Recombinant Surfactants Derived From the Naturally Occurring Protein Oleosin

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Abstract
Surfactants are molecules of key importance in the food, chemical and pharmaceutical industries due to their ability to stabilize interfaces and self-assemble in solution. Commercial surfactants are typically chemically synthesized making the material extremely difficult to functionalize for biological applications. Therefore there is a need to develop bioactive surfactants. A powerful alternative to chemical synthesis is the expression of recombinant protein surfactants through molecular biology. These proteins would be monodisperse and have the precise sequence dictated by the cognate gene. Recombinant protein production would permit the direct incorporation of specific motifs that mediate protein recognition. We have chosen the naturally occurring plant protein oleosin as a candidate. Oleosins are a family of plant proteins whose biological role is to stabilize oil bodies. They have two hydrophilic arms and a central hydrophobic domain. In this thesis, we have designed many variants of oleosin to self-assemble and/or stabilize interfaces. First we created a family of truncation mutants that assemble into sheets, fibers, or vesicles depending on the geometry of the protein and the solution chemistry. We further truncated this family to create protein variants that assembled into spherical micelles. All protein variants were confirmed using mass spectroscopy and the secondary structure was analyzed through circular dichroism. Protein aggregate size and shape were analyzed through light and X-ray scattering and directly visualized through cryogenic transmission electron microscopy. Oleosin variants were employed to stabilize interfaces in two applications. The first was the stabilization of microbubbles generated in a microfluidic device for ultrasound contrast and therapy. Bubbles were extremely stable over time, easily functionalized with an eGFP-oleosin fusion, and were echogenic. We further applied our variants to stabilize an oil-in-water emulsion for the creation of iron oxide nanoclusters as contrast agents for magnetic resonance imaging. Particles size was measured using light scattering and directly measured using electron microscopy. Oleosin stabilized iron oxide clusters were non-toxic and simple targeting to Her2/neu+ cells was achieved by blending a Her2/neu affibody-Oleosin fusion protein into the shell. These results set the foundation to further engineer oleosin for self-assembly and interface stabilization and functionalization.

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Daniel A. Hammer

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RECOMBINANT SURFACTANTS DERIVED FROM THE NATURALLY OCCURRING PROTEIN OLEOSIN

Kevin Bryce Vargo

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in

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Presented to the Faculties of the University of Pennsylvania

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Degree of Doctor of Philosophy

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RECOMBINANT SURFACTANTS DERIVED FROM THE NATURALLY OCCURRING PROTEIN OLEOSIN

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Kevin Bryce Vargo
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ABSTRACT

RECOMBINANT SURFACTANTS DERIVED FROM THE NATURALLY OCCURRING PROTEIN OLEOSIN

Kevin Bryce Vargo
Daniel A. Hammer

Surfactants are molecules of key importance in the food, chemical and pharmaceutical industries due to their ability to stabilize interfaces and self-assemble in solution. Commercial surfactants are typically chemically synthesized making the material extremely difficult to functionalize for biological applications. Therefore there is a need to develop bioactive surfactants. A powerful alternative to chemical synthesis is the expression of recombinant protein surfactants through molecular biology. These proteins would be monodisperse and have the precise sequence dictated by the cognate gene. Recombinant protein production would permit the direct incorporation of specific motifs that mediate protein recognition. We have chosen the naturally occurring plant protein oleosin as a candidate. Oleosins are a family of plant proteins whose biological role is to stabilize oil bodies. They have two hydrophilic arms and a central hydrophobic domain. In this thesis, we have designed many variants of oleosin to self-assemble and/or stabilize interfaces. First we created a family of truncation mutants that assemble into sheets, fibers, or vesicles depending on the geometry of the protein and the solution chemistry. We further truncated this family to create protein variants that assembled into spherical micelles. All protein variants were confirmed using mass spectroscopy and the secondary structure was analyzed through circular dichroism. Protein aggregate size and
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Chapter 1

Introduction

Surfactants are molecules of key importance in the food, chemical and pharmaceutical industries.\textsuperscript{1,2} Certain families of surfactants can be exploited for their ability to self-assemble due to the asymmetric polarity of the molecules. When the chemistry and geometry of the surfactants are controlled, precise and complex self-assembly can be achieved. If these structures are assembled from biologically relevant materials, the resulting structures can be considered part of the expansive field of biomaterials.

Biomaterials are materials that are engineered for medical or biological applications. The research field covering biomaterial is expansive and includes tissue engineering, implants, and drug delivery to name a few. Biomaterials are used in applications such as joint replacements, bone cements, artificial ligaments, and heart valve replacements.\textsuperscript{3-6} Biomaterials must possess important properties in order to be effectively used in the body. One must consider the host response to the material, materials biocompatibility and toxicity, and mechanical properties.
when engineering new materials. With the current technology, designer biomaterials are being developed with specific biological functionality.

Biological functionality is a broad term implying that the material or structure directly interacts with biology such as controlling cell fate, adhesion, or targeting. At a high level, these interactions are typically mediated through specific proteins but when meticulously investigated, it is found that many interactions are typically arbitrated by only a few amino acids in the protein structure. This can be thought of as a lock and key interaction where the key is an initiator molecule or ligand and the lock is a docking station or receptor. These interactions have evolved over time to control many biological functions. Therefore, understanding and incorporating functional domains into self-assembled biomaterials is a promising method to directly interact with biology.

The task of assembling functional biomaterials from surfactants can be approached in two ways. The first method uses chemical synthesis to create surfactant molecules. This method is robust and well characterized and has laid the groundwork for decades of future surfactant research. But, due to the syntheses methods, functionalization of these materials can be difficult. In many cases, the surfactant is synthesized and the ligand or key is added to the material in a separate step. This two-step process can lead to heterogeneous functionalization. Instead, one can exploit the precision of nature’s synthesis methods through recombinant biotechnology by using a host organism to create the desired surfactants. In this
method, surfactants would be monodisperse in molecular weight and functionalization of these sequences can be completed by altering the cognate gene in the organism. In simple terms, if it is desired to create biomaterials that directly interact with biology, a logical route is to assemble the materials from the same building blocks used in nature, amino acids, and to use the tools that nature has evolved to synthesize the materials.

This work addresses the need for a robust recombinant surfactant for the assembly of functional biomaterials. For these needs, we are inspired by the naturally occurring plant protein oleosin. The geometry and architecture of oleosin are unique and rarely observed in nature. The structure of oleosin mimics a chain-like surfactant making it an ideal candidate for engineered self-assembly. In this work, we attempt to apply the theory of polymer and lipid assembly to design variants of oleosin that assemble into desired structures. The specific aims of this work are as follows:

1. Develop a recombinant protein system that self-assembles into bilayer vesicles
2. Engineer soluble recombinant surfactants from the base protein oleosin
3. Develop oleosin mutants to stabilize and target clinically relevant biomaterials

This work is composed of seven chapters. **Chapter 2** describes background work and our perspective on creating recombinant surfactants. This chapter
provides an overview on self-assembly, surfactants, oleosin, current progress in the field, and the techniques used to complete this work. Chapter 2 is intended to provide sufficient information so that the reader has a context to understand the research described in this thesis.

Chapter 3 of this work explores the self-assembly behavior of oleosin truncation mutants. Families of mutants with varying molecular weight and hydrophilic fraction were designed and expressed. A specific family of mutants was found to self-assembled through an emulsions template into fibers, sheets, and vesicles depending on the geometry of the protein and the solution chemistry. To our knowledge, this was the first report of a bilayer vesicles assembled from recombinant protein.

Chapter 4 of this work looks to create soluble surfactants from oleosin. To achieve this goal, the hydrophobic domain of oleosin was heavily truncated eliminating the majority of the secondary structure in the protein. These protein mutants assemble into spherical micelles above a critical concentration and were functionalized with a cell-binding motif for targeted internalization.

Chapters 5 and 6 of this work focus on using oleosin as a surfactant to stabilize interfaces. In Chapter 5, we use oleosin to stabilize monodisperse microbubbles for ultrasound imaging and therapy. In this study, the bubble size and stability were monitored over time. Functionalization and ultrasound response was investigated.
In Chapter 6, we utilized an ionic mutant developed in Chapter 4 for the stabilization of superparamagnetic iron oxide nanoparticles for magnetic resonance imaging. Particle stability and magnetic properties are measured and the particles are targeted to cancer cells overexpressing a specific receptor.

Chapter 7 of this work comprises concluding statements and extensive future work. Preliminary results are included for future projects.
References


Chapter 2

Background

2.1 Self-assembly

Self-assembly is the process in which molecules align and orient themselves into a higher order structure without the guidance from an outside source.\(^1\) Assembly can be controlled through various interactions including hydrogen bonding, hydrophobic forces, electrostatic interactions, Van der Waals forces, and coordination bonds over many length scales.\(^2\) At the nanoscale, assembly is based on the information in the individual molecules such as shape, charge, and polarity. Self-assembly is considered a bottom-up approach because the ordered structures are assembled from random, disordered monomers opposed to a top-down approach, where particles are manufactured from larger bulk materials.\(^3\) The bottom-up approach allows for the manufacture of large quantities of assembled particles but lacks the ability to create all shapes and sizes accessible to top-down assembly methods. Bottom-up assembly is very well understood and can be precisely controlled allowing for the fine-tuning of functional properties in
nanomaterials. By engineering the structure, chemistry, and function of molecules, we can exploit the tools of molecular self-assembly to develop aggregates with desired sizes, geometries, and functional motifs.

2.2 Surfactants

Any molecule that adsorbs to an interface and lowers the surface pressure between the two phases is considered a surfactant. Common surfactants include lipids, polymers, dendrimers, polypeptides, proteins, or combinations therein. Traditionally, a surfactant has a hydrophilic head group and a hydrophobic tail. This asymmetric polarity leads to molecular assembly driven by the hydrophobic effect; the entropic aggregation of nonpolar domains in aqueous solution and the exclusion of water from these domains.

The most common surfactant self-assembly is that of non-ionic chain surfactants such as lipids or polymers. The assembly of these systems is dictated by their geometry. The ratio of the effective volume of the hydrophilic head group to the hydrophobic tail is defined as the packing parameter:

\[ p = \frac{v}{a_o l} \]  

(2.1)

where \( v \) is the volume of the hydrophobic tail, \( a_o \) is the effective head group area, and \( l \) is the length of the hydrophobic tail. The value of the packing parameter can be used to predict the aggregate morphology of surfactants in aqueous environments (Figure 2.1). A value of \( p<1/3 \) implies a cone shaped surfactant leading to the formation of high curvature spherical micelles. As the head group volume becomes comparable to the tail
volume, $p$ increases allowing for the formation of lower curvature structures such as cylindrical micelles ($p=1/3-1/2$), vesicles ($p=1/2-1$), and bilayers ($p \sim 1$).

$$p = \frac{v}{a_o l_c}$$

Figure 2.1: Schematic depiction of the packing parameter ($p$). $p$ defines the ratio of the volume of the hydrophobic tail to the volume of the hydrophilic head group. The packing parameter can be used to engineer surfactants to assemble into the desired morphologies in solution. Reprinted from Progress in Polymers Science, 37, Zhang, J. X., Li, X. D. & Li, X. H., Stimuli-triggered structural engineering of synthetic and biological polymeric assemblies, 1130-1176, Copyright 2012, with permission from Elsevier.

2.3 Nanoparticles

A nanoparticle can be defined as a particle or assembly with at least one length scale between 1 and 100 nm. This unique size range offers a bridge between classical and quantum material behaviors presenting new properties compared to the properties observed in the bulk material. Nanoparticles can be assembled from wide range of materials including polymers, lipids, dendrimers, proteins, silica, metals, carbon, and Quantum Dots.
The field of nanotechnology has had a major impact into medicine and biology over the past few decades. Nanoparticles have been employed as biological sensors in various applications with detection based on optics, magnetic fields, or electrochemical reactions. A common approach for optical sensing is the use of gold nanoparticles functionalized with ligands to directly interact with biology. The optical properties of gold is highly tunable and is controlled by the surface ligand as well as the size of the nanoparticles. Fluorescent nanoparticles represent another major optical sensor. Traditional organic dyes and fluorescent proteins offer a simple, non-destructive sensing methods. However, these particles have short fluorescent lifetimes and show low absorption coefficients. Fluorescent nanoparticles provide advantages over organic dyes due to their sensitivity and stability. The size of nanoparticles allows for a high signal to noise ratio and signal amplification and minimizes physical interactions in biological systems.

