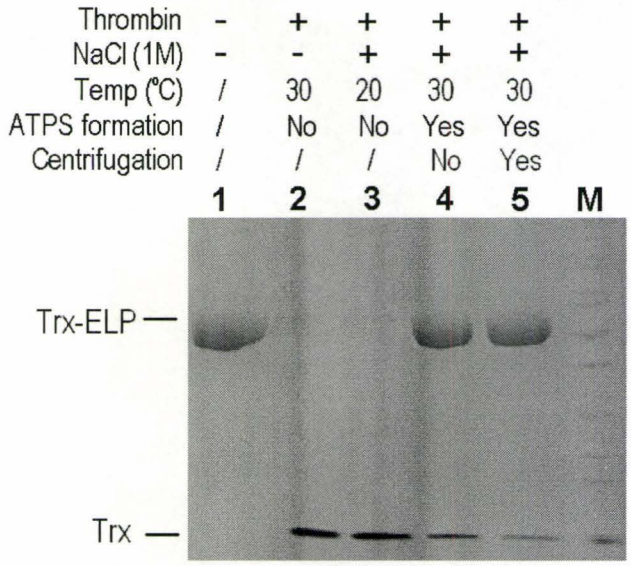

Figure 5.11 Protection from protein proteolysis with ATPS formation. Proof-of-concept experiments using thrombin cleavage. Lane 1: Initial Trx-ELP fusion protein; Lane 2: same as in lane 1 with incubation with thrombin for 2 hours and 30 °C (no ATPS formed); Lane 3: same as in lane 1, with addition of NaCl (1 M) and incubation with thrombin at 20 °C (below T_t, no ATPS formed); Lane 4: same conditions as for lane 3, but incubation at 30 °C (above T_t, ATPS formed); Lane 5: same conditions as lane 4, except that centrifugation was used to promote droplet coalescence before adding thrombin; Lane M: molecular markers. **Note:** free ELP tags are not visible in the gel because they are poorly stained with Coomassie Blue.

Changing the interfacial surface area of the Trx-ELP rich phase should change the rate of proteolysis. Centrifugation was applied to a sample (above T_t and 1M NaCl), to cause coalescence of the Trx-ELP droplets, resulting in a large decrease of the interfacial area. After incubation with thrombin, the amount of thioredoxin released by thrombin cleavage (lane 5) in the centrifuged sample is only ~1/3 of that observed when small droplets where present (lane 4). It was demonstrated that *in vitro*, ATPS greatly decreased the rate of proteolysis by excluding the proteolytic enzyme (in this case thrombin) from the volume where the protein targeted for proteolysis is present. We

believe that similar results could be obtained *in vivo* and could be useful for expressing recombinant proteins that are subjected to high levels of proteolysis and degradation.

5.5 Conclusions

The following conclusions can be drawn from this work:

1. Inverse phase transition of ELP tagged proteins results in the formation of an aqueous two-phase system (ATPS).
2. Phase transition occurs *in vivo*, resulting in the formation of aqueous droplets in the cytoplasm of cells.
3. These droplets exclude the ribosomes present in the cytoplasm of the cell, thereby physically separating recombinant protein synthesis from recombinant protein storage.
4. Formation of ATPS *in vitro* allows protection of ELP tagged proteins against proteolytic activity by excluding the proteolytic protein from the ELP fusion protein rich droplets.

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Chapter 6

Protein Based Aqueous-Multiphase Systems

6.1 Abstract

This chapter reports the formation of aqueous multiphase systems (AMPS) exclusively made with proteins. All these systems were generated using elastin-like polypeptides (ELP), which have the ability to undergo reversible phase transitions. Manipulating variables such as the molecular weight of ELP, composition of ELPs (using different amino-acid sequences or by fusing the ELP with different functional proteins), allowed us to manipulate the temperature at which phase transition takes place, the number of phase that are formed, as well as the composition of the multiple aqueous phases. Using these variables, both isotropic hybrid colloids with tunable functionality and anisotropic colloids with variable morphologies could be generated. Formation of aqueous multi-phase systems (AMPS) and anisotropic nanocolloids have been reported in the literature using synthetic polymers, but to our knowledge this is the first report of generating such systems using proteins.

6.2 Introduction

Controlling material distribution in ultra-small scales is the key challenge in nanotechnology. There are few reports on synthesis of multi-compartment nanoparticles,

such as biphasic and triphasic Janus droplets by simultaneous microfluidic jetting of parallel polymer solutions in an electric field (Roh 2005, Roh 2006) or through sheath flowing (Nisisako 2007). To date, all fabricated anisotropic nanoparticles are composed of polymers and no report could be found on building these structures with proteins. Protein based materials have significant advantages over chemically synthesized polymers: (1) the polypeptide sequence and length can be precisely controlled at the genetic level (Yu 1997); (2) protein based materials can be produced with very specific and predefined points for chemical conjugation to other functional molecules; (3) multiple functionality can be incorporated in a single molecule at the gene level by expression of chimeric proteins; (4) biocompatibility, which is an essential requirement for biomedical applications such as drug delivery (Meyer et al., 2001a), regenerative tissue engineering (Holmes 2000, Ellis-Behnke 2006), and stem cell differentiation (Silva 2004); (5) since proteins are expressed in living cells, then these proteins can be used *in vivo,* in applications such as microcompartmentalization of living cells (Chapter 5). Due to these advantages, protein based materials are being increasingly used in nanotechnology (Sarikaya 2003, Banta 2007).

Elastin-like polypeptides (ELP) are proteins biopolymers composed of repetitive sequences of five-amino-acids, VPGXG, where X, the guest residue, can be any amino acid except proline. Elastin-like polypeptides have a unique property – reversible inverse phase transition. Below a certain temperature, called phase transition temperature (T_t), aqueous solutions of ELPs are clear and homogeneous. Upon increasing the temperature of the solution above T_t, the solution becomes turbid (Urry 1992, Urry 1997) due to

coacervation of the ELP into droplets. Dynamic light scattering studies revealed that the size of these droplets range from ~200 nm to a few micrometers (Meyer et al., 2001b; Meyer and Chilkoti, 2002). The size and size distribution of elastin droplets can be manipulated by changing the protein concentration and temperature (Kaibara 2000, Meyer 2002). After coacervation, the ELP droplets will spontaneously aggregate to form aqueous two-phase systems (ATPS), and the kinetics of this process was recently studied and it was found that addition of surfactants such as SDS can stabilize the emulsion for several hours (Zhang 2003). The reversibility of the phase transition behavior of ELP has the potential to serve as a tool for fabrication of protein-based colloids, that are responsive to external stimuli and that can be formed in a reversible manner.

