J|A|C|S ARTICLES

Published on Web 06/04/2009

In Vivo Formation of Protein Based Aqueous Microcompartments

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Abstract: In this paper, we report the formation of protein based liquid droplets resulting in the formation of *in vivo* microcompartments in *E. coli* or tobacco cells. These microcompartments were 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 fluorescence recovery after photobleaching (FRAP) experiments at different stages of cell cultivation. In the initial phases of cell growth, the fusion protein concentration is low and is not sufficient 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 poles of the *E. coli* cells. FRAP experiments with cells at different growth stages reveals that the protein in these ELP based droplets is comprised of aqueous and not solid aggregates, as seen in typical inclusion bodies. Staining of the ribosomes and coimaging of the ELP−GFP 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. Additional studies are still required to test this possibility *in vivo*. To the best of our knowledge, this is the first report characterizing the formation of an engineered extra aqueous phase in a living organism.

Introduction

Aqueous two-phase systems (ATPS) can be obtained by the presence of two or more polymers, or one polymer and a chaotropic salt.[1] 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,[2] to mimics of cytoplasmic organization[3,4] since ATPS systems share a common feature with biological systems: compartmentalization. Keating's group described the use of an ATPS of PEG/dextran pairs to generate droplets and encapsulation of these droplets with lipid bilayers to generate vesicles that mimic molecular crowding inside cells.[3−5] 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 could be formed in a controlled manner in living cells.[6] Such systems could then be used to create different phases in complex *in vivo* environments. A more practical use of these systems would be the expression of toxic proteins, since these proteins could be directed toward a second aqueous phase formed in the cell's interior. This second phase would be composed of the toxic protein and water and could function as a protein storage compartment.

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 referred to as elastin-like polypeptides (ELP) because their sequence occurs in all mammalian elastin proteins.[7−9] 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

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 10.1021/ja902890r CCC: $40.75

reversible inverse phase transition resulting in ATPS formation.[10,11] Phase transition can be triggered using a variety of stimuli, such as temperature and the addition of chaotropic salts,[12−14] and is affected by the ELP's sequence, chain length, and concentration.[14,15] ELP tags have been mainly used for bioseparations for nonchromatographic purification of recombinant proteins.[16−18] Protein fusions to ELP tags have been shown to significantly enhance the accumulation of a range of different recombinant proteins in bacteria[19] and plants.[20] In this paper, we show that ELP induces the formation of ATPS *in vivo* in *E. coli* and tobacco cells. These results demonstrate that ELPs are ideal for bridging synthetic ATPS based systems, currently used to study the importance of molecular crowding in biological systems, by allowing for the generation of ATPS *in vivo*. The results also suggest that ATPS formation *in vivo* may be a valuable tool for high-level expression of proteins that are easily degraded by proteolysis, but *in vivo* studies are still required to test this possibility.

Experimental Section

Construction of Bacterial Expression Vectors. The elastin-like polypeptide (ELP) used in the bacterial work was comprised of 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio 5:2:3. The starting plasmid, pTrx−ELP,[16] was digested by *Bsu*RI, and the 1.3 kb ELP fragment was gel purified and inserted into the *Sma*I site of pUC19 (Invitrogen) to obtain pUC19−ELP. The ELP sequence containing *Eco*RI and *Hind*III sticky ends was cut out of pUC19−ELP by double digestion and subcloned into the corresponding sites of pET28b (Novagen) resulting in pET28b−ELP. The GFPuv4 gene[21] was PCR amplified from the pCA24N template[22] using 5′-gtacctgcaggggccgcagtaaaggagaagaa-3′ and 5′-catggcatggatgaactata-caaataagcttgcgcc-3′ 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 pELP−GFP bacterial expression construct.

Protein Expression and Purification. The plasmids coding for ELP−GFP were transformed into *E. coli* strain BLR (DE3) (Novagen), which is used for expressing recombinant proteins with tandem repeats, because of its deficiency in homologous recombination. For hyperexpression of ELP−GFP, the noninduction method was used.[14,19,23] Transformed BLR (DE3) were cultured for 36 h and 37 °C in 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) 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, pH 7.4) 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 an ice−water bath. ELP−GFP was purified using inverse transition cycling (ITC).[16] The purity of the proteins was determined with SDS-PAGE (Coomassie Blue staining), and the concentration of purified ELP−GFP was measured by its absorbance at 280 nm using a spectrophotometer (Varian Cary 100) using an extinction coefficient of 62 730 M^{-1} cm^{-1} calculated from the amino acid sequence.