Self-assembled organic nanoparticles are commonly assembled from surfactants. Lipids represent the most common material used for nanoparticle self-assembly due to their versatility as depicted in Figure 2.2. Lipids offer an advantage over many other materials due to the low cost and the large library of chemistries available. Lipid vesicles, or liposomes, have been used extensively in drug delivery and imaging applications. Liposomes can be functionalized by embedding proteins into the lipid bilayer including ion channel proteins, pore forming proteins, and active enzymes such as tissue factor. Native liposomes have low circulation times due to clearance by the reticuloendothelial system leading to the use of polymer coatings,
specifically polyethyleneglycol, to provide stealth capabilities to the nanoparticle.\textsuperscript{25,26}

Although liposomes are one of the most popular drug delivery vehicles, they are unstable in serum and mechanically weak, but certain chemistries allow for covalent cross-linking of the membrane potentially mitigate these issues.\textsuperscript{25,26}

Figure 2.2: Schematic depiction of liposome versatility.  a) Standard liposomes are vesicles with a lipid bilayer membrane. Hydrophilic molecules can be encapsulated in the lumen (green star) and hydrophobic molecules can be trapped in the membrane (red spheres). b) Stealth liposomes are created by grafting an inert PEG chain to the outside of the vesicle. c) Cationic lipids can be used to create “onion” liposomes and encapsulate DNA (purple cylinders) for gene therapy. d) Vase shaped lipids have been shown to form vesicles demonstrating the diversity of chemistries possible in lipid system. Reprinted by permission from Macmillan Publishers Ltd: Nature\textsuperscript{27}, copyright 2012.
To overcome the mechanical weakness of liposomes, diblock copolymer vesicles, or polymersomes, were invented. Polymersomes are bilayer vesicles assembled from diblock copolymers with blocks of opposing polarities. Polymer vesicle membrane thickness is dictated by the hydrophobic block molecular weight allowing for controlled mechanical properties (Figure 2.3 A-C). Polymersomes have been developed for various applications including drug delivery, advanced imaging, controlled release, and sensing. Polymer vesicles have been decorated with adhesion ligands, but these proteins were cross-linked to the membrane using biotin/avidin interactions potentially leading heterogeneous functionalization and additional purification steps post assembly (Figure 2.3 D). Polymer vesicles are inherently polydisperse in size likely stemming from the molecular weight distribution observed for the single polymer chains. Vesicle size is typically controlled post assembly with extrusion. Advances in polymer synthesis have lead to fine control over the polymer molecular weight distributions, which has been shown in polymersomes to correlate with a predicted average diameter based on the preferred curvature of the molecule. Diblock copolymers have been used for a variety of other applications including emulsification and lithography.
Figure 2.3: Polymersome membrane control and functionalization. Cryo-TEM micrographs showing polymersomes assembled from various molecular weights. Membrane thickness is dictated by the molecular weight of the polymer, specifically the hydrophobic block. A) OB2 (EO_{20}-BD_{46}), 3600 g/mol. B) OB18 (EO_{80}-BD_{115}), 10,400 g/mol. C) OB19 (EO_{150}-BD_{250}), 20,000 g/mol. Scale bars are 100 nm. Reprinted with permission from Macromolecules, 35, 8203-8208, Bermudez, H., Brannan, A. K., Hammer, D. A., Bates, F. S. & Discher, D. E., Molecular weight dependence of polymersome membrane structure, elasticity, and stability. Copyright 2002 American Chemical Society. D) Polymersomes can be functionalized through biotin-avidin bridges to link specific biological motifs to the membrane. Reproduced from reference with permission of The Royal Society of Chemistry.
Dendrimers have been developed as synthetic surfactants with precise molecular weight. A dendrimer is a molecular that is constructed from branched monomer units. Dendrimer amphiphiles have been shown to self-assemble into helical pores, micelles, and vesicles, called dendrimersomes. Dendrimersome diameter size has been shown to be dependent on the bulk material d-spacing and the concentration of the dendrimer in solution. Controlling the size of the aggregated structure poses a major advantage over polymersome systems. Recently, a family of amphiphilic glycodendrimers was synthesized with monosaccharides D-mannose and D-galactose and the disaccharide D-lactose in their hydrophilic head group. These amphiphilic glycodendrimers self-assembled into vesicles, worm-like micelles, and cubosomes and the self-assembled structures were shown to directly interact with sugar binding proteins. Recently, dendrimers have been developed to control the size and number of bilayers in each assembled dendrimersome. These “onion” like vesicles offer the possibility of kinetically controlling extended release with each layer providing a sequential release. Dendrimersomes are an interesting platform for drug delivery and imaging because the synthesis methods allow for complete control over the chemistry and molecular weight of the amphiphilic dendrimer, and simple methods can be used to control the dendrimersome diameter, a major advantage for controlled in biodistribution and circulation time.
2.4 Peptide and Protein Nanoparticles

A common limitation of many nanoparticles is their inability to directly interact with biology without the addition of a peptide or protein. Biological interactions are highly specific. For example, it is well known that the integrin binding sequence RGDS loses its binding affinity when just two of the amino acids are scrambled.\(^58\) It is therefore advantageous to create nanoparticles from naturally occurring biological molecules such as polypeptides or proteins. Proteins and polypeptides are polymers assembled from the twenty naturally occurring amino acids arranged in a specific order. What separates proteins from polypeptides is structure. Proteins have local secondary structure such as alpha helical or beta sheet domains. These local domains fold into a molecular structure called the tertiary structure dictating the overall shape and size of the protein and, in many cases, biological function. Polypeptides are linear polymer chains of amino acids that can display local secondary structure but do not fold into higher order structures.

Polypeptides have been of great interest for creating self assembled nanoparticles. Seminal work has shown block-copolypeptides can assemble into various structures, such as vesicles\(^59,60\) and micelles\(^61\). Stupp and coworkers have created various peptide amphiphiles that assemble similar to traditional surfactants.\(^62\) Additionally, very short peptide amphiphiles have been shown to assemble into nanotube or nanovesicles depending on the peptide sequence (Figure 2.4 A).\(^63\) Fletcher and coworkers have developed coiled-coil peptides that can be engineered to form cages, mimicking viruses.\(^64\) Deming and coworkers have produced libraries of diblock copolypeptides comprising of
two amino acids, a charged amino acid repeated for the head group and a nonpolar amino acid for the tail group (Figure 2.4 B). Different amino acids have been blended into each block with set average composition, but the precise sequence of amino acids in each block could not be controlled. The diblock copolypeptides have been used to stabilized nano-emulsions and engineered to assembled into hydrogels for tissue engineering and prolonged drug delivery. Lecommandoux and coworkers invented a zwitterionic pH switchable polypeptide system with poly(L-glutamic acid)-b-poly(L-lysine) (PGA-b-Plys) (Figure 2.4 C). Like polymers, most polypeptides are chemically synthesized, which can lead to polydispersity in the surfactant molecular weight and limits the ability to incorporate biologically active domains within the sequence.

An alternative is the expression of recombinant surfactants by molecular biology. These surfactants would be monodisperse and have the precise sequence dictated by the cognate gene. Unlike synthetic methods, recombinant production permits the direct incorporation of specific motifs that mediate protein recognition. Embedding recognition sequences, either at the ends of proteins or deep within the sequence, is straightforward. Mastrobattista and coworkers have invented small self-assembling recombinant oligopeptides with the defined sequence Ac-Ala-Ala-Val-Val-Leu-Leu-Leu-Trp-Glu-Glu-COOH. The oligopeptides vesicles are assembled through relatively weak forces, but the mechanical stability can be improved with intermolecular crosslinking through disulfide bonds. The assembled vesicles have been used as passive cellular delivery vehicles but the small sizes of these peptides preclude the incorporation of biologically relevant motifs within the assemblies.
Elastin-like-polypeptides (ELPs) are recombinantly produced polypeptide repeats of the sequence (VPGXG) where X is a guest amino acid to control the polarity of the repeats (Figure 2.5 A). ELPs display a lower critical solution temperature (LCST), above
which the polypeptide precipitates out of solution (Figure 2.5 B). Diblock elastin-like polypeptides (ELPs) remain the main building block for the formation of nanoparticles from recombinantly produced polypeptides.\textsuperscript{76-79} ELP diblocks are commonly created by fusing two blocks together with varying LCSTs. Manipulating the chemistry and length of the blocks creates a temperature window where one block precipitates out of solution while the other remains soluble leading to the formation of nanoparticles. Recent work has shown that assembly can be controlled through the conjugation of hydrophobic moieties to an asymmetric ELP diblock.\textsuperscript{80} In this work, molecules with varying hydrophobicity were linked to the C-terminus of the ELP chain. It was found that a minimum hydrophobicity was needed to assemble these asymmetric surfactants into spherical micelles(Figure 2.5 C).\textsuperscript{80} In many cases, the core of ELP micelles is highly solvated potentially limiting the hydrophobicity of the cargo.\textsuperscript{81} The specific repeat sequence in ELP diblocks and the cloning methods used to create them\textsuperscript{79,82} make it difficult to incorporate biological functionality into the core of the peptide sequence.

Self assembled protein nanoparticles are found in nature in various systems. The milk protein casein forms spherical micelles to encapsulate hydrophobic components in solution.\textsuperscript{83} These micelles are considered associated colloids, which are formed by highly phosphorylated caseins interacting with calcium phosphate. Micelles have an average diameter of 200 nm in milk but can be engineered to form much smaller structures.\textsuperscript{84,85} Casein micelles have been used extensively for oral drug delivery but must be cross-linked for stability.\textsuperscript{86-89}
Figure 2.5: A) Schematic depiction of the versatility of elastin like polypeptides. Left: Single chain ELPs will reversibly aggregate in solution as a function of temperature, pH, or ionic strength. Middle: ELP diblocks can be designed such that a change increase in temperature will lead to the aggregation of one block while the other block remains soluble creating spherical micelles in solution. Right: Reactive crosslinking sites can be cloned into the ELP backbone allowing for the formation of ELP hydrogels upon reaction with a cross linker. Reproduced from reference 78. Copyright © 2010 Wiley Periodicals, Inc.  
B) Thermal scans of an ELP diblock at various concentrations reveal the lower critical solution temperature and the effect of concentration on the aggregation of the polypeptides. Reprinted with permission from reference. 81 Copyright 2008 American Chemical Society.  
C) Cryo-TEM micrograph of ELP micelles assembled from an asymmetric ELP diblock. The hydrophilic block consisted of a long hydrophilic ELP and the hydrophobic block was a paclitaxel conjugated peptide sequence. The conjugation of paclitaxel forced the assembly of the hydrophobic core. Reproduced from reference. 80 Copyright © 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
Another common scaffold for new materials is the self-assembling shell from protein capsids termed virus-like particles.\textsuperscript{90} Protein capsids form a single layer of protein able to entrap hydrophilic compounds inside but unable to store hydrophobic compounds. These structures offer uniform sizes and can be created recombinately.\textsuperscript{91} They are assembled from many copies of the same protein monomer. This allows for highly controlled assembly but limits functionalization due to the precise sequence needed for assembly. Therefore, much of the functionalization on the interior or exterior of the shell is done through chemical methods.\textsuperscript{91,92}

There are very few naturally occurring protein surfactants. For stability purposes, proteins generally embed their hydrophobic amino acids in the core of the molecular and expose polar and charged amino acids at their surface. A few commonly used proteins have been shown to lower the surface pressure at interfaces such as lysozyme\textsuperscript{93} and bovine serum albumin (BSA)\textsuperscript{94}. These proteins are thought to adsorb to the interface, which induces stress on the protein structure causing slow denaturation. This denaturation allows for hydrophobic amino acids to partition into the nonpolar phase, lowering the surface pressure. The majority of surfactant proteins display a hydrophobic patch such as hydrophobin\textsuperscript{95}, or they rearrange at an interface to expose a hydrophobic domain such as latherin\textsuperscript{96} and ranaspumin\textsuperscript{97}. Although these proteins display surfactant characteristics, they are not ideal to engineer for self-assembly due to their globular shape. Ideally, a chain like protein would be desired to mimic the well-studied assemblies of lipid and polymers. The only known chain like natural surfactant protein is oleosin\textsuperscript{98}.
2.5 Oleosin

Oleosin is a naturally occurring plant protein that stabilizes oil bodies in seeds.\textsuperscript{98} The native structure is defined in three domains: an N-terminal hydrophilic arm, a central hydrophobic domain, and a C-terminal hydrophilic arm (Figure 2.6).\textsuperscript{99,100} A proline knot in the core of the hydrophobic domain forces a 180° turn leading to a hairpin like structure.\textsuperscript{98,101-103} The secondary structure of oleosin has been contradictory in literature.\textsuperscript{99,101,104-107} Modeling has shown the hydrophobic domain to fold into a coiled-coil domain\textsuperscript{101}, but recent work suggests that oleosin maintains a beta sheet structure in oil bodies.\textsuperscript{105} It is unclear what structure oleosin takes in aqueous environments due to its low solubility.