Changing external conditions or the internal composition of ELP molecules can modify the phase transition temperature of ELP. The external conditions effecting T_t include salt type and concentration (Urry 1992, Yamaoka 2003), pH (Urry 1992), pressure (Urry 1992) and ELP concentration (Yamaoka 2003, Meyer 2004). The T_t of ELP can be changed by fusing the gene for ELP to the gene for a protein partner (Trabbic-Carlson 2004), by changing the length of ELP (Meyer 2002, 2004) and/or by changing the amino acid sequence of ELP, especially at the guest residue site (Urry 1991, Meyer 2002). It is possible to use all these tools to generate ELPs with a large variety of T_t. Based on this fact, we hypothesized that aqueous multiple phase systems can be formed by mixtures of different ELPs, or in other words, it should allow for the generation of protein based anisotropic colloids. In this chapter, we report that isotropic hybrid colloids with varied functional density, and anisotropic colloids with different

morphologies could be generated in a controllable fashion (following the scheme shown in Figure 6.1). To our knowledge, this is the first report on the formation of polypeptide based aqueous multi-phase systems and anisotropic colloids.

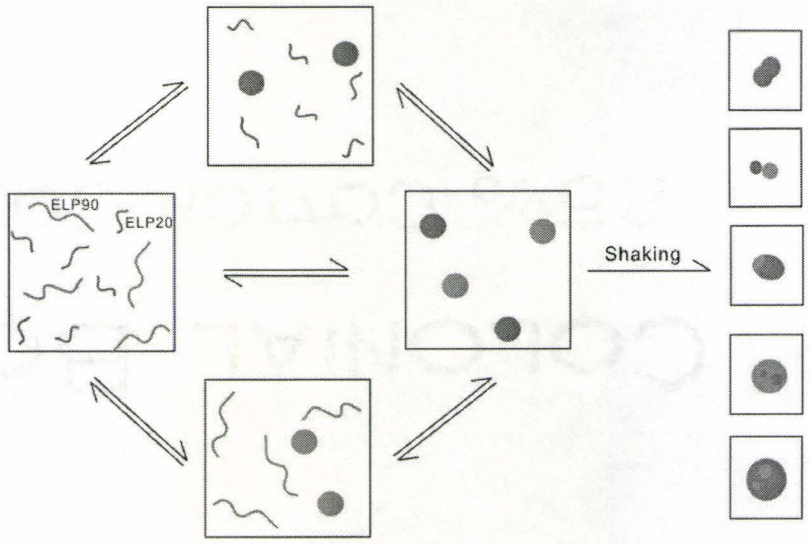

Figure 6.1 Scheme showing aqueous multiple phase system based on the Elastin-Like Polypeptides

6.3 Material and Methods

6.3.1 Plasmid

The plasmids that encode for Trx-ELP20 and Trx-ELP90 (Meyer 2001b) are based on the pET-32b expression vector (Novagen). The fusion proteins expressed with using these plasmids consist of thioredoxin (Trx), and ELP20 or ELP90 polypeptides. The ELP20 polypeptide consists of 20 repeats of the pentapeptide Val-Pro-Gly-Val-Gly. The ELP90 polypeptide consists of 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio of 5:2:3. Standard molecular biology

protocols were applied to assemble other plasmids used in this study. Briefly, pTrx-ELP20 was digested with *Nde*I (Fermentas), and the larger fragment was gel purified (Qiagen) and self- circularization with T4 ligase (Fermentas) resulting in the plasmid pELP20 that carries sequence for expression of ELP20 without thioredoxin. The same approach was used for pELP90, which encodes for the ELP90 polypeptide without thioredoxin. The plasmid for expression of green fluorescence protein fused to the C terminus of ELP90 (pELP90-GFP) was also constructed, where GFP is the variant GFPuv4, that has enhanced fluorescence excitation at 488 nm (Ito 1999). To generate pELP90-GFP, the plasmid pTrx-ELP90 was digested with *Bsu*RI and the 1.3kb fragment (ELP gene) was recovered and subcloned into pUC19. The ELP90 sequence with *Eco*RI and *Hind*III sticky ends was generated by double digestion of pUC19-ELP90, and subcloning into pET28b to give pET28b-ELP90. The gene of GFPuv4 was PCR amplified using pCA24N (Kitagawa 2005) as the template and 5'-gtacctgcaggggccgcagtaaaggagaagaa-3' and 5'-catggcatggatgaactatacaaataagcttgcgcc-3' as the primers. The PCR product was digested with *Pst*I and *Hind*III, and inserted into the corresponding sites on pET28b-ELP90 to finally obtain pELP90-ELP. All the plasmids were analyzed by restriction mapping and confirmed by DNA sequencing (Mobix, McMaster facility).

6.3.2 Production and purification of elastin-like polypeptides and their fusion proteins

E. coli strain BLR (DE3) (Novagen) was used as the host cells to produce ELP and ELP fusion proteins, since its *recA* deficiency helps stabilizing target plasmids

containing repetitive sequences. The plasmids were transformed into the competent cells through the $CaCl_2$ treatment method. The transformed cells were cultivated in rich media, Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 2.31 g/L KH_2PO_4, and 12.54 g/L K_2HPO_4). For protein expression, isopropyl β-thiogalactopyranoside (IPTG) was not used, since it has been shown that ELP and ELP fusion proteins are expressed to very high levels in the absence of IPTG, and by extending the cultivation period to 24 hours (Guda 1995; Chow 2006). The cells were harvested by centrifugation and the pelleted cells were resuspended in PBS at a 1:25 ratio. Ultrasonication was applied for cell disruption, followed by centrifugation to obtain cell lysate. From the cell lysate, ELP proteins were purified through three rounds of inverse transition cycling (Meyer 2001b). The purity of the separated ELP proteins was verified by SDS-PAGE. The concentrations of purified ELP proteins were measured by UV measurements at 280 nm, using extinction coefficients calculated using the amino-acid composition of the expressed proteins. Typical yields of ELP proteins were more than 50 mg/L of culture media.

6.3.3 Characterization of phase transition by turbidity measurement as a function of temperature

The thermal response of the ELPs, ELP fused proteins, or their mixtures, was determined using a Cary 100 spectrophotometer equipped with a temperature controller (Varian Instruments). The turbidity of the samples was monitored as a function of solution temperature at a wavelength of 350 nm. The rate at which temperature was

increased in all experiments was set to 0.5 °C/min. The phase transition temperature (T_t), was defined as the temperature at which the turbidity of the sample attained 50% of its maximum value.

6.3.4 Preparation of nanocolloid samples and microscopic imaging

A variety of samples consisting of mixtures of ELP /ELP fusion /NaCl /PBS in different proportions and concentrations were prepared and kept on ice. Before imaging, these samples were exposed to room temperature for at least 5 minutes. An aliquot consisting of 5 µl of each sample was pipetted to a glass slide for imaging. The images were taken using a Leica TCS SP5 confocal microscope (Leica Microsystem) and using Leica Application Suite Advanced Fluorescence software. Excitation for visualization of GFP was achieved using a 100 mW 488 nm Argon ion laser source. Visible light microscopy was done using differential interference contrast (DIC) channel.