Sample Preparation for Imaging. For the *in vitro* studies, a sample containing 100 μM of purified ELP−GFP and 1.5 M NaCl was prepared, which resulted in the phase transition of the ELP−GFP fusion protein. This turbid solution was transferred to a microscope slide, and fluorescence microscopy was used to collect stack images in the *Z*-direction and to perform fluorescence recovery after photobleaching (FRAP) experiments. This solution 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 and 200 rpm. Cells were sampled at various cultivation times, and imaging was completed within 1 h of sampling. FRAP experiments were done *in vivo* using ELP−GFP expressing *E. coli* cells grown for 24 h. 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 were collected by centrifugation, and the pelleted cells were resuspended in 330 μL of H_2O with the addition of 165 μL of 30%/0.8% acrylamide/biacrylamide, 3 μL of 10% ammonium persulfate, and 0.3 μL of TEMED. After mixing, 5 μL of the mixture were immediately transferred onto microscope slides and covered with a cover glass. As soon as polymerization took place, the edges of the cover glass were sealed with nail polish to prevent drying. Imaging the distribution of cell membranes and nucleic acids within *E. coli* was done using lipophilic styryl dye FM 4-64 (Molecular Probes) and SYTO 62 (Molecular Probes), respectively. *E. coli* cells were cultivated for 24 h, collected by centrifugation, and resuspended in PBS (for FM 4-64) or 150 mM NaCl (for SYTO 62). The final concentration for both dyes was 1 μM, and the cells were stained for 20 min before imaging.

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. 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 measured period of time had elapsed after photobleaching.

Phase Transition. The phase transition behavior of ELP at different concentrations (500, 200, 100, and 50 μM and in PBS) was monitored using a Varian Cary 100 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

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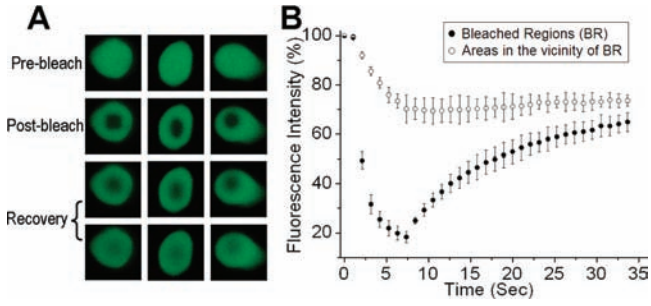

Figure 1. *In vitro* fluorescence recovery after photobleaching (FRAP) analysis of ELP−GFP rich phase droplets: (A) Fluorescent imaging performed at different stages of the FRAP experiments; (B) Quantitative FRAP analysis of bleached regions and the areas in the vicinity of the bleached regions.

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.

Results and Discussion

In Vitro Formation of Aqueous Two-Phase System. For our studies, we used an elastin-like polypeptide (ELP) consisting of 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly (Xaa being Val, Ala, and Gly in the ratio 5:2:3) fused to green fluorescent protein (GFP). The first step was to clearly demonstrate that this fusion protein (ELP−GFP) results in the formation of ATPS *in vitro*. The key features of aqueous two-phase systems are that both phases are composed mainly of water and each of the phases is relatively rich in one of the solute components.[1] To probe the fluidity of the solute (ELP−GFP) rich phase, NaCl (1.5 M, final concentration) was added to a sample of ELP−GFP (100 μM, final concentration), which immediately caused the solution to become turbid. This sample was then used to perform quantitative fluorescence recovery after photobleaching (FRAP) experiments using a confocal microscope. As shown in Figure 1, 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 surrounding areas decreases, due to the mobility of ELP−GFP in the entire droplet. Due to the intrinsic nature of FRAP experiments, not all the fluorescence of the ELP−GFP rich phase can be recovered, because part of the GFP undergoes fluorescent microphotolysis (which is irreversible). Regardless, the results show that the fluorescence intensity of the bleached area increases rapidly and approaches the intensity observed in the areas surrounding it toward the end of the experiment. The data shown in Figure 1 (along with the data shown in Supporting Information Figure S1) clearly demonstrate that the ELP−GFP fusion protein allows for the formation of aqueous two-phase systems *in vitro*.