The surfactant nature of oleosin has been exploited in many applications. Oleosin has been expressed recombinantly and utilized for its surfactant behavior,\textsuperscript{108-113} but little engineering has been completed to optimize the protein for self-assembly. The main application for oleosin variants has been the stabilization of emulsions, termed artificial oil bodies.\textsuperscript{98,113-115} Their ability to stabilize oil-in-water emulsions has led to the development of oleosin fusions for protein purification.\textsuperscript{109} Additionally, targeted oil bodies have been established as hydrophobic drug delivery agents by fusing a Her2 affibody to oleosin and stabilizing artificial oil bodies with the fusions.\textsuperscript{110,111} Oleosin has not been exploited as a stabilizing agent for various other interfaces, an area that this work looks to explore. Also, few studies have explored engineering oleosin into a
surfactant protein for self-assembly. Tzen and coworkers have created truncation mutants of oleosin by eliminating portions of the hydrophobic domain from the N- and/or the C-termini but focused on the mutant’s ability to stabilize artificial oil bodies. Oleosin is a prime candidate for engineered self-assembly due to its native hydrophobicity, block-like structure, and known expression in bacterial hosts.

Figure 2.6: Schematic depictions of oleosin. A) Block primary structure of oleosin shows the hairpin-like structure of the protein. The cartoon indicates that the two hydrophilic arms directly interact with the phospholipid membrane while the hydrophobic block could be in a beta-sheet structure into the TAG matrix. Reproduced from Huang, A. H. C. Oil Bodies and Oleosins in Seeds. B) Cartoon depiction of the proline knot and oil body membrane. Oil bodies are 1-3 µm emulsions found in seeds. Oleosin is thought to stabilize these emulsions and protect from aggregation and ripening. Reproduced from indicated source. Copyright 2004, American Society of Plant Biologists.

2.6 Protein and Nanoparticle characterization

There are two families of characterization techniques that were employed in this work: protein and nanoparticle. Protein characterization assessed the purity, identity, molecular
weight, and secondary structure of the molecules. Nanoparticle characterization primarily focused on shape and size of the particles either through scattering techniques or direct imaging using electron microscopy.

### 2.6.1 Protein Characterization

SDS-PAGE is used to access the purity and estimated molecular weight of a protein. A gel stained with Coomassie Blue will indicate all protein in the sample. Using image analysis software, the intensity of a lane can be plotted and the area under curve can be calculated. By calculating the relative area under the curve, the individual bands can be assessed for percent purity (>95% purity is expected from recombinant purification methods). Protein identify can be confirmed through western blotting. In western blotting, a specific antibody is bound to the protein entrapped in a membrane. This antibody is fluorescently identified through the binding of a secondary antibody. The confirmation of protein identity is of utmost importance because of the heterogeneous mixture in the cell lysate from which the target protein is purified. Protein molecular weight can be confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF). This technique ionized a dried spot of protein that is entrapped in a host matrix using a high power laser and the time it takes the ionized protein to travel from the plate to the detector is measured.\(^{117}\) The time is correlated to standards and a mass spectra is generated. MALDI-TOF provides the molecular weight of the entire protein compared to other mass spectroscopy methods where enzyme cleavage is needed to obtain a spectra. Finally, the overall secondary structure of the
proteins can elucidated through circular dichroism\textsuperscript{118,119}. Due to the chirallity of the peptide bond, proteins adsorb circularly polarized light. Pluses of left- and right-handed circularly polarized light are alternated through an aqueous solution over a range of wavelengths. The difference between the absorbance of the left and right handed polarized light is plotted as a function of wavelength. Standard CD spectra for an alpha helix, beta sheet, or unordered structure are shown in (Figure 2.7). It should be noted that CD provides the secondary structure of the entire protein. For this reason, CD is useful for examining how mutations affect the overall secondary structure but can not be used to determine specific regions of secondary structure.\textsuperscript{118,119} CD can also elucidate changes in the secondary structure as a function of concentration or temperature during self-assembly.

Figure 2.7: CD spectra of pure secondary structure domains. The red curve is indicative of a random coil, the green curve an alpha helix, and the blue curve a beta sheet. Reproduced from the University of Leeds, CD facility.
2.6.2 Nanoparticle characterization

The nanoparticle characterization techniques employed in this work were scattering methods and direct imaging through electron microscopy. Scattering methods provide population averages for size whereas direct imaging can confirm size and provides direct evidence of the shape and size of the particle through a visual micrograph. Two types of scattering were employed in this work, light and X-ray. Dynamic light scattering is used to measure the hydrodynamic diameter and the distribution of the diameter in nanoparticle solutions. The Brownian motion of particles causes laser light to be scattered at different intensities and the fluctuations in intensity can yeild the particle velocity. Employing the Stokes-Einstein equation, the particle size can be determined. For protein solutions, the raw intensity data were analyzed because an accurate reading for the refractive index and absorbance, which is needed to convert to percent number or percent volume, was not availible. Small angle X-ray scattering can be used to measure characteristic lengths in a nanostrcutured material. In this technique, concentrated solutions of protein are subjected to the bombardment of X-rays at a known wavelength. X-rays are elastically scattered and the 2-D scattering profile is detected at a known distane from the sample. The profile is integrated as a function of the scattering angle. The spectra can be fit to known fuctions to determine the size and shape of the nanostructures.

Cryogenic transmission electron microsocpy (Cryo-TEM) is used to directly image soft matter nanoparticles in solution. Cryo-TEM is the superior method for soft matter imaging because unwanted rearrangement of solution structure is avoided
through rapid vitrification. Freeze fracture TEM fails to identify membranes, and TEM with negative staining is associated with potential staining artifacts.\(^{123}\) In cryo-TEM, a sample in the form of a liquid drop is placed on a lacey TEM grid and blotted with filter paper to create liquid films with film thicknesses between 10-500 nm, but films between 50-100 nm are ideal for imaging.\(^{124}\) The film is plunged into a bath of liquid cryogen, typically ethane due to its high specific heat. The grid is then stored in liquid nitrogen and imaged under the electron beam at or below 180°C (Figure 2.8).

![Figure 2.8: Cryo-TEM sample preparation. Sample in the form of a liquid drop (a) is deposited on a carbon coated TEM grid (b). The grid is blotted and plunged into liquid ethane (c). (d and e) Cartoon depictions of the grids after blotting. Reprinted from Current Opinion in Colloid & Interface Science, 17, Newcomb, C. J., Moyer, T. J., Lee, S. S. & Stupp, S. I., Advances in cryogenic transmission electron microscopy for the characterization of dynamic self-assembling nanostructures, 350-359, Copyright 2012, with permission from Elsevier.\(^{125}\)](image)
Artifacts can still exist, and one must study multiple images to understand the architecture of the assemblies. Figure 2.9 shows the projection of structures in the grid onto the 2-D micrograph. Vesicles show distinct membranes at the edges of the structures but can easily be confused with disks or circular worm-like micelles if careful analysis is not taken. Worm like micelles are long strands with equal intensity from enter to edge. Spherical micelles are shown as single circular dots on the micrograph. Examples of representative images are shown in Figure 2.9.

Figure 2.9: A) A schematic of the 2-D projections of various structures in micrographs. Reprinted from Colloids and Surfaces A: Physicochemical and Engineering Aspects, 174, Mats Almgren, Katarina Edwards, Göran Karlsson, Cryo transmission electron microscopy of liposomes and related structures, 3-21, Copyright 2000, with permission from Elsevier. B) Diblock copolymer vesicles. C) Diblock copolymer worm-like micelles. D) Protein spherical micelles. All scale bars are 200 nm.
Artifacts observed in cryo-TEM arise from various sources such as strong sheer forces from blotting, the confinement of structures into the film, and solvent freezing. The main artifacts observed are from the formation of ice crystals within the film or on the film surface. Ice contamination can look like vesicles or micelles in many cases but can be distinguished by melting with the electron beam. Figure 2.10 shows typical ice contaminates.

Figure 2.10: Cryo-TEM ice artifacts. A) Large ice crystals. B) Hexagonal ice crystals. C) Porous dry film likely caused by over blotting. When a film is over blotted, the sample is very prone to evaporation during the plunging step. D) Polygonal ice crystals formed from thawing of the film. Scale bars: 200nm. Reproduced from reference with permission of The Royal Society of Chemistry.
2.7 References


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Chapter 3

Protein self-assembly through emulsion templating


Abstract

The self-assembly of suprastructures from recombinant amphiphilic proteins would allow precise control over surfactant chemistry and the facile incorporation of biological functionality. We used cryo-TEM to confirm self-assembled structures from recombinantly produced mutants of the naturally occurring sunflower protein, oleosin. We studied the phase behavior of protein self-assembly as a function of solution ionic strength and protein hydrophilic fraction, observing nanometric fibers, sheets, and
vesicles. Vesicle membrane thickness correlated with increasing hydrophilic fraction for a fixed hydrophobic domain length. The existence of a bilayer membrane was corroborated in giant vesicles through the localized encapsulation of hydrophobic Nile red and hydrophilic calcein. Circular dichroism spectra revealed that changes in nanostructure morphology in this family of mutants was unrelated to changes in secondary structure. Ultimately, we envision the use of recombinant techniques to introduce novel functionality into these materials for biological applications.
3.1 Introduction

Self-assembled vesicles are of great interest in drug delivery and imaging, given their ability to sequester large payloads of hydrophilic or hydrophobic agents. Vesicles made from biologically-relevant phospholipids\textsuperscript{1} are currently employed for drug delivery\textsuperscript{2}, but they are mechanically weak and difficult to functionalize. These limitations have prompted an extensive effort to make vesicles from other macromolecular surfactants including block co-polymers\textsuperscript{3,4} and Janus amphiphilic dendrimers\textsuperscript{5}, but these materials remain difficult to functionalize and are often not biocompatible.

An alternative is the assembly of materials purely from polypeptides. Seminal work has shown that block-copolypeptides can assemble into various structures, such as vesicles.\textsuperscript{6,7} However, these surfactants consist of amino acids polymerized into polydisperse blocks and then appended to make copolymers. Different amino acids have been blended into each block with set average composition, but the precise sequence of amino acids in each block could not be controlled.\textsuperscript{8} Amino acid copolymerization prevents the direct incorporation of specific peptide sequences, which are required for recognition and targeting in biology. Incorporation of such motifs would be limited to the ends or between these peptide blocks.

A powerful alternative is the expression of recombinant surfactant proteins by molecular biology. These proteins would be monodisperse and have the precise sequence dictated by the cognate gene. Unlike synthetic methods, recombinant protein production
would permit the direct incorporation of specific motifs that mediate protein recognition. Embedding recognition sequences, either at the ends of proteins or deep within the sequence, is straightforward. Smaller self-assembling oligopeptides can be produced recombinantly but the small sizes of these peptides preclude the incorporation of biologically relevant motifs. Elastin-like polypeptides have been assembled into various structures including micelles and vesicles, although the direct visualization of a bilayer membrane or vesicular encapsulation has not been explicitly shown.

While a number of naturally occurring proteins, such as hydrophobins, oleosins, latherin, and ranaspumin, are known to stabilize interfaces, only oleosins are structurally reminiscent of a chain surfactant. Oleosins are a family of plant proteins whose biological role is to stabilize oil bodies. They have an N-terminal hydrophilic segment, followed by a hydrophobic core (among the longest natural hydrophobic stretches) and another hydrophilic segment at the C-terminus. Although the crystal structure is unknown, the molecule is believed to resemble a hairpin with the hydrophobic domain bifurcated by a proline knot, a stretch containing three prolines that induce a 180° turn in the chain. The two legs of the hairpin are helical, possibly forming a coiled-coil. Recently, oleosin has been shown to stabilize artificial oil bodies and emulsions. We postulated that the surfactant-like block architecture of oleosin would make it a logical starting point for the creation of tunable self-assembled protein suprastructures.
We describe the expression of sunflower oleosin mutants in bacteria, their purification, characterization, and assembly into supramolecular structures at the nano- and micro-scales. Depending on hydrophilicity and the ionic strength of the buffer, these proteins assemble into sheet, fibers, or vesicles. Self-assembly of the proteins was investigated with cryo-transmission electron microscopy (cryo-TEM) and laser scanning confocal microscopy (LSCM).