6.4 Results and Discussion

6.4.1 Modulating Tt required for colloid formation using mixtures of ELP based proteins

It has been previously shown that simultaneous phase transition occurs in mixtures of ELP and ELP fusion proteins. This has been used in recovery of recombinant protein present at ultra low concentrations (Ge and Filipe, 2006, Christensen et al., 2007), but its effect to modulate the phase transition temperature, T_t (*i.e.* colloid formation temperature), has not been studied. If two ELP based proteins, each of them having a different T_t are mixed, we hypothesize that the overall T_t required to result in phase

transition should be a function of the proportion in which these two proteins are present. We tested this hypothesis by using mixtures of a fusion of ELP90 to thioredoxin (Trx-ELP90) and ELP90 without Trx. A fusion of ELP with Trx was used since it has been previously demonstrated that fusing Trx to ELP acts to increase the phase transition temperature (T_t) as compared to the non-fused ELP tag (Meyer and Chikoti, 1999). The turbidity profiles as a function of temperature (Figure 6.2) show that a solution containing 25 μM of ELP90 in PBS has a T_t 47 °C, whereas a 25 μM Trx-ELP90 sample in PBS results in a T_t of 56 °C. This difference is possibly due to the hydrophilic nature of Trx (Trabbic-Carlson et al., 2004), which acts to increase T_t.

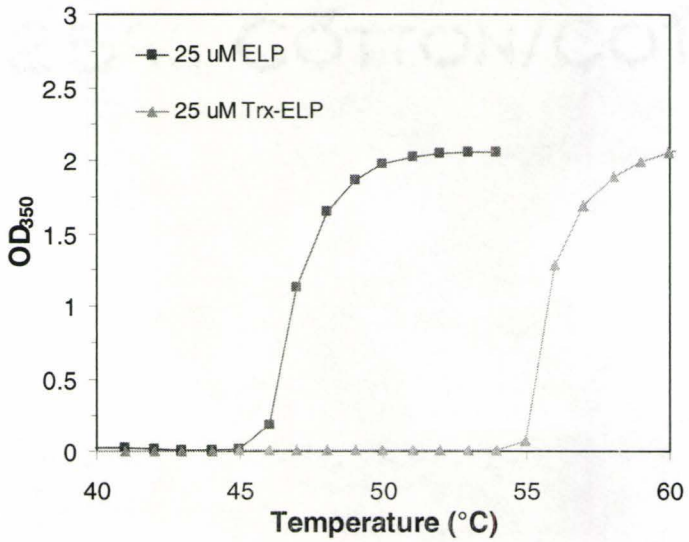

Figure 6.2 Phase transition behavior of two samples: ELP90 at a concentration of 25 μM and Trx-ELP90 at a concentration of 25 μM. Both samples were prepared in PBS buffer. The inverse phase transition for the free ELP90 and for Trx-ELP90, occurs at 47°C and 56°C, respectively.

The T_t of samples with different proportions of Trx-ELP90 and free ELP90 were measured, and the change of turbidity as a function of temperature is shown in Figure

6.3A. In all experiments, the total concentration of ELP90, either free or fused to Trx, was maintained at 20 μM and all samples were prepared in PBS buffer. Figure 6.3B shows the value of T_t (defined as the temperature at which 50% of the maximum turbidity for that sample is observed) as a function of the molar percentage of Trx-ELP90 in the sample. As seen in Figure 6.3, it is straightforward to very precisely manipulate T_t, by changing the proportion of the two ELP90 based molecules. The T_t can be progressively increased from the T_t associated with free ELP90, to the T_t associated with Trx-ELP90. This result provides yet another alternative, to the currently existing methods to "dial in" T_t, such as changing the amino-acid sequence of the ELP (Urry 1991; Meyer, 2002) and changing the ionic strength of the solution in which the ELP molecules are present (Urry 1997). It should be possible to extend the range over which T_t can be modulated by selection of different protein partners to ELP, to maximize the differences of T_t.

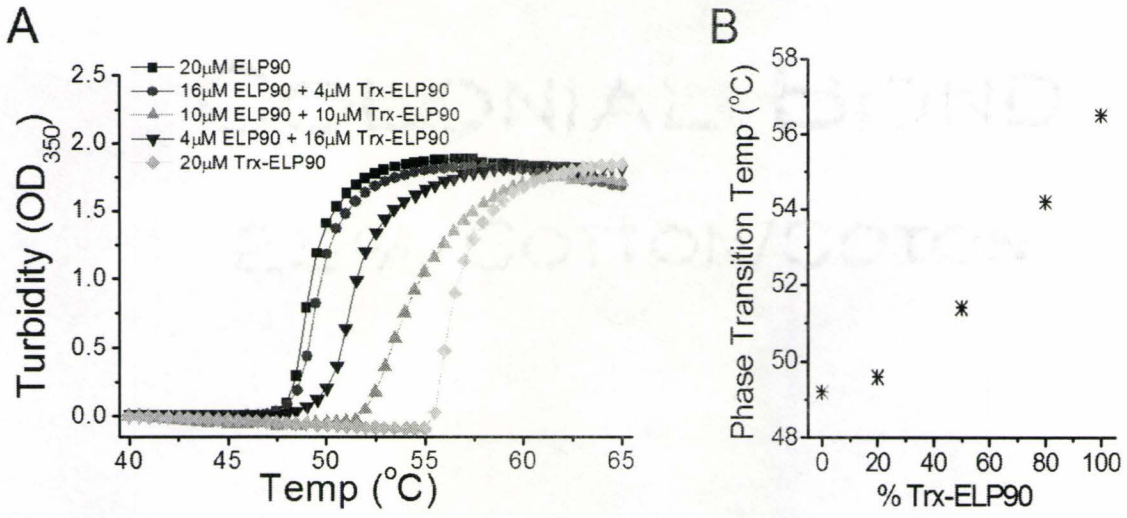

Figure 6.3 Phase transition behavior of mixtures of Trx-ELP90 and ELP90 at different molar ratios. (A) Phase transition profiles; (B) T_t as a function of the Trx-ELP molar ratio. For all samples, the total concentration of ELP (free and fused to Trx) was 20 μM and all samples were prepared in PBS buffer.

6.4.2 Forming ELP based isotropic hybrid colloids with varying levels of functionalization

Although the results from Figure 6.3 suggests that a turbidity increase occurs, it does not tell us that a single elastin-rich phase is formed, , it is necessary to directly probe the material distribution within single droplets of the emulsion. It may be possible that a second phase transition event (associated with Trx-ELP90) could take place and it could be masked due to the high background in turbidity associated with droplets formed from the free ELP90. Moreover, the dependence of the sample turbidity on temperature does not provide absolute proof that hybrid aggregates (composed of ELP90 and ELP90 fusion protein in the same droplet) are formed. It could be possible that two separate populations of droplets, one formed by the free ELP and the other formed by ELP fusion protein, may exist.