ATPS Formation in Vivo. The formation of ATPS is dependent on the concentration of the solute.[15,24] Results from phase transition temperature measurements (Supporting Information Figure S2) show that, for ELP concentrations higher than 200 μM, ATPS formation occurs under physiological conditions (in PBS and 37 °C). Extremely high expression levels have been reported for proteins fused to ELP with as much as 1.6 g of purified ELP fusion protein being obtained per liter of culture.[19] Since the entire mass of ELP fusion protein is contained within the small volume associated with the *E. coli* cytoplasm, the local concentration of ELP fusion proteins inside the cell must be at levels well above those required for the formation of ATPS. By imaging cells at different culturing time points, three distinct stages of ATPS formation could be observed *in vivo* (Figure 2).

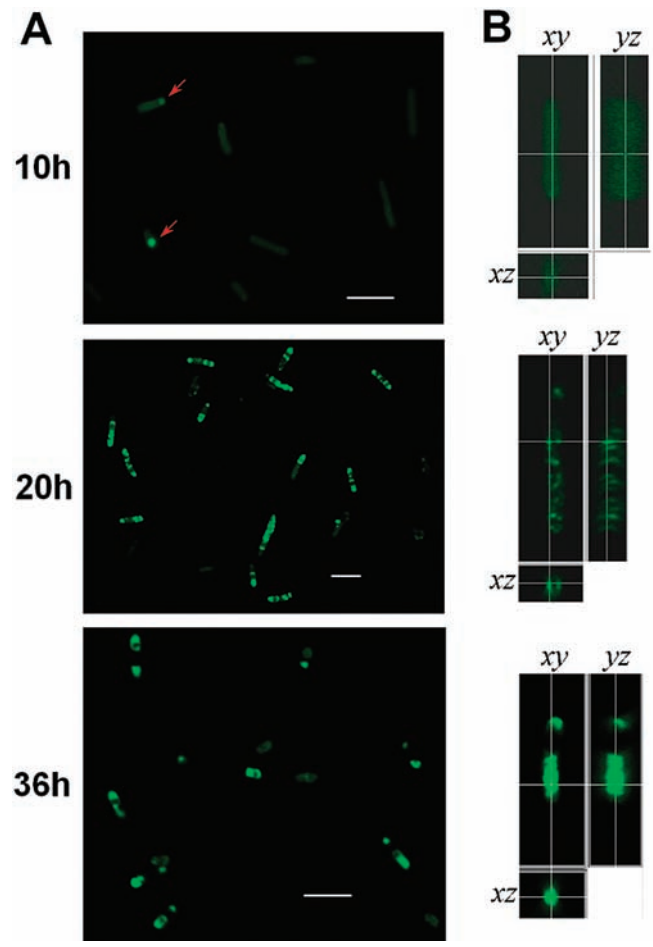

Figure 2. Increasing the ELP−GFP concentration by extending cultivation time leads to the formation of ELP−GFP rich ATPS compartments inside *E. coli*: (A) Fluorescent imaging for different cultivation times: 10 h (top), 20 h (middle), and 36 h (bottom); (B) 3D-section fluorescence images of individual cells at various cultivation times, for the same samples as shown in panel A. Bars represent 4 μm.

During fairly short cultivation times, the amount of ELP−GFP produced is still low, resulting in an intracellular concentration that is not sufficient to drive the formation of ATPS. Figure 2A and 2B (showing sections in the *xyz* directions) reveal that, after 10 h 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−GFP concentration in the cells is not high enough to induce the formation of ATPS. The arrows point to the poles of some cells where ATPS starts to form, as protein is accumulated.

After 20 h of cultivation, small droplets started to appear within the cells, clearly showing the formation of ATPS *in vivo* as the intracellular concentration of ELP−GFP continued to increase. Sections of the cells were imaged, and it can be seen that these droplets are isolated from each other.