3.2 Materials and Methods

*Oleosin Gene Creation and Expression*

The sunflower seed oleosin gene was provided as a gift by Dr. Beaudoin at Rothamsted Research, Hampshire, England. Standard molecular biology techniques were used to create the modified genes in the expression vector pBamUK, a pET series derivative, that was constructed by the van Duyne laboratory (SOM, Penn). PCR primers 42S (5’-AGA TAT ACC CAT ATG GCC ACC ACA ACC TAC GAC C) and 63AS (5’- TTT CTC ACC CTC GAG TTT CCC CCC TTC TTT TCG CCC TTC) were used to amplify the gene and add NdeI and XhoI restriction sites to the ends creating 42-87-63 (Oleo-WT).

The P=65 family was cloned into Avi-pBamUK. A gene for a biotin binding site was created with the primers Avi S (5’- TAT GGG TCT GAA CGA CAT CTT CGA GGC TCA GAA AAT CGA ATG GCA CGA AG) and Avi AS (5’- GAT CCT TCG TGC CAT TCG ATT TTC TGA GCC TCG AAG ATG TCG TTC AGA CCC A). This gene was digested with NdeI and BamHI and ligated into pBamUK to create Avi-pBamUK.
43-65-33 was created with the primers 43S (5’ - AAG GAG ATA GGA TCC TAC CGC CAT GAT CAA CAC ACC) and 33AS (5’ - TAT ATG AAT CTC GAG CTG GCC CAA GTC CTT CG); 33-65-23 was created with the primers 33S (5’ - AAG GAG ATA GGA TCC CTC ACC CCA CAG C) and 23AS (5’ - TAT ATG AAT CTC GAG ATA CTC CCC CAC ATC C); 28-65-18 was created with the primers 28S (5’ - AAG GAG ATA GGA TCC CGC CAG CAA CAA GG) and 18AS (5’ - AAG GAG ATA GGA TCC CGC CAG CAA CAA GG); 23-65-13 was created with the primers 23S (5’ - AAG GAG ATA GGA TCC CCC TCA ACC GGC AAG ATA ATG G) and 13AS (5’ - TAT ATG AAT CTC GAG CAC ATA ATC CCT CTG G). The P=65 family was digested with BamHI and XhoI and ligated into Avi-pBamUK. Mutants were confirmed through DNA sequencing prior to expression. pBamUK adds a 6-Histidine tag to the C-terminus of the protein for IMAC purification if needed. The E. coli strain BL21 DE3 (Stratagene) was used with induction controlled by the lac promoter. Cultures were grown at 37°C in Luria Bertani (LB) Broth with kanamycin at a final concentration of 50 µg/ml until OD$_{600}$=0.4. Expression of the protein was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. Cells were harvested by centrifugation; cell pellets were frozen at -20°C prior to purification.

**Extraction and Purification of Oleosin Proteins**

B-PER Protein Extraction Agent (Thermo Scientific) was used to lyse the cells using a modified protocol for inclusion bodies. A 500 ml culture cell pellet was resuspended with 10 ml B-PER. DNase was added to a final concentration of 0.5 µg/ml and incubated at room temperature for 20 minutes. The solution was centrifuged at 15,000 g for 15
minutes and the supernatant was discarded. The resulting pellet was resuspended in 10 ml B-PER. Lysozyme was added at a final concentration of 0.2 mg/ml and incubated at room temperature for 5 minutes. The suspension was diluted with 25 ml of 1:10 B-PER in water and centrifuged at 15,000 g for 15 minutes and the supernatant was discarded. The resulting pellet was washed three times with 25 ml of 1:10 B-PER resulting in a purified inclusion body pellet. The resulting inclusion body pellet was washed three times with 10 ml 200 mM Na₂CO₃ (pH 11). Oleosin was extracted from the inclusion bodies using an organic solvent mixture.²⁷,²⁸ The pellet was resuspended in 10 ml 200 mM Na₂CO₃ (pH 11). Chloroform:methanol mixtures were added to the suspension yielding monophasic solutions of Na₂CO₃:chloroform:methanol with compositions corresponding to 1:1:8 (v/v/v) (organic solution A) or 1:2:7 (v/v/v) (organic solution B). The solutions were centrifuged and the protein rich supernatant was retained.

**SDS-PAGE**

Protein samples in organic solution A were dried overnight under vacuum. The protein was suspended in 8M urea, 50 mM phosphate and used for electrophoresis. SDS-PAGE gels were run on NuPAGE Novex 4-12% Bis-Tris Mini Gels (Invitrogen). Following electrophoresis, the gel was stained with SimplyBlue SafeStain (Invitrogen). The gel was destained and imaged with a Kodak Gel Logic 100 Imaging System.

**Western Blot Analysis**

Western blot analysis was completed according to Li-Cor Biosciences Western Blot Analysis protocol. Specifically, after SDS-Page electrophoresis, the samples were
electroblotted onto a PVDF membrane. The membrane was washed overnight with blocking buffer at 4°C. The membrane was then washed with wash buffer (PBS +0.01% Tween 20) five times and incubated with anti-6xHis anti-mouse antibody for 1 hour at room temperature (Antibody dilution of 1:2000). The washing step was repeated and the membrane was incubated with the secondary antibody IRDye 700x conjugated goat polyclonal anti-mouse (Bio-Rad) for 1 hour at room temperature (Antibody dilution 1:5000). The washing step was repeated and the membrane imaged on an Odyssey Infared Imager (Li-Cor Biosciences, Lincoln, NE).

Mass Spectroscopy
Protein pellets were solubilized in 50:50 (v/v) TFE in water. Protein samples were sent to The Wistar Institute Proteomics Facility (Philadelphia, PA) for mass analysis using matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy.

Nano-vesicle preparation
Protein solutions in organic solution A ranged from 0.25 to 0.35 mg/ml measured using a Nanodrop 100 (Thermo Scientific). Solutions were injected (10% volume fraction) into various buffers created from dilutions of 1X PBS pH 7.4 (0.01 M Phosphate buffered saline, 0.138 M NaCl, 0.0027 M KCl, Fisher Scientific). Ionic strength of dilutions: 1X PBS-140 mM ionic strength, 0.5X-70 mM, 0.25X-35 mM, and 0.1X-14 mM. Solutions were sonicated in a Branson 3510 bath sonicator for 10 seconds and gently swirled by hand until the solution turned clear. Solutions were open to atmosphere for >6 hours to allow for organic evaporation and stored for >12 hour at 4°C prior to imaging.
**Cryo-TEM**

Vesicle samples were deposited on lacey formvar/carbon mesh grid (Ted Pella) and added to a cryoplunger (Gatan Cp3, Gatan, Pleasanton, CA). The sample was blotted by hand and plunged into liquid ethane. Samples were transferred to the cryoholder (Gatan CT3500TR, Gatan, Pleasanton, CA) and the cryoholder was immediately inserted into a JEOL 2010 TEM (JEOL, Tokyo, Japan) operating at 200kV. Micrographs were imaged with an Orius SC200 digital camera.

**Cryo-TEM: Membrane Thickness**

Vesicles appear on the micrographs as circles with a darker membrane. The inside of the vesicles will be darker compared to the bulk solution. This is due to electron scattering through the vesicle shell. The edges of the vesicles are darker due to the increased number of protein molecules that the electrons must transmit through. The membrane thickness is measured in ImageJ.\(^{29}\) Ten measurements were made on each vesicle and averaged to get an overall vesicle membrane thickness. Six vesicle membrane thicknesses were then averaged to get the membrane thickness for a specific mutant and plotted in Figure 2G.

**Circular dichroism**

Far-UV CD spectra were collected at 25° C on an AVIV 410 spectrometer (AVIV Biomedical Inc, Lakewood, NJ) using a 1 mm quartz cell. Protein concentrations ranged from 9-12 µM in 50:50 (v/v) TFE in water. Data was analyzed using DichroWeb
software\textsuperscript{30} using the CDSSTR method\textsuperscript{31} and Reference Set 7 containing 48 known protein structures\textsuperscript{32}.

\textit{Giant vesicle preparation}

Protein concentrations in organic solution B ranged from 0.25 to 0.5 mg/ml measured using a Nanodrop 100 (Thermo Scientific). The monophasic solutions were injected (5-10\% volume fraction) into 1X PBS, which resulted in phase separated aqueous in oil in aqueous double emulsions. The excess organic solvent was allowed to evaporate at room temperature yielding stable vesicles. Giant vesicles were dyed by adding Nile Red (Sigma) and calcein (Invitrogen) to the injection mixture.

\textit{Confocal Microscopy}

Laser scanning confocal microscopy (LSCM) was used to expose giant protein bilayer vesicles to light at 488 nm. An Olympus Fluoview FV1000 confocal microscope (Center Valley, PA) with a UPLFLN 40x objective lens was used to image the vesicles with a scan speed of 4.0 \mu s pixel\textsuperscript{-1} (4.426 s frame\textsuperscript{-1}). Nile Red signal was collected between 600-650 nm and calcein was collected between 500-520 nm.

3.3 Results and Discussion

3.3.1 Protein Modifications

Structurally, oleosin is comprised of three connected segments – a hydrophilic block at the N-terminus, a center hydrophobic block with a proline turn, and a second hydrophilic
block at the C-terminus. We use the nomenclature N-P-C to describe the variants in this chapter, where N is the number of amino acids in the hydrophilic N-terminus, P is the number of amino acids in the hydrophobic core, and C is the number of residues in the hydrophilic C-terminus (Figure 3.1). Wild type oleosin is denoted 42-87-63. A library of mutant proteins with variable hydrophobicities and molecular weights was created by deleting amino acids from either hydrophilic arm or the hydrophobic segment. Without a defined crystal structure, we could not truncate based on secondary structure motifs. Although we ultimately have the potential to introduce point mutations anywhere within the protein, in this work we chose to simply truncate the hydrophobic block and hydrophilic arms creating families of various hydrophilic fractions; future publications will describe point mutations and replacements. We created two families of mutants, with two different sized hydrophobic blocks – that of the wild type (P = 87), and one in which the hydrophobic block was truncated at the hydrophobic-hydrophilic junction by removing a total of 22 amino acids, decreasing the hydrophobic block size by 25% (P=65). Using the P=87 and P=65 hydrophobic blocks as templates, the hydrophilic arms were systematically truncated by 5 amino acids creating a large family of proteins. Members of the P=65 family included 43-65-33, 33-65-23, 28-65-18, and 23-65-13 self-assembled into a variety of nanostructures in aqueous solution. Members of the P=87 family remained very surface active but were not found to self-assemble. These proteins could be useful for surfactant stabilization at interfaces but pose difficulty due to their limited solubility in common buffers.
Figure 3.1: Schematic representing protein vesicle formation. a) Oleosin protein mutants were expressed recombinantly in *E. coli*. An organic solution was used to extract and purify protein from the inclusion bodies. Purified protein was injected into aqueous solutions yielding self-assembled protein suprastructures. b) The P=65 family of mutants was found to self-assemble upon solvent injection. This family contains identical hydrophobic blocks and the length of the hydrophilic arms varies as indicated. Protein variants are named by N-P-C, where N is the number of amino acids in the hydrophilic N-terminus (blue N-terminal arm), P is the number of amino acids in the hydrophobic core (red), and C is the number of residues in the hydrophilic C-terminus (blue C-terminal arm). Proteins are drawn to scale based on the number of amino acids in each domain.
3.3.2 Protein expression, purification, and identification

Oleosin genes were created using standard recombinant techniques and cloned into the expression vector, pBamUK for expression in the BL21 (DE3) strain of *Escherichia coli* following induction with IPTG. Recombinant protein was found in inclusion bodies, which were isolated using standard extraction techniques and repeatedly washed with a high pH buffer to remove bound DNA from the positively charged hydrophilic arms. Protein was extracted using monophasic mixtures of sodium carbonate (pH 11), chloroform, and methanol as previously reported\(^{27,28}\). Decreasing the size of the hydrophobic core to P=65 amino acids from P=87 amino acids increased protein expression 10-fold, and we obtained between 35-100 mg purified protein per liter of culture, with yield increasing when N and C were longer. Protein purity exceeded 95%, as assessed by SDS-PAGE followed by Coomassie staining (Figure 3.1a). Protein homogeneity was examined by Western blotting using an anti-6x-His tag antibody (Figure 3.1b) and in all cases the bands displayed the expected molecular weight. Molecular weights were confirmed by MALDI-TOF (Figure 3.2 C-G). The dual peaks observed in mass spectroscopy were due to incomplete loss of the initiation methionine.\(^{33}\) Expression of oleosin in inclusion bodies presumably inhibits methionine aminopeptidase from accessing the initiation methionine within the aggregated protein leading to partial removal. Expected molecular weights with and without the methionine are compared to the mass spectroscopy peaks in Table 3.1. In all cases, the expected protein mass with and without the methionine matches very closely with the two peaks in the MALDI-TOF spectra. No contamination peaks were observed in the spectra.
Figure 3.2: Protein identification and MALDI-TOF spectra. a) Protein purity was measured after organic solvent purification to be >95% with SDS-PAGE. b) Oleosin mutant identity was confirmed through Western blotting using an anti-6-His antibody. The lanes for both gels are (1) 42-87-57 (wild-type oleosin), (2) 43-65-33, (3) 33-65-23, (4) 28-65-18, (5) 23-65-13. c-g) MALDI-TOF confirmed the molecular weights of the protein mutants: c) 42-87-63 (wild-type oleosin), d) 43-65-33, e) 33-65-23, f) 28-65-18, g) 23-65-13. Peak closely match expected weights of the protein mutants as seen in Table 3.1.
3.3.3 Surfactant protein self-assembly