To address these two possibilities and to study the material distribution within the droplets, ELP90 was fused with green fluorescent protein (GFP), and confocal microscopy was used to investigate the droplets in emulsions formed by using mixtures of ELP90 and ELP90-GFP in various molar ratios. The total molar concentration of ELP90 (either free or fused to GFP) was 40 μM for all samples, and the ELP90-GFP molar ratios studied were 25%, 50%, 75% and 100%. For all samples, NaCl was added to a final concentration of 1.5 M, to lower T_t below room temperature. Images of the droplets in the emulsions are shown in Figure 6.4A. The results reveal that, for each sample, all the droplets have green fluoresce and that no colorless droplets could be

found in these samples (DIC images not shown). The fluorescent intensity (FI) of the droplets was measured, and it is found that FI is approximately identical for all droplets in the same sample. These results demonstrate the formation of a single population of droplets and unequivocally demonstrate simultaneous phase transition of the ELP and ELP fused with GFP. The images also show that the FI is uniform within individual droplets indicating that the simultaneously phase transition leads the formation of isotropic hybrid nanocolloid composed of free ELP and ELP fusion protein.

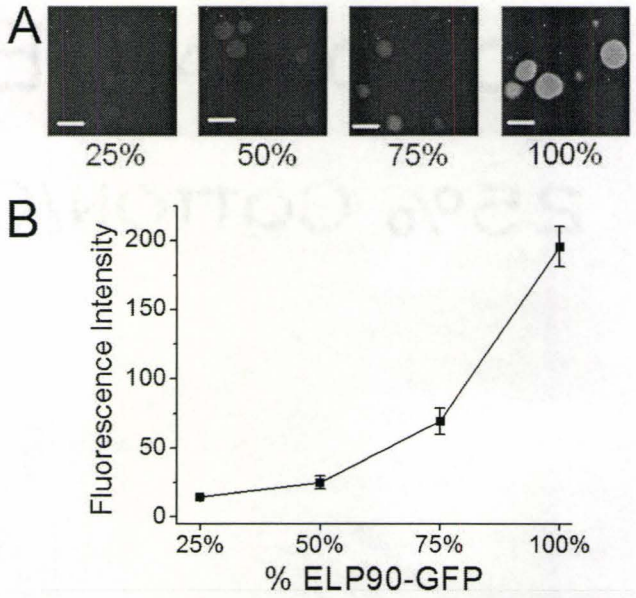

Figure 6.4 Isotropic hybrid colloid formation with ELP90 and ELP90-GFP. (A) homogenous ATPS droplets using different ELP90-GFP molar ratios (bar=5 μm); (B) average fluorescence intensity of the droplets as a function of the ELP90-GFP molar ratio. For all samples, the total concentration of ELP (free and fused to GFP) was 40 μM and [NaCl]= 1.5 M.

The average FIs of the droplets are plotted in Figure 6.4B as a function of ELP90-GFP molar ratio. It can be seen that the FI of the droplets progressively increases as the

molar ratio of ELP90-GFP is increased. If FI represents a functional property to be modulated, the finding described herein provides a way to easily modulate the level of functionalization of a droplet at a given set of experimental conditions. By fusing/conjugating ELP to different proteins or other molecules, it should be easy to different levels of functionilzation of the droples that are formed.

6.4.3 Separate phase transition in mixtures of ELP with different sequences and lengths

In Figure 6.3 and 6.4, we describe the simultaneous phase transition and the formation of isotropic colloids. In these experiments, the free ELP and the ELP present in the fusion protein had the same sequence and the same length. We were interested in knowing if mixtures of ELPs with different lengths and sequences could lead to the formation of aqueous multiple-phase system and anisotropic colloids. As a preliminary study, we used mixtures of ELP20 and ELP90, which have 20 repeats of the pentapeptide Val-Pro-Gly-Val-Gly, and 90 repeats of Val-Pro-Gly-Xaa-Gly (Xaa is Val, Ala, and Gly in the ratio of 5:2:3), respectively. The turbidity of the mixtures was monitored as a function of temperature. A few mixtures of different ELP20 and ELP90 concentrations were tested and it was found that the combination of 60 μM ELP20 and 5 μM ELP90 with addition of 1.5 M NaCl gave the best result in terms of high levels of turbidity as well as a wide T_t difference for the ELP20 and ELP90 samples. The phase transition profiles of 60 μM ELP20 and 5 μM ELP90 on itself were also determined and used as reference points.

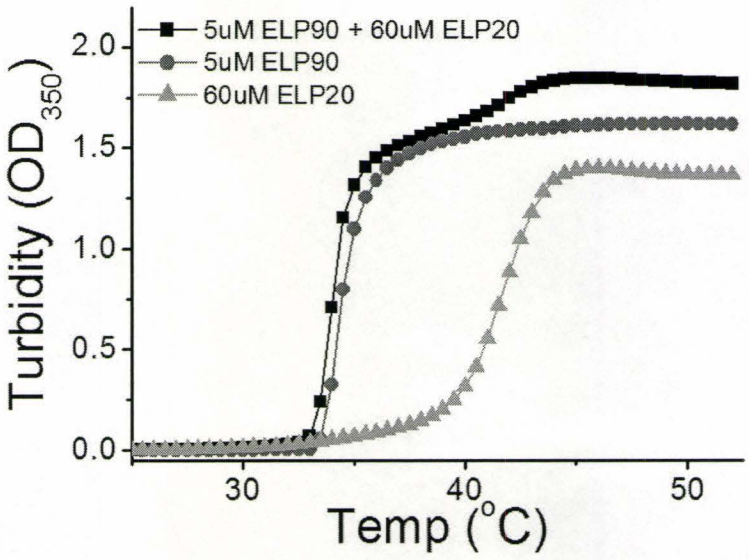

Figure 6.5 Two phase transition events take place for a mixture of ELP20 and ELP90.

As shown in Figure 6.5, the 5 μM ELP90 and 60 μM ELP20 samples exhibit single sharp increases in the OD at 350 nm, with T_t of 34.7 °C and 41.8 °C, respectively. The phase transition profile of the mixture of 5 μM ELP90 and 60 μM ELP20 clearly shows two discrete increases of OD_{350}, revealing that two separate phase transition events took place. The first increase in OD_{350} started at ~33 °C and ends at ~37 °C, having a T_t of 34.3 °C, which is very close to T_t of a sample consisting of 5 μM ELP90 only. Moreover, the manner in which the turbidity changes with temperature and the absolute value of OD_{350} at the end of this first stage, closely matches that of the 5 μM ELP90 sample. This indicates that the first increase in turbidity is due to ELP90. A second increase in turbidity occurs between ~39 °C and ~43 °C, with a T_t at 41.5 °C, which is very close to that of the sample containing 60 μM ELP20 only. For temperatures higher than 40 °C, the absolute value for OD_{350} for the mixture is much less than the sum of the

two individual readings associated with ELP90 and ELP20 alone. This is due to the non-linearity of the Beer-Lambert law when the absorbance of the sample is very high. These results prove that two phase transition events took place, individually and sequentially for ELP90 and ELP20.