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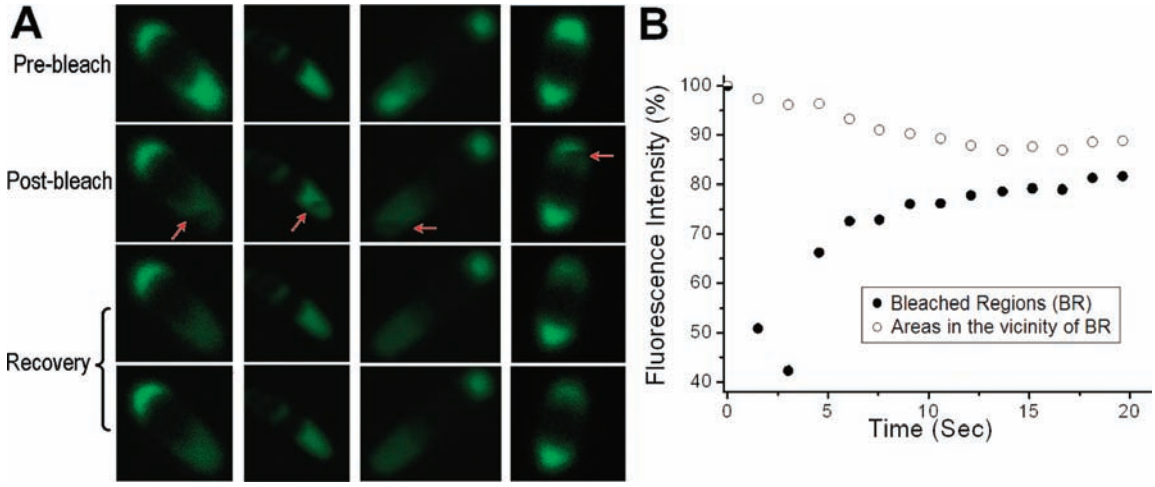

Figure 3. FRAP analysis of ELP−GFP rich phase compartments *in vivo*: (A) Fluorescent images of *E. coli* cells (24 h cultivation) during FRAP experiments: prebleach (top row), immediately after bleaching (the second row), and recovery (bottom two rows). The bleached regions in each cell are indicated with arrows; (B) Quantitative data for a FRAP experiment.

After 36 h 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 2A). Due to the high intracellular concentration of the fusion protein, the small droplets merge together resulting in a smaller number of very large droplets (~1.5 μm in length). From Figure 2A, 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 2B) reveal that the ELP−GFP rich phase consists of a single homogeneous phase that extends to the entire height of the cytoplasm. We also tested and confirmed that ELP induces the formation of an ATPS in eukaryotic plant cells (Supporting Information Figure S3).

We decided to probe the nature of the intracellular droplets shown in Figure 2 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.[26] Imaging the ELP−GFP rich compartments with transmitted light (Differential Interference Contrast, DIC) and fluorescence imaging showed that these compartments were optically clear without any opacity (Supporting Information Figure S4), which suggests that they are liquid in nature and not typical solid inclusion bodies. To further confirm that these compartments were liquid, quantitative FRAP experiments were used to analyze cells cultivated for 24 h (Figure 3). 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 of the poles) indicating that ELP−GFP is highly mobile within one ATPS compartment and not between compartments. Recovery rates varied for different cells, due to the 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 prevents all cell movement during imaging. The polyacrylamide gel is transparent to the optical light and laser beam, and no detectable refraction was observed. This technique should be very useful for accurate FRAP measurements and 3D Z-stack imaging of highly mobile organisms.

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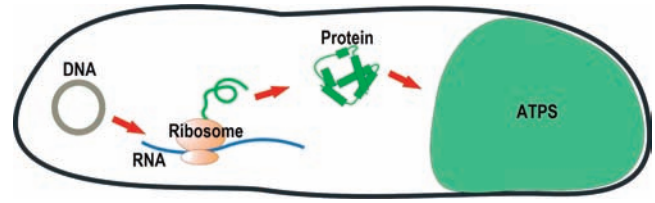
Scheme 1. Functional Intracellular Partitioning: Intracellular Compartments for Recombinant Protein Synthesis and Recombinant Protein Storage



Functionalized Microcompartments. The formation of ATPS compartments *in vivo* suggests that functional compartmentalization of cells may be possible. Scheme 1 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 accumulate in the second phase, which acts as a separate compartment to store protein. To locate the ELP−GFP storage compartments in living cells, a lipophilic styryl dye (FM 4-64) was used to stain the cell membrane and thus define the boundary of the cell. This showed that the ELP−GFP rich compartment is located at the cells' poles (Figure 4).