The large hydrophobic core of oleosin limits its solubility in water, but since it is a surfactant, the protein can stabilize emulsions. Following published procedures\textsuperscript{27,28}, we solubilized the recombinant proteins in an organic phase, choosing chloroform for its volatility and consequent ease of removal. Injection of the protein in Na\textsubscript{2}CO\textsubscript{3}:chloroform:methanol (1:1:8 v/v/v) into phosphate buffered saline (PBS) created an oil-in-water single emulsion stabilized by the oleosin mutants. Brief sonication was used to reduce the emulsion droplet size. As the chloroform phase in the emulsion diffuses through the aqueous phase and evaporates, the local concentration of protein in the emulsion increases forcing spontaneous protein budding and assembly similar to polymer systems\textsuperscript{34,35}. Cryo-TEM provided conclusive evidence of the morphology in solution due to the instant vitrification of the sample (Figure 3.3). The buds self-assemble to form higher surface area structures determined by protein sequence and solution composition. By controlling the solvent ratios, buffer solution, and protein chemistry, we were able to assemble nearly water-insoluble compounds into various self-assembled structures in an aqueous environment through kinetic pathways. We found protein

Table 3.1: Protein mutant properties and mass spectroscopy information

<table>
<thead>
<tr>
<th>Protein</th>
<th># Amino Acids</th>
<th>Estimated pI</th>
<th>Expected MW (Da)</th>
<th>Expected MW (-Met) (Da)</th>
<th>MS peak 1 (Da)</th>
<th>MS peak 2 (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42-87-63 (WT)</td>
<td>192</td>
<td>9.95</td>
<td>20785</td>
<td>20653</td>
<td>20778</td>
<td>20672</td>
</tr>
<tr>
<td>43-65-33</td>
<td>139</td>
<td>9.232</td>
<td>15406</td>
<td>15274</td>
<td>15399</td>
<td>15268</td>
</tr>
<tr>
<td>33-65-23</td>
<td>121</td>
<td>6.851</td>
<td>13082</td>
<td>12951</td>
<td>13083</td>
<td>12955</td>
</tr>
<tr>
<td>28-65-18</td>
<td>111</td>
<td>9.501</td>
<td>11942</td>
<td>11811</td>
<td>11939</td>
<td>11815</td>
</tr>
<tr>
<td>23-65-13</td>
<td>101</td>
<td>6.408</td>
<td>10820</td>
<td>10688</td>
<td>10832</td>
<td>10709</td>
</tr>
</tbody>
</table>
concentrations in the organic injection mixture exceeding 0.4 mg/ml led to aggregation of the protein rather than self-assembly -- possibly because protein-protein interactions at these concentrations overwhelmed the surface instabilities that initiate budding.

![Image](108x401 to 539x610)

Figure 3.3: Cryo-TEM micrographs of protein membrane formation. a) Cryo-TEM micrograph of 23-65-13 emulsion droplet with vesicular buds on the surface in PBS. b) Cryo-TEM micrograph of 33-65-23 showing budding from dried bulk protein in PBS. Scale bars are 200 nm.

### 3.3.4 Morphology control

The morphology of the structures in solution was investigated using cryo-TEM. Cryo-TEM is a superior method for soft matter imaging \(^{36-38}\), because unwanted rearrangement of solution structure is avoided through rapid vitrification; whereas, freeze fracture TEM fails to identify membranes and TEM with negative staining is associated with potential staining artifacts \(^{39}\). Protein suprastructures were observed in phosphate buffered saline (PBS), a physiological buffer with an ionic strength of 140 mM. The cryo-TEM
micrographs in Figure 3.4 (a-d) show a dark membrane and a lumen that is darker than the surrounding solution representative of a bilayer vesicle \(^4\). The smallest and most hydrophobic mutant, 23-65-13, as well as a slightly less hydrophobic variant, 28-65-18, were found to form both sheets and vesicles upon injection. The small size of the hydrophilic head groups of these molecules lead to planar and curved lamellar packing enabling the coexistence of sheets and vesicles. Increasing the head group size by an additional five amino acids in each hydrophilic arm (33-65-23) led to the formation of only vesicles, suggesting that the larger head group induced sufficient curvature to prevent the formation of planar bilayers. The addition of ten more amino acids to each hydrophilic arm, 43-65-33, also yielded vesicles; the increased hydrophilic head group maintained curvature and did not hinder vesicle formation.
Figure 3.4: Cryo-TEM micrographs indicate vesicle formation across the entire P=65 family. All scale bars are 200 nm. a) 23-65-13 and b) 28-65-18 coexist as sheets (Figure 7) and vesicles in PBS (140 mM) after injection. c) 33-65-23 and d) 43-65-13 form vesicles in PBS (140 mM).

The membrane thickness of vesicles formed by the proteins injected into PBS was measured by cryo-TEM. We found that the membrane thickness increases as a function of the total molecular weight of the surfactant (Figure 3.5), even though the length of the hydrophobic block remained unchanged. This effect is different than increasing membrane thickness by increasing the hydrophobic block, which has been seen previously in polymer systems. In the absence of a crystal structure, modeling suggests that the length of the hydrophobic domain of the wild type protein is ~6.0 nm; the hydrophobic block of the P=65 family may be estimated to be ~5.2 nm long. The measured membrane thickness of 23-65-13 is 6.2 ± 0.1 nm, implying that the two hydrophobic domains are highly interdigitated, similar to a zipper. A further increase in
the size of the hydrophilic arms (43-65-33) increased the membrane thickness to 8.6 ± 0.4 nm. In various macromolecular amphiphilic systems, the hydrophilic arms are well-solvated and are not directly visible in cryo-TEM due to the lack of contrast. Our system, however, is composed of protein hydrophilic arms possessing secondary structure, which potentially provides contrast through phase-contrast mechanisms in cryo-TEM. Further, the hydrophilic arms contain multiple electron-dense Tyr and Met residues, which could enhance mass-thickness contrast. It is unclear if the electron-dense amino acids combined with secondary structure in the hydrophilic arms contribute visible contrast in the micrographs or if the hydrophilic arms are well-solvated and not visible in the micrographs. If the hydrophilic arms contribute contrast, an increase in membrane thickness with increasing hydrophilic block size is easily rationalized. If the hydrophilic arm is well-solvated and diffuse, it is conceivable that the hydrophobic core becomes less interdigitated (i.e., expands) as the hydrophilic arms increase in size. Either or both of these explanations could lead to increased apparent total membrane thickness. Distinguishing the relative contributions of these two possibilities would need further work, perhaps using scattering methods.
Figure 3.5: Increasing the molecular weight of protein at constant hydrophobic block length increases the hydrophilic block fraction and leads to thicker vesicle membranes as measured through Cryo-TEM. Protein hydrophilic fraction: 23-65-13: 35.4%, 28-65-18: 41.4%, 33-65-23: 46.3%, and 43-65-33: 53.9%. Error bars represent one standard deviation (N=6).

The phase behavior of the protein surfactants likely depends on the total molecular weight, hydrophilic block fraction, ionic strength and pH of the buffer, secondary structure of the protein, specific amino acid interactions, and protein concentration. The hydrophilic block fraction of each protein was estimated by the number of amino acids in the hydrophilic arms divided by the total number of amino acids in the molecule. Increasing ionic strength of the solution will screen electrostatic interactions. Since the hydrophilic arms contain distributed positive and negative amino acids throughout the sequence, it is difficult to predict the effectiveness of charge shielding, but the phase
behavior can be readily assessed by experiment. We detected a general trend in the structural transition of self-assembled oleosin structures from lamellar phases to vesicles as the ionic strength of the surrounding solution was increased (Figure 3.6). In deionized water, 23-65-13 and 28-65-18 formed exclusively sheets while 33-65-23 and 43-65-33 formed only fiber-like structures. The fiber structures were >20 nm in thickness indicating that the packing was similar to the previously reported lamellar packing in block copolypeptide fibers \(^{41}\) rather than that in high-curvature worm-like micelles. 43-65-23 and 33-65-23 display a coexistence of fibers and vesicles when the ionic strength is increased to 35 mM and a single vesicle phase in 70-140 mM ionic strength solutions. For the smaller head group sized proteins, 23-65-13 and 28-65-18, it was not until the ionic strength reached 140 mM that vesicles were seen. This finding reasonably suggests that proteins with smaller head groups prefer to pack into lower curvature sheets.

Representative micrographs of the three phases are shown in Figure 3.6 (b-d). Cryo-TEM micrographs for each point in Figure 3.6 (a) can be found in Figure 3.7. The large regions of coexistence between phases suggest the multiple effects of the many parameters that affect assembly such as molecular weight, hydrophilic fraction, isoelectric point, pH, and protein concentration. We have shown how hydrophilic block fraction and ionic strength can be varied to systematically alter the structure of the assemblies.
Figure 3.6: a) The phase behavior of the P=65 family was explored as a function of hydrophilic fraction and ionic strength of the buffer. The diagram shows three phases, vesicles, sheets, and fibers, as well as coexistence phases. Figure 3.7 shows cryo-TEM micrographs for each phase point on the diagram. b,c,d) Representative micrographs of fibers (43-65-33 in DI water), sheets (23-65-13 in 1x PBS), and vesicles (43-65-33 1x PBS) are shown. All scale bars are 200 nm.
Figure 3.7: Phase behavior of the -65- family in various ionic strength buffers. a-f) 43-65-33 and g-l) 33-65-23 shift from fibers in DI water and 14 mM ionic strength solution to fibers and vesicles in 35 mM ionic strength solution to only vesicles in 70-140 mM ionic strength solutions. m-r) 28-65-18 and s-x) 23-65-13 exist as sheets in 0 mM, 14 mM, 35 mM, and 70 mM ionic strength solutions and coexist as sheets and vesicles in 140 mM ionic strength solutions. Background colors match phase mapping in Figure 3.6. Scale bars are all 200 nm.
3.3.5 Protein secondary structure

Modeling has suggested that the hydrophobic block of oleosin is helical, possibly a coiled-coil\textsuperscript{20}. Helical polypeptides prefer to align along the helical axes\textsuperscript{42}, creating rigid chain conformations. The resulting rigid packing of the hydrophobic block as well as interactions between chains leads to the formation of low curvature structures explaining the lack of higher curvature spherical and worm-like micelles\textsuperscript{43}. This suggests that increases in ionic strength would decrease the rigidity of the helical blocks by softening interactions in the hydrophilic arms, allowing proteins to shift from planar lamellar alignments in the fibers and sheets to curved lamellar alignments in vesicles.

Given the variety of assemblies formed by the mutant oleosins, we investigated whether the structure of the assembly could be related to the secondary structure of the constituent proteins. We analyzed secondary structure using CD spectroscopy. Since the proteins are insoluble in a pure aqueous phase, and chloroform could not be used as a co-solvent because of its absorption at far-UV wavelengths (thus interfering with the spectroscopy) protein samples were resuspended in 50/50 trifluoroethanol:water (v/v). CD data were fit to a reference set of 48 proteins\textsuperscript{31} using Dichroweb\textsuperscript{30} software and the CDSSTR analysis method\textsuperscript{32} (Figure 3.8 a). The fits to the spectra were very good and provided an estimate of the relative contributions of different characteristic secondary structural motifs (Figure 3.8 b). Spectral curves show that all mutant proteins studied exhibited secondary structure after exposure to organic solvents during purification. Decreasing the length of the hydrophilic arms and the hydrophobic core leads to
increasing alpha-helical structure compared to the wild-type protein, but all the members of the P = 65 family retained similar secondary structure. Since members of this family exhibited widely variable self-assembly, this suggests that changes in secondary structure are not responsible for the changes in the structure of the corresponding suprastructures in this family of molecules.

Figure 3.8: a) Far-UV circular dichroism spectra of wild-type oleosin and the P=65 mutants. Solid lines represent fits calculated with DichroWeb software. The fits match experimental data very well allowing for prediction of overall secondary structure. b) Estimation of secondary structure of wild type oleosin and the P=65 mutants. The P=65 family show similar secondary structure, although mutants show increased alpha-helical structure compared to wild-type oleosin. Morphological differences seen between protein mutants are not attributable to changes in the secondary structure of the proteins across the P=65 family.