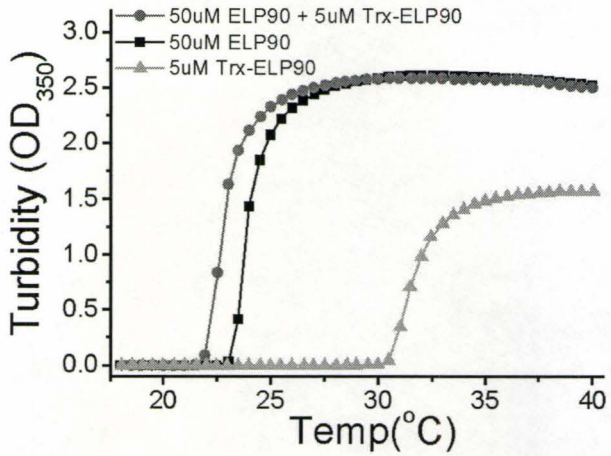

Figure 6.6 Simultaneous phase transition of a mixture of ELP90 and Trx-ELP90.

When a mixture of ELP90 and Trx-ELP90 was used (Figure 6.6), a single phase transition event was observed. In this case the ELP in the fusion protein has the same length and sequence as the free ELP. The T_t observed for this mixture indicates that the ELP in the Trx-ELP90 interacted in a cooperative manner with the free ELP90, resulting in a T_t lower than the T_t observed when either the ELP90 or Trx-ELP were present on their own. It is therefore possible to form aqueous multiple phase system, or generate anisotropic nanocolloids, by using ELPs with difference lengths and sequences.

6.4.4 Formation of ELP based aqueous multiple phase systems

There are still two possibilities associated with the second phase transition shown in Figure 6.5: the formation of a different population of droplets (formed by ELP20) or an increase of the size of droplets by wrapping a ELP90 core with an ELP20 coat. This is a critical issue for understanding the formation of this aqueous multi-phase system and the composition/morphology of the colloids formed. To test these two possibilities, emulsions of ELP90-GFP and ELP20 mixtures were prepared and imaged with confocal microscopy. Since imaging was done at room temperature (RT, ~25°C), a set of experimental conditions (protein concentration and NaCl concentration) were identified such that one ELP component would undergo phase transition at RT but the other ELP component would not, or that both ELP components underwent phase transition at RT. Table 6.1 shows the 4 combinations used and their associated T_t. For example, in sample A, ELP20 is present at a concentration of 250 μM in 1.5 M NaCl and its phase transition occurs at 23.4°C (which is below RT), while ELP90-GFP, at a concentration of 2 μM in 1.5 M NaCl, undergoes phase transition at 30.4°C (which is well above RT). Similarly, in sample C, 10 μM ELP90-GFP in 1.5 M NaCl undergoes phase transition at RT, but 60 μM ELP20 in 1.5M NaCl does not. By modulating the protein concentration, the sequence of phase transition events as the temperature is raised can be reversed, as shown in the difference between sample A and sample C (Table 6.1). While in samples B and D the proteins concentrations are the same as in samples A and C, the NaCl concentration was increased to 2.0 M, thereby lowering the T_t of both ELP90-GFP and ELP20 and making them undergo phase transitions at RT.

Table 6.1 Composition of sample a-d, and associated phase transition temperatures (°C)

			1.5M NaCl		2.0M NaCl
250 μM	ELP20	A	23.4 °C	B	15.5 °C
2 μM	ELP90-GFP		30.4 °C		22.5 °C
60 μM	ELP20	C	32.0 °C	D	23.3 °C
10 μM	ELP90-GFP		23.8 °C		18.1 °C

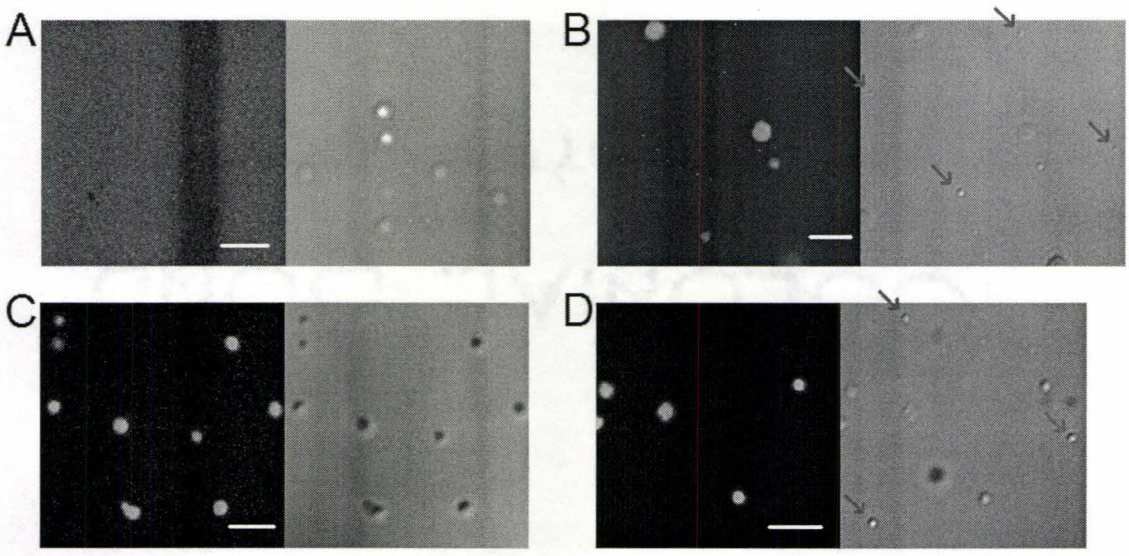

Figure 6.7 Confocal microscopy imaging showing selective formation of multiple phases. The emulsions were formed with mixtures of ELP90-GFP and ELP20 and different NaCl concentrations (see Table 1). Both the green channel and visible DIC channel are shown. The arrows indicate the droplets formed by ELP20. Bar=5 μm.

Figure 6.7 shows the imaging results in both the fluorescent channel (GFP) and optical channel for all samples (A through D) described in Table 6.1. For the experimental conditions used for sample A (Figure 4A), all the droplets are colorless while the solution has a uniform green florescence, indicating that ELP20 forms droplets while the ELP90-GFP remains soluble in solution. This result shows that separate phase

transitions can be used to maintain one component in solution while the other undergoes phase transition forming a colloid. Since the phase transition temperature can be manipulated by changing the protein concentration, the phase partitioning can be reversed. For the combination used in sample C (Figure 6.7C), all the droplets are fluorescent while the solution has no fluorescence, indicating that ELP90-GFP forms droplets while ELP20 remains in solution. This demonstrates that it is possible to have a mixture of two ELP based molecules, selectively choosing one component in the mixture to undergo phase transition and result in the formation of a nanocolloid, while the other component remains in solution. When the NaCl concentration is increased to 2.0 M, the T_t of both ELP90-GFP and ELP20 are below room temperature (combination B and D in Table 6.1). The imaging results (Figure 6.7B, 6.7D) show that in addition to the ELP90-GFP droplets (green), ELP20 droplets (colorless droplets) are also formed, as pointed by the arrows in Figure 6.7B and 6.7D. This is direct evidence that two populations of droplets co-exist in the emulsion, indicating the formation of a protein-based aqueous triphasic system. It is expected that the finding demonstrated herein can be expanded to multiple phasic systems by introducing other species of ELP.