SYTO 62 is a red fluorescent dye whose fluorescence is enhanced upon binding to nucleic acids. It is widely used to

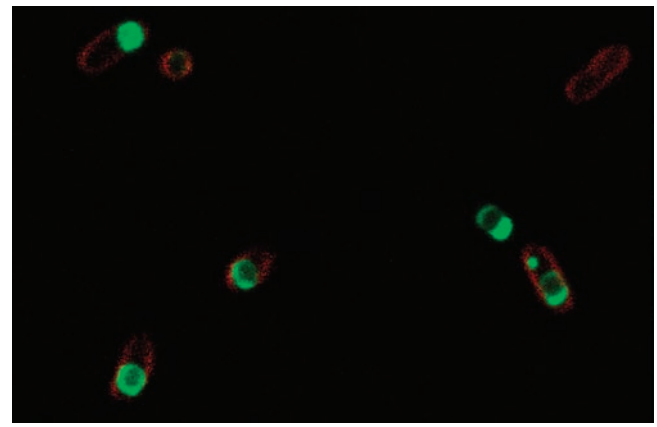


Figure 4. Accumulation of ELP−GFP as compartments at the poles of *E. coli* (cultivated for 24 h). The cellular membrane was stained with a red fluorescent lipophilic styryl dye (FM 4-64).

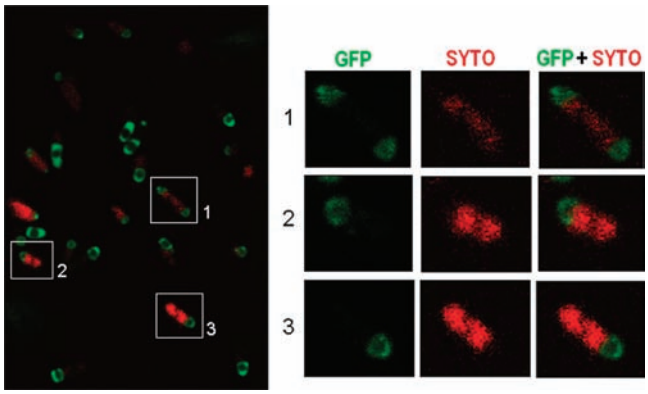


Figure 5. Nucleic acids and ELP−GFP are physically separated inside *E. coli* (cultivated for 24 h). Nucleic acids were stained by SYTO 62. Featured cells are enlarged and shown in the right panel.

stain DNA, RNA and to visualize ribosomes in living cells.[27] Cells expressing ELP−GFP were cultured for 24 h, stained with SYTO 62, and imaged (Figure 5). Enlarged images of selected (boxed) cells are shown in the right panel of Figure 5. The green and red fluorescence do not overlap. A control experiment was done *in vitro* to demonstrate that the SYTO 62 dye can permeate through ELP−GFP droplets (Supporting Information Figure S5). Taken together, these results show that exclusion of the SYTO 62 stain from the ELP−GFP compartments found *in vivo* is not due to immiscibility of the dye in ELP−GFP rich compartments but happens because nucleic acids and ribosomes were excluded from these compartments. Therefore, it is 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. This has potential value for the expression of aggregation prone proteins, because it minimizes exposure of nascent and incompletely folded proteins to an environment (cytoplasm) with a very high protein concentration, by directing the ELP tagged proteins to a separate compartment.

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[18,28] from complex mixtures, such as cell lysate using Inverse Transition Cycling (ITC).[16] 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 be a successful protein purification method. We hypothesized that formation of ELP based droplets would exclude proteolytic enzymes from these droplets, thereby protecting the ELP fused proteins from proteolytic attack and excluding the protein from normal physiological turnover. This could partially explain the very high expression levels that have been reported when expressing proteins fused to ELP,[19,23,29,30] since we show that ATPS formation takes place inside the cells.