3.3.6 Giant oleosin vesicles

In order to visualize the membrane better and to investigate the ability of the vesicles to sequester solutes, giant vesicles (> 1 µm in diameter) were created with recombinant 33-65-23 using phase-separated double emulsions. Protein solubilized in Na₂CO₃:chloroform:methanol (1:2:7 v/v/v) was injected into PBS, resulting in
spontaneous water-in-oil-in-water (W/O/W) double emulsions confirmed by dual encapsulation of Nile Red and calcein that were added to the injection mixture (Figure 3.9 a,b). As organic solvent evaporated from the double emulsion, the hydrophobic dye Nile Red sequestered into the hydrophobic core of the membrane whereas the hydrophilic calcein remained in the hydrophilic lumen (Figure 3.9 c,d). Laser scanning confocal microscopy was used to image the original double emulsions and the vesicles created after solvent evaporation. The ability to encapsulate hydrophobic dye in the membrane and hydrophilic dye in the lumen suggests the assembly of bilayer vesicles on the micron scale, consistent with the formation of vesicles on the nanometer scale.

Figure 3.9: a,b) Giant bilayer vesicles evolve from phase separated double emulsions. Protein solutions in organic mixture B (see Methods) were injected into PBS resulting in a phase separated water-in-oil-in-water (W/O/W) double emulsion. Nile Red and calcein are loaded into the organic injection mixture and partition into the appropriate phases. Scale bars = 50 µm. c,d) The organic middle phase evaporates leading to sequestering of Nile Red into the hydrophobic membrane and calcein into the hydrophilic lumen. Scale bars = 5 µm.
3.4 Conclusions

Recombinant proteins hold the promise of making designer surfactants with precise chemical sequences. We employed emulsions of tailored composition to self-assemble largely water-insoluble amphiphilic proteins through kinetic pathways. Such a strategy could be used to assembly other low solubility surfactants. Using recombinant oleosins, we have demonstrated that engineered recombinant surfactant proteins can yield a fascinating variety of self-assembled structures in solution, including vesicles, at both the nano- and micro-scales. In making vesicles, we found that increasing the length of the hydrophilic arms while keeping the length of the central hydrophobic block constant altered the membrane thickness of the vesicles. By varying both the ionic strength of solution and the molecular weight (hydrophilic fraction) of the protein, we could control the phase behavior of the assemblies. The oleosin mutants possess helical hydrophobic blocks which likely drive lamellar membrane packing. However, significant changes in suprastructure morphology are seen without changes in the protein secondary structure. Giant vesicles were also made, creating a platform for macroscopic measurements in future studies, such as micropipette aspiration to assess membrane material properties. We envision using oleosin and its mutants to make a wide variety of materials with biological activity: examples include adding terminal adhesive domains that bind to cell surface receptors for vesicle targeting; protease recognition sites that could mediate protease-induced disintegration; and self-assembly motifs driven by coiled-coil assembly and ionic concentration. Therefore, recombinant oleosin has significant potential for making assembled structures of designer biofunctionality.
3.5 References


17 Beaudoin, F. & Napier, J. A. Targeting and membrane-insertion of a sunflower oleosin in vitro and in *Saccharomyces cerevisiae*: the central hydrophobic domain contains more than one signal sequence, and directs oleosin


Chapter 4

Spherical Micelles Assembled from Soluble Recombinant Oleosin Mutants


Abstract

An emerging field in biomaterials is the creation and engineering of protein surfactants made by recombinant biotechnology. Biomaterials made from self-assembled recombinant proteins allow for complete control of the surfactant chemistry. The proteins are uniform and monodisperse in molecular weight and functionalization with amino acid
sequences is straightforwardly achieved by genetic engineering. We modified the naturally occurring amphiphilic plant protein oleosin by truncating a large portion of its central hydrophobic block creating a soluble triblock surfactant. Additional mutants were constructed to eliminate secondary structure and create ionic surfactants. Oleosin surfactant mutants assembled into spherical micelles with a diameter ~21 nm above a critical micelle concentration. We found that the critical micelle concentration could be manipulated through changes in the protein backbone and were correlated with changes in the protein secondary structure. Micelle size and shape is characterized with dynamic light scattering (DLS), small angle X-ray scattering (SAXS), and cryogenic transmission electron microscopy (cryo-TEM). Micelles were functionalized with the integrin-binding domain, RGDS, leading to a 2.9 fold increase in uptake in Ovcar-5 cells after 12 hours. Oleosin surfactants present a promising platform for micellar assembly because of the ability to precisely modify the protein backbone through molecular biology allowing for control over the CMC and addition of functional domains into the material.
4.1 Introduction

Surfactants are molecules of key importance in the food, chemical and pharmaceutical industries. Some families of surfactants are known to self-assemble into various structures in solution as a function of molecular weight, chemistry, and solvent. One such structure, the spherical micelle, provides an ideal system for the entrapment of hydrophobic compounds in their hydrophobic core, which is surrounded by a solvated corona. Micelles are traditionally assembled from amphiphilic surfactants such as lipids or diblock copolymers, but herein we describe an approach of assembling spherical micelles from protein amphiphiles made by recombinant biotechnology.\(^1,2\)

Molecular biology provides the ability to control the precise sequence of amino acids in the surfactant. Such precision is tantamount to control of the type and position of monomers in a polymer chain, while simultaneously ensuring that each polymer is the same molecular weight. Also, the sequence of amino acids is a unique identification sequence for lock and key binding, and recombinant methods allow for direct incorporation of biological motifs at any point in the protein backbone.

Diblock elastin-like polypeptides (ELPs) have been used as building blocks for the formation of nanoparticles from recombinantly produced polypeptide.\(^3-6\) ELPs display a lower critical solution temperature (LCST), above which the polypeptide precipitates out of solution. ELP diblocks are created by fusing two blocks together with differing LCSTs. Manipulating the chemistry and length of the blocks can yield a material that
when exposed to an intermediate temperature, one block precipitates out of solution while the other remains soluble, leading to the formation of nanoparticles. Recent work has shown that assembly can be controlled through the conjugation of hydrophobic moieties to an asymmetric ELP diblock. In many cases, the core of the ELP micelle is highly solvated, as shown by a pyrene assay, potentially limiting the hydrophobicity of the cargo.

Here we present an alternative, using nature as a template we engineered the natural surfactant protein oleosin to self-assemble in solution. Oleosin is a protein from plants that stabilizes oil bodies in seeds. Oleosin has been expressed recombinantly and used as a surfactant, but little engineering has been completed to optimize it for self-assembly. The native structure is defined in three domains: an N-terminal hydrophilic arm, a central hydrophobic domain, and a C-terminal hydrophilic arm. A proline knot in the core of the hydrophobic domain forces a 180° turn leading to a hairpin like structure. Models have estimated the hydrophobic core to have significant helical structure. Previously, we have shown that a family of oleosin mutants produced by recombinant methods, where the center block was truncated, and the hydrophilic arms varied in length will self-assemble from the surface of an emulsion into fibers, sheets, or vesicles, based on the hydrophilic fraction of the protein and the solution ionic strength. Thus, oleosin has proven a versatile surfactant that can be expressed recombinantly and is amenable to engineering.
Our goal in the current work was to remove the secondary structure of oleosin to create a surfactant that possesses the structure of a random coil and is capable of self-assembly in solution. Previous work has shown that oleosin remains surface active at oil/water interfaces when large portions of the hydrophobic domain are removed.\textsuperscript{22} In order to abolish the secondary structure of the protein, we removed a large portion of the hydrophobic domain including the elimination of cross chain hydrogen bonds through threonines, which have been hypothesized to generate secondary structure in previous models.\textsuperscript{19} Three additional mutants were made from the base protein to explore the function of secondary structure and charge on the self-assembly behavior. The soluble mutants self-assemble into spherical micelles as a function of concentration, and pyrene was used to determine the critical micelle concentration. Micelle size was determined with dynamic light scattering and small angle X-ray scattering. Assemblies were directly visualized with cryogenic transmission electron microscopy. We demonstrate the simplicity of functionalizing the micelles by adding the integrin binding peptide, RGDS, to the C-terminus. This peptide increased the uptake of micelles three fold in Ovcar-5 cells after a 12 hour incubation. These studies demonstrate the potential for oleosin mutants to have a major impact in the design of functional protein micelles.

4.2 Materials and Methods

*Gene creation and expression*

The sunflower seed oleosin gene was provided as a gift from Dr. Beaudoin at Rothamsted Research, Hampshire, England. Sequential rounds of PCR were used to create the mutated genes. Mutants were created using sequential PCRs. Primers can be found in
Appendix 1. Oleosin-30 was created using 42-87-63 as a template. Oleosin-30G and oleosin-30-RGDS was created using oleosin-30 as a template. The five glycines were added in using the following oligo as a PCR template: 5’ —

CTCGCTCTCGGTGCGACTCCGCTGGTTGTGTATAGGTTTTCAGCCCTGTTATT
GTTCCAGCGATGGGTATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTCAG — 3’.

Oleosin-30 and oleosin-30G were created by combining individual PCR products for each domain: the N- and C-terminal arms and the hydrophobic core. The charged mutants were created using Oleosin-30G as a template. The charged mutants and oleosin-30-RGDS were created using extending sequential PCRs. The genes for were inserted into the expression vector pBamUK, a pET series derivative constructed by the Duyne Laboratory (SOM, Penn). Mutants were confirmed through DNA sequencing prior to protein expression. pBamUK adds a 6-Histidine tag to the C-terminus of the protein for IMAC purification. Protein was expressed in the E. Coli strain BL21 DE3 (Stratagene) controlled by the lac promoter. Cultures were grown at 37°C in Luria Bertani (LB) with kanamycin at a final concentration of 50 µg/ml until OD$_{600}$≈0.7.

Protein expression was induced with isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1.0 mM. Cells were harvested by centrifugation; cell pellets were frozen at −20 °C prior to purification.

Protein Purification

Oleosin-30G(−) was the only mutant expressed solubly; Oleosin-30, Oleosin-30G, Oleosin-30G(+), and Oleosin-30-RGDS were all expressed in inclusion bodies. B-PER protein extraction agent (Fisher Scientific) was used for protein purification following the
manufactures’ protocol for soluble or inclusion body purification respectively. Briefly, pellets were resuspended in B-PER (30 ml B-PER per liter of culture) and DNase was added to a final concentration of 0.7 µg/ml. The resuspended pellets were centrifuged at 15,000 g for 15 minutes. The supernatant of Oleosin-30G(-) was collected and added to an equilibrated Ni-NTA column (Hispur Ni-NTA resin, Thermo Scientific) and allowed to bind for >1 hour while the supernatant of the other mutants was discarded. Inclusion body purification only: The remaining inclusion body pellet was suspended in denaturing buffer (8M urea, 50 mM phosphate buffer, 300 mM NaCl). The solution was centrifuged at 15,000 g for 15 minutes and the supernatant was added to an equilibrated Ni-NTA column. Protein was allowed to bind to the column for >1 hour and washed three times with denaturing wash buffer (denaturing buffer with 20 mM imidazole). Protein refolding was accomplished by diluting the column 50 times with refolding buffer (50 mM phosphate buffer, 300 mM NaCl, 5% by volume glycerol, 4°C) and rocked at 4°C for >1 hour. All mutants: Ni-NTA columns were washed extensively with wash buffer (50 mM phosphate buffer, 300 mM NaCl, 20 mM imidazole) and eluted in fractions with elution buffer (50 mM phosphate buffer, 300 mM NaCl, 300 mM imidazole). The concentration of each elution fraction was measured with a Nano-Drop 1000 (Thermo Scientific). Buffer exchanges were completed with dialysis or using centrifugal filters (Amicon Ultra, 3 kDa, Millipore). All analysis was completed in 50 mM phosphate, 140mM NaCl unless otherwise noted.

SDS-PAGE
SDS/PAGE gels were run on NuPAGE Novex 4–12% Bis-Tris mini gels (Invitrogen) in MES buffer. Following electrophoresis, the gel was stained with SimplyBlue SafeStain (Invitrogen). The gel was destained and imaged with a Kodak Gel Logic 100 Imaging System.

Matrix Assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF)

Protein molecular weight was confirmed with MALDI-TOF. Sample spots were created with 0.5 µl protein in 1x PBS and 0.5 µl saturated sinapinic acid solution (50/50 acetonitrile/water + 0.1% TFE). Spectra were collected on an Ultraflextreme MALDI-TOF (Bruker, Billerica, MA).