6.4.5 Morphology of ELP based anisotropic colloids

Finally, we tested the possibility of generating anisotropic colloids by shaking emulsions composed of both ELP90-GFP and ELP20 droplets. Varied concentrations of ELP90-GFP and ELP20 were used to form the emulsions and intensive vortex was applied before imaging. The results are shown in Figure 6.8.

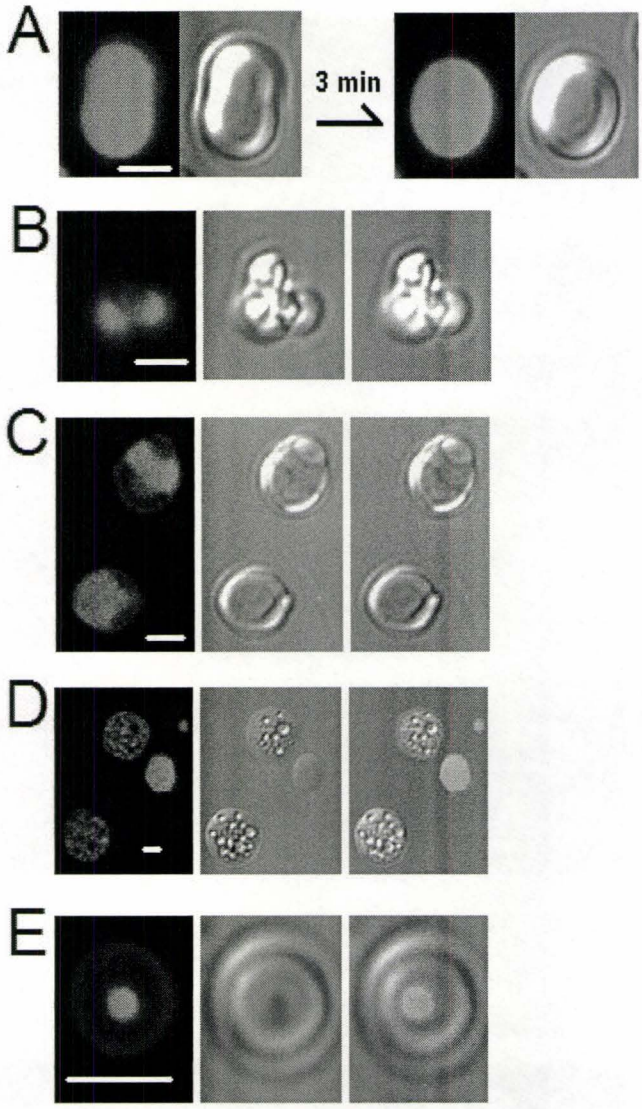

Figure 6.8 Different morphological shapes are formed after vortexing emulsions composed of ELP20 ELP90-GFP droplets. Concentration of ELP20 / ELP90-GFP in samples: (A), 0 μM / 10 μM; (B) and (C), 350 μM / 8 μM; (D), 60 μM / 10 μM; (E), 400 μM / 1.5 μM. For all sample NaCl concentration was 2.0 M. Bar=2 μm.

In contrast to the miscibility of two ELP90-GFP droplets (Figure 6.8A), it was found that ELP90-GFP and ELP20 droplets are immiscible (Figure 6.8B-E). Figure 6.8 clearly shows the formation of anisotropic colloids composed of protein-based materials.

Different morphologies of colloids are generated after vortexing an emulsion consisting of ELP90-GFP and ELP20 droplets. These morphologies include droplets touching droplets (Figure 6.8B), immiscible compartments partitioning in one fused droplet (Figure 6.8C) and droplet-in-droplet (Figure 6.8D and 6.8E). These results demonstrate generation of anisotropic colloids with two immiscible phases partitioning in compartments. It may be possible to create multi-phasic colloids, by applying several different ELP species.

Comparing samples C, D, E, the only differences among them are the concentrations of ELP20 / ELP90-GFP, in the following ratios: (C), 350 μM / 8 μM; (D), 60 μM / 10 μM; (E), 400 μM / 1.5 μM. Previous studies have shown that the concentration of ELP has an effect on droplet size and size distribution (Kaibara 2000). Thus, the results shown in Figure 6.7 imply that the different morphologies can be attributed to the different concentrations and proportions of the ELP species. When the concentrations of both ELP90-GFP and ELP20 are high (sample C), both ELP90-GFP droplets and ELP20 droplets formed have a relatively large size. After energy is introduced in the system (shaking) anisotropic nanocolloids with relatively large compartments are formed. When the concentration of one ELP molecule is high and the concentration of the other ELP molecule is low (sample D, E), droplets with different sizes are formed, with large droplets formed by the ELP molecules initially present in high concentration, and small droplets being associated with the ELP molecules initially present at low concentration. After shaking, the large droplets aggregate and form larger droplets, and during this process the small droplets are buried inside of them. Therefore,

the morphologies and the relative size of compartment in anisotropic nanocolloids can be modulated by concentration/ratio of ELP species.

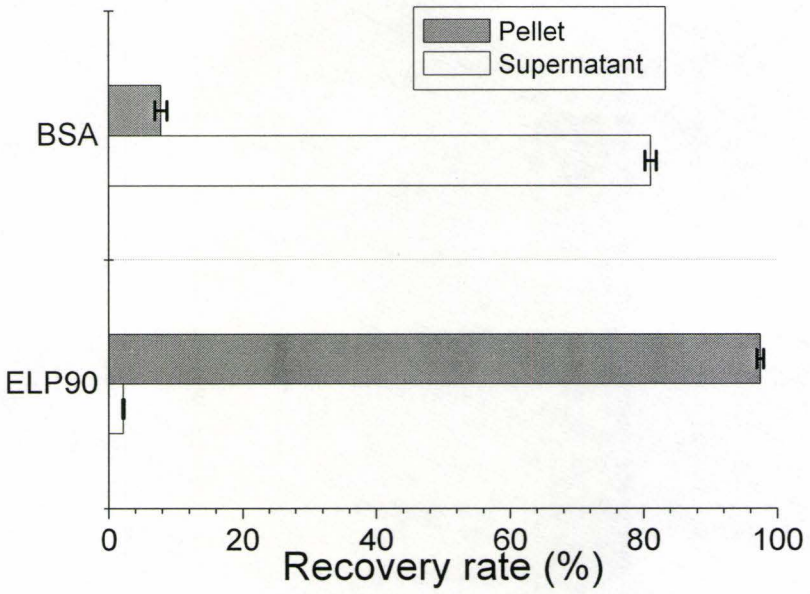

Figure 6.9 Recovery of 1 μM Trx-ELP20 when present in a solution containing BSA or ELP90. Experiments were repeated twice.