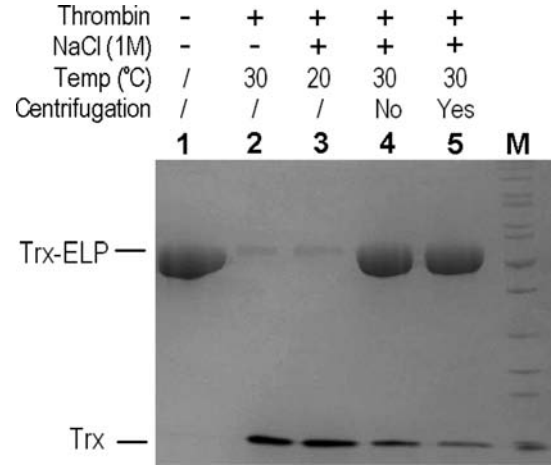


Figure 6. ATPS formation protects against protein proteolysis. Proof-of-concept experiments using thrombin cleavage. Lane 1: Initial Trx−ELP fusion protein; Lane 2: same as in lane 1 with incubation with thrombin for 2 h at 30 °C (no ATPS formed); Lane 3: same as in lane 1, with addition of NaCl (1 M) 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 weight markers. Note: free ELP tags are not visible in the gel because they are poorly stained with Coomassie Blue.

We tested this hypothesis *in vitro*, but future work should be done to test it *in vivo*. Thioredoxin−ELP fusion protein (Trx−ELP) with a thrombin cleavable linker between the Trx and ELP tag, was expressed and purified as described elsewhere.[14,16] 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 1 M NaCl were maintained at either 20 °C (below Tt) or 30 °C (above Tt). Thrombin was added to these samples in a ratio of 2 U per mg of Trx−ELP fusion, and the samples were incubated for 2 h. The results are shown in Figure 6. 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 and 5). These results demonstrate that Trx−ELP, without ATPS formation, is readily accessible and cleaved by thrombin (lane 3), but is protected from thrombin when ATPS is formed (lanes 4 and 5). By excluding the thrombin from the Trx−ELP droplets, the cleavage reaction can only happen at the interface of the two phases, thereby 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, as no detectable difference in cleavage efficiency was found between samples at low and high NaCl concentrations, without ATPS formation (lanes 2 and 3).

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 1 M NaCl), to cause coalescence of the Trx−ELP droplets, resulting in a large decrease of the interfacial area. As shown in lane 5 of Figure 6, coalescence driven by centrifugation further protected

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Trx−ELP from thrombin attack: the amount of cleaved thioredoxin from the centrifuged sample was only $\sim^1/_3$ of that observed when small droplets were present (lane 4). It is important to note that since the majority of the Trx−ELP fusion was not cleaved in these experiments (lanes 4 and 5) due to ATPS formation, the level of proteolytic activity is best reflected by examining the amount of thioredoxin cleaved from the fusion and not the amount of Trx−ELP remaining (which was close to the initial amount used). Overall, it was demonstrated that, *in vitro*, ATPS greatly decreased the rate of proteolysis by excluding the proteolytic enzyme (in this case thrombin) from the phase 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 prone to high levels of proteolysis and degradation.

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) *in vitro* and *in vivo* (demonstrated for bacterial and plant cells).
2. These droplets exclude the ribosomes present in the cytoplasm of the cell, thereby physically separating recombinant protein synthesis from recombinant protein storage.
3. Formation of ATPS *in vitro* may result in the protection of ELP tagged proteins against proteolytic activity by excluding the proteolytic proteins from the ELP fusion protein rich droplets.

Acknowledgment. We thank Dr. Robert Pelton for insightful discussions and Dr. Tony Collins for his help on the use of the Leica SP5 microscope. This work is funded by a NSERC Discovery Grant and the Canadian Foundation for Innovation (CFI) to C.F. We thank the Ontario Graduate Scholarship for giving financial support to X.G. We thank Dr. Ashutosh Chilkoti, from the Department of Biomedical Engineering, Duke University, for the donation of the plasmid pTrx−ELP.

Supporting Information Available: Supporting Information is available as noted in the text. It includes additional data demonstrating ATPS formation *in vitro*, the experimental procedures used and the results demonstrating ATPS formation in plant cells, a figure showing that intracellular accumulation of ELP−GFP does not result in typical inclusion bodies, and a figure demonstrating that SYTO 62 dye can permeate through the ELP−GFP droplets. This material is available free of charge via the Internet http://pubs.acs.org.

JA902890R