Circular Dichroism (CD)

Far-UV CD spectra were collected at 25 °C on an AVIV 410 spectrometer (AVIV Biomedical Inc.) using a 1 mm quartz cell. Protein concentration was 15 µM in 50 mM phosphate, 140 mM NaF. NaF was used to replace NaCl due to the strong absorbance of the Cl– ion.

Dynamic Light scattering (DLS)

Dynamic light scattering of protein solutions was performed on samples at 30 µM using a Malvern Zetasizer Nano ZS (Westborough, Massachusetts). Each sample was run in triplicate.

Small Angle X-Ray Scattering (SAXS)
Protein micelle solutions were loaded into 1 mm glass capillaries (Charles Supper Company, Natick, MA) at a concentration of 15 mg/ml and sealed on both ends. The X-ray scattering setup uses Cu Kα X-rays from a Nonius FR-591 rotating-anode generator operating at 40 kV and 85 mA. Osmic Max-Flux optics and triple pinhole collimation were used to obtain a highly collimated beam under vacuum. Data were collected using a Bruker Hi-Star multiwire detector with a sample detector distance of 150 cm for 2.5 hours each. The data reduction and analysis were performed using Datasqueeze software. Background scattering from a capillary filled with pure buffer was subtracted from the sample scattering.

**Pyrene Assay (CMC)**

The fluorescence probe pyrene was used to determine the critical micelle concentration (CMC). 7 µl of a 12 mM pyrene (Sigma-Aldrich; St. Louis, MO) in ethanol was added to 20 ml of 50 mM phosphate, 140 mM NaCl. 100 µl of the phosphate pyrene solution was added to low volume cuvettes. High concentration protein stock solutions were used to create a dilution series. Pure buffer was added to each cuvette to bring the total volume to 700 µl maintaining an equal pyrene concentration in each cuvette. Cuvettes were scanned with an excitation of 334 nm and an emission of 360-400 nm (excitation slit: 3 nm, emission slit: 3 nm) using a Fluoromax-4 spectrophotometer (Horiba Jobin Yvon). The intensity of the first and third peak, I₁ and I₃, was determined as the maximum from 369-374 nm and 380-384 respectively. The ratio I₁/I₃ was plotted as a function of oleosin concentration. The CMC was determined as the inflection point of the best-fit sigmoidal curve.
Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryogenic transmission electron microscopy was performed at the University of Pennsylvania in the Nanoscale Characterization Facility (Philadelphia, PA). Lacey formvar/carbon grids (Ted Pella) were washed in chloroform to remove the formvar template and carbon coated with a Quorum Q150T ES carbon coater (Quorum Technologies, United Kingdom). Grids were cleaned with hydrogen/oxygen plasma for 15 seconds using the Solarus Advanced Plasma System 950 (Gatan, Pleasanton, CA). A sample in the form of a 2 µl drop was deposited onto the grid and added to a Gatan Cp3 cryoplunger (Gatan, Pleasanton, CA). The samples were blotted by hand and plunged into liquid ethane. Grids were transferred to a Gatan CT3500TR cryoholder (Gatan, Pleasanton, CA) and immediately inserted into a JEOL 2100 HRTEM (JEOL, Tokyo, Japan) operating at 80 keV. Micrographs were imaged with an Orius SC200 digital camera.

Protein labeling and calibration curve

Protein was directly labeled with the amine reactive fluorophore Dylight 488 NHS-ester (Pierce/Thermo Scientific) using the manufacturer’s protocol. Excess dye was removed using centrifugal filters (Amicon Ultra, 3 kDa, Millipore) until the flow through fluorescence was undetectable. Labeled protein concentration was calculated according to the labeling protocol. A calibration curve was generated to correlate the fluorescent signal from Oleosin-30 and Oleosin-30-RGDS to protein concentration to account for differences in the degree of labeling. A dilution series was created in 50 mM phosphate,
140 mM NaCl. Fluorescent spectra were taken ($\lambda_{\text{ex}} = 493$ nm, $\lambda_{\text{em}} = 500 – 600$ nm) and the peak intensity was plotted as a function of concentration. The calibration curves were used to correlate fluorescence measurements taken of the lysed cells to the amount of protein in each cell.

**Cell Line**

NIH:OVCAR-5 (OVCAR-5) cells were obtained from the NIH and maintained in RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin (Invitrogen).

**In vitro cell internalization**

OVCAR-5 cells were plated in RPMI 1640 media (10% FBS, 1% Pen/Strep) and grown until they reached 60% confluence. Once confluent, the cells were rinsed with RPMI 1640 media (no serum) and treatment media containing either no micelles, 8 µM of Oleosin-30, or 8 µM Oleosin-30-RGDS in low serum RPMI 1640 media (5% FBS, 1% Pen/Strep) was added. Following the internalization period (3 or 12 hours), the cells were rinsed three times with RPMI 1640 media (no serum), trypsinized (0.05% trypsin, Invitrogen), and counted using a Z1Coulter Particle Counter (Beckman Coulter). The trypsinized cells were transferred to a conical tube and centrifuged at 1200 RPM for 5 minutes to pellet the cells. The cell pellet was collected and resuspended in 1mL of 50 mM phosphate, 140 mM NaCl. In order to obtain the complete unquenched fluorescent signal from internalized micelles, the cells were lysed by freezing in liquid nitrogen. After thawing the lysed cells, the fluorescent signal from the micelles was detected via
fluorescence ($\lambda_{ex} = 493$ nm, $\lambda_{em} = 500 – 600$ nm) using a Fluoromax-4 spectrophotometer (Horiba Jobin Yvon). The internalization study was performed in triplicate.

4.3 Results and Discussion

4.3.1 Gene design and expression

The naturally occurring 20-kDa wild-type sunflower seed oleosin has 87 amino acids in the hydrophobic block. The hydrophobic block was truncated by 57 amino acids leaving 30 amino acids in the hydrophobic core, creating a variant we designate as oleosin-30 (Figure 4.1). This family of molecules can be thought of as a triblock N-P-C surfactant protein, where N and C are hydrophilic and P is hydrophobic. Five glycines were added into the hydrophobic block of oleosin-30 creating oleosin-30G. The glycines were positioned such that a proline or glycine was present every four amino acids providing increased flexibility to the hydrophobic domain (Figure 4.1). Cationic and anionic mutants of oleosin-30G were cloned by mutating the hydrophilic arms to contain only positive or negative charges creating oleosin-30G(+) and oleosin-30G(-) respectively (Figure 4.1). The mutants were created such that the naturally charged amino acids were replaced to create all positive or all negative mutants. Tyrosines were also removed from the hydrophilic arms and replaced with Asn or Gln removing all aromatic groups from the hydrophilic portions of the protein. Charge was distributed throughout the hydrophilic arms with an average charged residue every six amino acids.
Figure 4.1: Top: Cartoon depicting mutations made to the oleosin hydrophobic core (note the N- and C-terminal domains are not shown). 42-87-63 depicts the wild-type molecule. 57 amino acids are removed from the hydrophobic block (transparent) creating oleosin-30. This mutation eliminated the two hypothesized hydrogen bond bridges (T63-T104 and T67-T97-T101). Additionally, five glycines are inserted into the hydrophobic block providing increased flexibility creating oleosin-30G. Bottom: Block diagrams of the primary sequences of the mutants created. Numbers in the block diagrams indicate the number of amino acids in each block. Each mutant has a 6-histidine tag at the end of the C-terminus hydrophilic arm.

Genes were cloned into the expression vector pBamUK for expression in the BL21 (DE3) strain of Escherichia coli. pBamUK adds a 6-histidine tag on the C-terminus for purification through immobilized metal affinity chromatography (IMAC). All mutants were expressed in inclusion bodies, except for oleosin-30G(-), which was expressed
solubly. Protein mutants were purified using IMAC. Protein yields were ~15 mg (oleosin-30), ~25 mg (oleosin-30G), ~18 mg (oleosin-30G(+)), and ~80 mg (oleosin-30G(-)) of purified protein per liter of culture. Mutants were highly soluble in buffered solutions (pH 7.4) and the charged mutants were extremely stable at high concentrations (>100 mg/ml). Protein purity was >95% as assessed by SDS-PAGE electrophoresis with Coomassie Blue staining (Figure 4.3 A). Molecular weights were confirmed with MALDI-TOF (Figure 4.2) (oleosin-30 expected: 14677 Da, measured: 14667 Da, oleosin-30G expected: 15025 Da, measured: 15022 Da, oleosin-30G(-) expected: 14995 Da, measured: 14954 Da, oleosin-30G(+) expected: 14977 Da, measured 14979 Da).

Figure 4.2: Mass spectra of A) Oleosin-30, B) Oleosin-30G, C) Oleosin-30G(-), and D) Oleosin-30G(+). Expected and measured masses can be found in Table 4.1.
4.3.2 Protein secondary structure

Reports on the secondary structure of oleosin have been contradictory.\textsuperscript{16,17,19,24-26} Modeling has shown the hydrophobic domain to fold into a coiled-coil domain.\textsuperscript{19} Recent work suggests that oleosin maintains a beta sheet structure in oil bodies.\textsuperscript{24} It is unclear what structure oleosin adopts in aqueous environments due to its low solubility. Previously, we demonstrated that truncating the hydrophobic block of oleosin from 87 amino acids to 65 amino acids creates a structure with high alpha-helical content in a buffer of 50:50 TFE:water, a buffer that promotes alpha helical structure. The role of the oleosin secondary structure in self-assembly is unclear, although it is hypothesized that strong helical character limited self-assembly to low curvature structures due to strong helix-helix interactions.\textsuperscript{16,27}

One goal of our work was to eliminate the secondary structure of oleosin to create a protein surfactant that acts as a random coil in solution removing the unknown effects of the secondary structure on assembly. The hairpin in oleosin has been hypothesized to fold due to hydrogen bonding between the two arms of the molecule.\textsuperscript{19} By significantly truncating the hydrophobic domain, we eliminated the hypothesized cross-chain hydrogen bonding (bridges T63-T104-T108 and T67-T97-T101) (Figure .41). Protein secondary structure was elucidated using circular dichroism (Figure 4.3 B). The spectra were fit with the CDSSTR analysis method using Dichroweb (Figure 4.3 C).\textsuperscript{28-30} Analysis of the CD spectra shows almost no helical character in the variants. The removal of 65% of the hydrophobic domain in oleosin-30 leads to ~50% unordered
structure with ~20% beta sheet remaining in the protein. The addition of five glycines into the hydrophobic block in oleosin-30G creates a random coil polypeptide with ~65% of the backbone in an unordered structure. Surprisingly, the cationic and anionic mutants show drastically different secondary structure. oleosin-30G(+) is ~60% unordered whereas oleosin-30G(-) has a large portion of its backbone committed to turns in the protein. The proline knot remains in all mutants, but it is unclear if a full 180° turn exists in the molecule. It is likely that without the hydrogen bonds in the hydrophobic core to stabilize the turn, the molecule exists in a more flexible form, rather than a hairpin seen in the naturally occurring protein, allowing for the high curvature assemblies.

![Image](image.png)

Figure 4.3: A) SDS-PAGE gel shows >95% purity after IMAC purification for all mutants. Lane 1: oleosin-30, lane 2: oleosin-30G, lane 3: oleosin-30G(-), lane 4: oleosin-30G(+). B) CD spectra of the four oleosin mutants at 15 μM. C) CDSSTR analysis of the CD spectra show that oleosin-30 and oleosin-30G(-) display more confined structures compared to the nearly unordered structures of oleosin-30G and oleosin-30G(+).

4.3.3 Pyrene Assay

The critical micelle concentration (CMC) for protein assemblies was determined using the fluorescent probe pyrene. Pyrene preferentially partitions into non-polar environments and the ratio of the first and third emission peaks (I1/I3) incrementally
decreases as a function of the local polarity.\textsuperscript{31,32} The I\textsubscript{1}/I\textsubscript{3} ratio decreased as a function of concentration for oleosin solutions indicating the formation of a hydrophobic core (Figure 4.4). The profiles were fit with a Sigmoidal-Boltzmann equation (SBE),

\[
\frac{I_1}{I_3} = \frac{(a_i-a_f)}{1+\exp[(x-x_0)/\Delta x]} + a_f
\]

where \(a_i\) and \(a_f\) are the initial and final asymptotes of the sigmoid respectively, \(x_0\) is the inflection point of the curve, and \(\Delta x\) is the independent variable interval \(x\).\textsuperscript{33} It has been shown that this method can yield two CMCs, one at \(x_0\) and one at \(x_0+2\Delta x\) depending on the ratio of \(x_0/\Delta x\). For all curves, the ratio \(x_0/\Delta x<10\), therefore the CMC is the concentration at the inflection point.\textsuperscript{34} oleosin-30 micelles display high thermodynamic stability with a CMC of 5.7 µM.