Another experiment was done to confirm the formation of hybrid droplets composed of both ELP90 and ELP20. Trx-ELP20 was radiolabeled with ^{125}I, and two samples were prepared: one sample consisted of 1 μM radioiodinated Trx-ELP20 with addition of 20 μM ELP90 in 2.5 M NaCl, and the second sample contained 1 μM radioiodinated Trx-ELP20 with addition of 20 μM bovine serum albumin (BSA) in 2.5 M NaCl. These samples were centrifuged at 16,500 g for 5 minutes. The radioactivity of the pellet and supernatant were measured and the recovery percentages were calculated based on setting the radioactivity levels of the samples before centrifugation as 100%. The

results (Figure 6.9) indicate that the presence of the ELP90 is essential for capturing 1 μM Trx-ELP20. When 20 μM BSA was used (to act as an inert supplement to also minimizes non-specific binding to the surface of the Trx-ELP20 to the walls of the tube of the test tube), it was found that 1 μM Trx-ELP20 alone could not be separated using centrifugation. These results are consistent with the results shown in Figure 6.7D explaining how co-aggregation takes place between different species of ELP and leading the process of co-precipitation.

Comparing the sample before (Figure 6.7) and after (Figure 6.8) shaking, it becomes clear that extra energy is needed to generate anisotropic nanocolloids. Future work should focus on how to precisely control the morphology of nanocolloids by using methods such as manipulating the amount and the rate at which energy is introduced to the system, or manufacturing devices for microfluid jetting or blending.

Geometric parallel patterns are the only morphologies discovered in synthetic polymer based biphasic or triphasic Janus nanoparticles, and this geometric features are a result of the fabrication methods used: microfluid jetting of parallel polymer solutions. A different morphology is created using the system reported in this paper: a sphere symmetrical structure, such as droplet-in-droplet. In addition, the reversible nature of ELP phase transition allows these structures to be assembled and disassembled in stimulus-responsive manner.

6.5 Conclusions

Polypeptide based aqueous multi-phase systems, and both isotropic and

anisotropic colloids can be formed in a controlled manner using elastin-like polypeptides and their fusion proteins. It was shown that that fusing ELP with partner proteins can: (1) Modify phase transition temperature, (2) Vary levels of functionalization of isotropic hybrid colloids. Changing the length and sequence of ELP can: (3) Form aqueous multiple-phase systems, (4) Fabricate anisotropic colloids with different morphologies.

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Chapter 7

Closure

7.1 Summary

The following main conclusions can be drawn from the work reported in this dissertation:

1. A method for recombinant protein purification was developed that does not require the use of chromatography or proteases. ELPs and inteins were used as parts of a single system for protein purification and their functions could be activated sequentially, in an independent and controlled manner. First the ELP tag was used to purify the fusion protein from a complex mixture (such as cell lysate) and then the intein was activated to cleave the ELP tag from the target protein. The attractive features of this technique are its simplicity and low cost. The materials required for purification consisted of a disposable membrane filter, common chemicals (such as NaCl, and DTT) and the purification could be completed in about 5 minutes.

2. The scalability of this technique was further demonstrated using a membrane-based process operated in a continuous manner. The microfiltration/ELP based purification method is suitable for production of recombinant proteins at the

industrial level. It also has great potential to be used in situations where a large number of protein samples need to be purified in parallel.

3. A highly efficient and specific method for purification of ELP fused recombinant proteins was developed, using free ELP as a coacervation additive. For this method, we demonstrated: (1) the efficiency of the method by capturing an ELP tagged recombinant protein present at different concentrations, ranging from μM to as low as pM; (2) the specificity of the method by purifying the ELP tagged protein present in cell lysate at a final concentration of 1 nM, which is approximately equal to the presence of a single molecule of the recombinant protein per single *E. coli* cell. This research could be particularly useful for high-throughput proteomic studies, where small amounts of poorly expressed proteins could be recovered for analysis. In a more general context, the concept presented in this study provides a method that is highly efficient, specific, and fully reversible, should have potentially wide ranging applications in many diverse areas.

4. The results reported in Chapter 5 clarify the conceptual blur associated with ELP phase transition of ELP. They also provide unequivocal evidence of formation of an ELP based aqueous two-phase system, using confocal imaging and FRAP experiments. ATPS formation was obtained *in vivo*, resulting in functional microcompartmentalization of a living cell: one area associated with protein

synthesis and a physically separated area for soluble protein storage. Expression of ELP and ELP fused proteins can be a very powerful approach to study molecular crowding *in vivo*, as well as for efforts directed towards the formation of synthetic cells. Formation of ATPS *in vitro* protected a protein from proteolysis, an observation that could serve as the basis for high-level production of proteolitycally labile recombinant proteins.

5. It was demonstrated, for the first time, the generation of aqueous multi-phase systems exclusively composed of proteins. This demonstration consisted of generating isotropic colloids with tunable levels of functionality, and anisotropic colloids with pre-defined morphologies.

7.2 Proposed Work

7.2.1 Protein Expression and ELP fusions

When GFP-ELP was expressed in *E. coli*, the fusion protein was concentrated at the poles of the cell, and no GFP could be seen in the cytoplasm. There was clear physical separation between the protein production machinery (ribosome), and the aqueous phase where the ELP tagged protein was stored (ELP rich microcompartment). We hypothesize that this could be useful for expressing aggregation prone proteins; as proteins are being produced, they can be continuously removed from the cytoplasm into the ELP rich compartment, leading to small target protein concentrations in the cytoplasm. This should minimize the potential for aggregation. This is totally different from the formation of

traditional inclusion bodies (Baneyx, 1999), because in ELP based ATPS systems the protein is still soluble. We could test this concept using the enzyme ANT(2"). This enzyme covalently modifies aminoglycosides, allowing organisms harboring this gene to have resistance against 4,6-disubstituted aminoglycosides containing one hydroxyl group in the 2" position, which accounts for antibiotics such as kanamycin and gentamicin. This protein was selected because (1) it is clinical relevance; (2) it has not been purified in its native form and no crystal structure is available; (3) extensive data on refolding is available (Wright and Serpersu, 2004); and (4) it is easy to assay protein activity (Ekman et al., 2001). This protein has only been generated initially as inclusion bodies and then refolding. We could attempt to use ELP fusions to express the protein in soluble form, by storing it in a second phase *in vivo*. An additional tool that can be explored is the use of a dual expression system, consisting of two plasmids. One encodes for the formation of ELP, and another encoding for the production of ELP-ANT(2"). The expression of ELP will be turned on at the beginning of cultivation to generate ELP based droplets in the cytoplasm. Later in the growth phase, expression of the ELP-ANT(2") will be turned on and we anticipate that the fusion protein will accumulate in the previously formed ELP droplets. This avoids the need to build up the concentration of ELP-ANT(2") in the cytoplasm of the cell, which is necessary for the formation of a second aqueous phase. This should further minimize the potential for protein aggregation.

7.2.1.2 ELP droplets and protection against proteases

Some data indirectly suggests the possibility of protection of ELP fusion proteins against the action of proteases: extremely high levels of protein expression that have been reported with ELP fusion systems (Chow et al., 2006; Guda et al., 1995; Patel et al., 2007); the product purity obtained with ITC results in highly pure product. ELP based purification of the proteins works indicates that proteases are indeed excluded from ELP based droplets. Preliminary studies were done *in vitro* using Trx-ELP, with the thrombin cleavage site being used as the proteolysis target. Thrombin was added at a temperature below and above the Tt of Trx-ELP. The rates of protein cleavage were measured and results suggested the formation of ATPS could protect the fusion protein from protease attack. It is hypothesized that this mechanics could work *in vivo* as well. Therefore, we propose to select a proteolytically prone protein, fuse it with ELP and express the fusion protein, comparing the productivity / solubility of the protein with and without ELP fusion. Some antimicrobial peptides with potential for clinical applications could be of interest for this study. Most of peptide antibiotics currently found are small cationic peptides with molecular weight <5 kDa (Zasloff, 2002). Like most small peptide, they are often prone to proteolysis, thus impeding the development to be commercial products (Valore and Ganz, 1997; Haught et al., 1998; Skosyrev et al., 2003; Rao et al., 2004). If successful, this approach could be quite valuable from a production point of view, since it minimizes loss of product and the ELP tag could further be used for simple protein purification.

7.2.1.3 Multiphasic aggregation of the cytoplasm of living cells

Using different sequences and different lengths in ELP, it is possible to tune in the conditions (temperature, concentration, etc.) at which phase separation takes place. We propose to create a library of ELPs, with a variety of lengths and sequence, using previously published methods (Meyer and Chilkoti, 2002; 2004), to clone this library in two plasmids (with different origins of replication), one of them expressing the library as a fusion to GFP and the other plasmid as a fusion to yellow fluorescence protein. Fluorescence based microscopy will be used to assess the morphology and number of phases that can be obtained *in vitro* and then extend this work to *in vivo* experiments. We want to see if it is possible to generate different compartments and if these compartments can be generated *in vivo*.

7.2.2 ELP based detection systems

A variety of methods exist for detecting molecules present at very low levels in biological samples. The common theme in these methods is the use of DNA and PCR for signal amplification. Immuno-PCR, originally developed by Sano, Smith and Cantor (1992) and the DNA based barcodes developed by Mirkin's group (Nam et al., 2003) are two elegant implementations of this approach. We would like to avoid using PCR based detection, since it is not available in all cases. We propose to use the fact that ELP coacervates as the basis of a highly sensitive method that generates a visual signal in response to small concentrations of an analyte of interest. The method is presented in Figure 7.1.

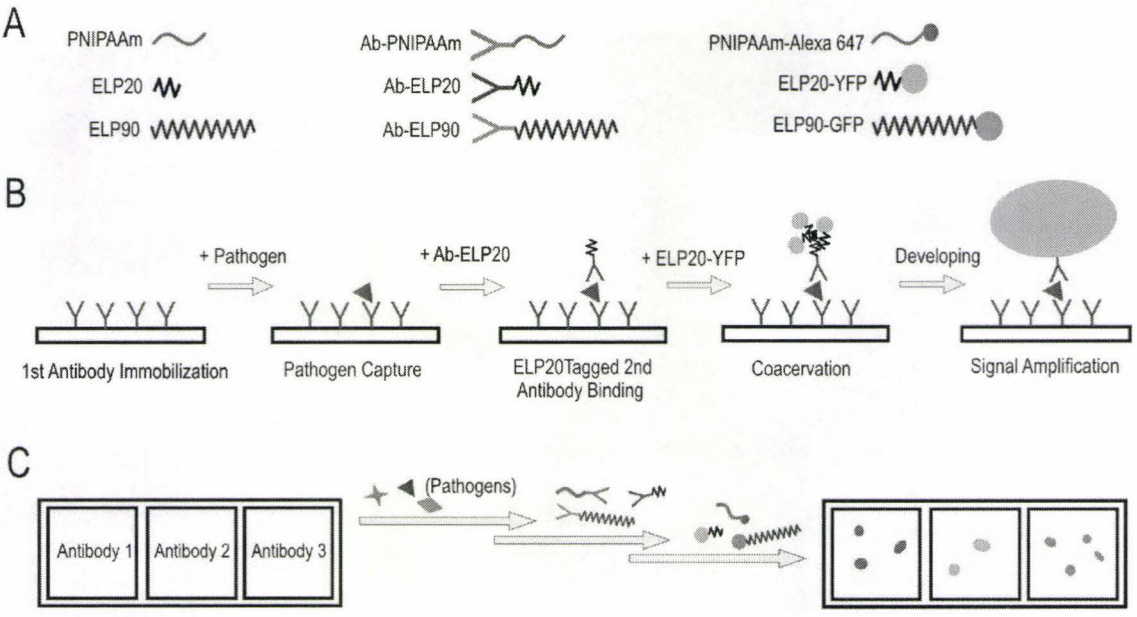

Figure 7.1 Proposed method for visual detection of molecules at low concentration. (A) Building blocks consisting of three thermally responsive polymers (PNIPAAM, ELP 20 and ELP 90) conjugated to an antibody and to three different fluorophores (red, blue and green); (B) capture of a pathogen by an immobilized antibody, coacervation of fluroresencently labeled ELP around an antigen/ELP-antibody conjugate for signal generation; (C) the method can be multiplexed.

We will select three thermally responsive polymers, with different Tt: poly-N-isopropyl-acrylamide (PNIPAAM), ELP20 (consisting of 20 repeats of the pentamer V_5) and ELP90 (consisting of 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio of 5:2:3). The polymers will be conjugated to an antibody. PNIPAAM will be conjugated to a red fluorophore, ELP 20 will be expressed as a fusion to yellow fluorescent protein (YFP) and ELP 90 will be expressed as a fusion to green fluorescent protein (GFP). The procedure is shown in Figure 7.1B. An antibody will be immobilized on a surface, very much in the same manner as when doing an ELISA and then the sample to be analyzed is loaded. The antigen, if present, will bind to

the immobilized antibody. To generate a signal, antibody conjugated to a thermally responsive polymer (in Figure 7.1B, ELP20 is shown) is added binding the antigen that had been captured by the primary antibody. This results in the formation of a "sandwich" with a polymer sticking towards the bulk of the solution. This polymer will serve as an anchor. Addition of a reporter molecule, in this case ELP20 fused with YFP, and changing the temperature to above T_t, will result in coacervation of the ELP-YFP fusion around the anchor polymer. We have been able to grow ELP droplets to diameters in the order of several millimiters, which can be clearly seen by naked eye. Theoretically, this method should allow detection and visual reporting of the presence of a single antigen molecule. This method could be easily multiplexed (Figure 7.1C).

7.3 Reference

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