The oleosin-30G mutant displays two aggregation concentrations. The lower concentration is thought to be a nucleation concentration where multimers assemble but do not pack efficiently enough to expel all of the water. This can be seen as the shoulder in the I\textsubscript{1}/I\textsubscript{3} curve for oleosin-30G (Figure 4.4 A). We hypothesize that the presence of the nucleation concentration is due to the increased disorder in the hydrophobic block from the glycine additions. As concentration is increased, the protein surfactants pack in a way to expel the majority of the water leading to the second aggregation concentration, termed the micelles maturation concentration.
Figure 4.4: Pyrene fluorescence is used to calculate the CMC for surfactant self-assembly. The ratio of the first and third fluorescence peaks (inset) is plotted as a function of concentration and the inflection point is the CMC. A) oleosin-30 (blue) shows a single inflection point whereas oleosin-30G (red) displays two inflection points, a nucleation point at a low concentration and a micellar maturation point at higher concentration. The addition of the five glycines is hypothesized to provide increased flexibility to the hydrophobic domain allowing for assembly at lower concentrations. B) As expected, the charged arms of oleosin-30G(+) (red) increase the concentration at which the micelle hydrophobic core matures as indicated by the broad shoulder in the curve. Surprisingly, the shoulder for oleosin-30G(-) (blue) is narrower than oleosin-30G indicating that the hydrophobic core develops at lower concentrations as seen by the shift in the second inflection point.

By charging the hydrophilic arms, it was anticipated that the maturation concentration would increase due to the electrostatic repulsions opposing the hydrophobic forces driving assembly. The ionic mutants show similar nucleation concentrations and as expected the maturation concentration of oleosin-30G(+) increased by 6-fold compared to oleosin-30G. Surprisingly, oleosin-30G(-) displayed a lower maturation concentration than oleosin-30G. The values for each inflection point can be found in Table 4.1.
Table 4.1: Protein mutant and micelle characterization

<table>
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<tr>
<th>Protein</th>
<th>Expected MW (g/mol)</th>
<th>Measured MW (g/mol)</th>
<th>Inflection point 1 (µM)</th>
<th>Inflection point 2 (µM)</th>
<th>Hydrodynamic Diameter (nm)</th>
<th>PDI</th>
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<tr>
<td>42-30-63</td>
<td>14677</td>
<td>14667</td>
<td>-</td>
<td>4.1</td>
<td>21.52</td>
<td>0.086</td>
</tr>
<tr>
<td>42-30G-63</td>
<td>15026</td>
<td>15022</td>
<td>0.09</td>
<td>11.3</td>
<td>20.77</td>
<td>0.139</td>
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<tr>
<td>42-30G-63 (-)</td>
<td>14955</td>
<td>14954</td>
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<td>7.2</td>
<td>20.68</td>
<td>0.165</td>
</tr>
<tr>
<td>42-30G-63 (+)</td>
<td>14977</td>
<td>14979</td>
<td>0.1</td>
<td>61.3</td>
<td>18.9</td>
<td>0.289</td>
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</tbody>
</table>

To investigate the discrepancy between oleosin-30G(-) and oleosin-30G, we studied the protein secondary structure as a function of concentration. The secondary structure of oleosin-30G(-) does not significantly change between 3-20 µM whereas the secondary structure of oleosin-30G becomes restricted losing much of its unordered character as the concentration is increased from 5 µM to 25 µM (Figure 4.5). Mutants that display restricted structure at low concentration, oleosin-30 and oleosin-30G(-), will mature into micelles at lower concentrations compared to proteins that display pure random coil structure at low concentrations, oleosin-30G and oleosin-30G(+), due to the additional conformation entropy in the random coil mutants. This indicates that the protein must conform to a more restricted structure to proceed through the maturation aggregation concentration and fully assemble into a micelle.
Figure 4.5: Concentration dependent CD spectra. A) CD spectra of oleosin-30G(-) at various concentrations. B) CDSSTR analysis of the CD spectra of oleosin-30G(-) indicates no change in structure as a function of concentration. C) CD spectra of oleosin-30G at various concentrations. D) CDSSTR analysis of the CD spectra of oleosin-30G indicates that the protein transitions from an unordered polypeptide to a more confined structure with increasing concentration. It is hypothesized that the confined structure is needed to mature the hydrophobic core of the micelles and is directly related to the pyrene data discussed in Figure 4.4.

The $I_{1}/I_{3}$ ratio is a direct indication of the polarity of the environment inside the micelle.\textsuperscript{32,33} The lower the $I_{1}/I_{3}$ ratio, the more hydrophobic the micellar core. Pure hydrocarbon solvents have an $I_{1}/I_{3}$ ratio of about 0.6, polar solvents vary between 1.25-2.00, and micellar systems have an $I_{1}/I_{3}$ ratio that range from 1.1-1.5.\textsuperscript{32,33} ELP spherical
micelles have an I/I₃ minimum of about 1.55 indicating a very polar core.⁸ The oleosin spherical micelles’ I/I₃ ratio falls to 1.05 indicating an extremely hydrophobic core creating a more stable environment for nonpolar encapsulates. The I/I₃ ratio minima difference between ELP and oleosin micelles can be attributed to the head group size.³²,³⁵ Diblock ELPs have much larger head groups than oleosin, which leads to lower interfacial compactness allowing for water to penetrate more deeply within the micelle. The oleosin head group is much smaller and composed of two arms likely leading to a more compact interface limiting water penetration.

### 4.3.4 Micelle characterization

Micelle size was measured with small angle X-ray scattering. The scattering profile of concentrated solutions of oleosin-30 micelles were fit to a Rayleigh function, which assumes spherical particles with uniform electron density. DataSqueeze was used to calculate the fits for the SAXS profiles.²³ Good agreement was found with a model consisting of the sum of a polynomial (representing a smooth background) and a Rayleigh function³⁶ (which models spherical particles with uniform electron density). The momentum parameter Q=(4π/λ)sin θ was used as the independent parameter for the least squares fits. Fit parameters can be found in Tables 4.2 and 4.3. The fit yielded an average diameter of 23.0 nm (Figure 4.6 A). Oleosin-30 micelles were directly visualized with cryogenic transmission electron microscopy (Cryo-TEM). Proteins inherently have low electron density due to the lack of high atomic number elements leading to low contrast in TEM. Therefore, samples were imaged at 80 keV to increase
contrast between the protein micelles and the external buffer solution at the expense of resolution. In agreement with X-ray scattering, micrographs show monodisperse spheres in solution with an average core diameter of 18.4±1.6 nm (standard deviation of the mean, n=30) (Figure 4.6 B). We expect the diameter measured from cryo-TEM micrographs to be slightly smaller due to any hydration of the shell and the resolution sacrifice from operating at 80 keV.

Table 4.2: Datosqueeze fit parameters for Oleosin-30

<table>
<thead>
<tr>
<th>Oleosin-30</th>
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<th>Value</th>
<th>One-Parameter Error Bars</th>
<th>Multi Parameter Error Bars</th>
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<td>Dispersion</td>
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<td>29.43669</td>
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Table 4.3: Datasqueeze fit parameters for Oleosin-30-RGDS

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<th>Value</th>
<th>One-Parameter Error Bars</th>
<th>Multi Parameter Error Bars</th>
</tr>
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<td>Upper Limit</td>
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<tr>
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<td>Dispersion</td>
<td>38.61803</td>
<td>7.83E-02</td>
<td>37.71027</td>
</tr>
</tbody>
</table>

Dynamic light scattering was used to confirm micellar populations in all mutants. Scattering spectra show monodisperse populations with an average hydrodynamic diameter of 21.5 nm (PDI=0.086) for oleosin-30 and 20.8 nm (PDI=0.139) for oleosin-30G. DLS confirmed similar sizes for the ionic mutants of 20.68 nm (PDI=0.165) and 18.9 (PDI=.289) for oleosin-30G(-) and oleosin-30G(+) respectively (Figure 4.6 C). Measurements were taken at 30 µM, above the maturation concentration of all mutants except for oleosin-30G(+). The hydrodynamic diameter of the oleosin-30G(+) aggregates were undistinguishable above and below the maturation concentration.
Figure 4.6: A) Oleosin-30 micelle size is measured with small angle X-ray scattering. Data were fit with a Rayleigh function and a first order polynomial to account for background scattering. Fits resulted in a diameter of 23.0 nm. B) Cryo-TEM micrograph of oleosin-30 micelles shows uniform, spherical particles in solution. C) Dynamic light scattering was used to confirm micellar structures in all mutants. Spectra show monodisperse peaks of ~20 nm indicative of micelle assembly. Curves are shifted for clarity.
4.3.5 Micelle functionalization

Targeted protein mutants were created by appending the integrin binding motif RGDS to the C-terminus of oleosin-30 creating oleosin-30-RGDS. The RGDS tag was appending after the 6-histidine tag allowing for terminal display of the peptide. oleosin-30-RGD was expressed and purified using the same method as oleosin-30; purity and MW were confirmed (Figure 4.7 A-B). The addition of the tag had no affect on the CMC or secondary structure of the protein (Figure 4.7 B-C). The addition of the RGD sequence has been shown to alter the morphology of certain block copolymer assemblies in aqueous environments. Light and X-ray results indicate that the addition of the integrin-binding domain, RGDS, does not affect the size of the oleosin micelles (Figure 4.7 D-E).

Figure 4.7: Conformation of Oleosin-30-RGDS. A) SDS-PAGE shows >95% purity for Oleosin-30 (lane 1) and Oleosin-30-RGDS (lane 2). B) A pyrene assay indicates no change in the CMS with the addition of the RGDS tag. C) MALDI-TOF confirms the molecular weight of the mutant. Expected: 14924 Da, measured: 14915 Da. D) CD spectra shows no change in secondary structure with the addition of the peptide. Dynamic light scattering (E) and SAXS (F) show no change in size of the micelles. Analysis indicates that the addition of RGDS does not affect micelle assembly.
Cellular uptake was analyzed in OVCAR-5 cells, a human epithelial carcinoma cell line of the ovary. Labeled micelles were delivered at a concentration above the CMC and allowed to incubate with the cells for varying periods of time. The addition of RGDS increased the cellular uptake of the micelles by a factor of 2.4 after 3 hours of incubation and 2.9 after 12 hours of incubation, compared to micelles made without the binding peptide (Figure 4.8). The protein concentration in cells was calculated with a calibration curve correlating the fluorescence intensity to the protein concentration (Figure 4.9). This example shows the simplicity of precisely controlling the chemistry of the protein backbone.

Figure 4.8: Top: Block diagram of the primary sequence of oleosin-30 and oleosin-30-RGDS. The 6-histidine tag is cloned before the RGDS ligand to allow for terminal display of the peptide. Bottom: The addition of the RGDS tag resulted in an increase in cellular uptake by 2.4 times after 3 hours and 2.9 times after 12 hours of incubation. Fluorescence is directly correlated to protein concentration through a calibration curve. *p=3.2E-4, **p=1.6E-5.
Figure 4.9: Calibration curves used to correlate the fluorescence intensity of the lysed Ovcar-5 cells to the amount of protein per cell. Both curves showed linear behavior at low concentration.

4.4 Conclusions

We report the creation of a soluble protein surfactant by mutating the naturally occurring protein oleosin. The oleosin variants are expressed in high quantity and are simply purified. By removing 65% of the hydrophobic domain, the helical secondary structure has effectively been abolished and the addition of five glycines into the hydrophobic block creates a random coil tri-block surfactant protein. These mutants self-assembles into spherical micelles in solution above a critical concentration. Mutating multiple
charges into the hydrophilic arms can create ionic assemblies. It was found that the secondary structure of the protein directly affected the concentration at which assemblies form. This finding could lead to structure driven assembly at controlled concentrations. The base protein was functionalized through molecular biology to add the integrin-binding domain, RGDS, to the C-terminus. This addition did not affect the CMC or the morphology of the micelles. The RGDS mutant displayed a significant increase in uptake after 12 hours of incubation with Ovcar-5 cells. Oleosin surfactants present a promising platform for micellar assembly because of the ability to precisely modify the protein backbone through molecular biology allowing for the control over CMC and addition of functional domains into the material.
4.5 References


Chapter 5

Oleosin Stabilized Microbubbles

The work presented in this chapter is submitted for publication with the following reference: F. E. Angilè, K. B. Vargo, C. M. Sehgal, D. A. Hammer, & D. Lee, Recombinant Protein-Stabilized Monodisperse Microbubbles With Tunable Size Using a Valve-based Microfluidic Device. Submitted for Publication 2014. A patent application on this technology is submitted.

Abstract

Microbubbles are used as contrast enhancing agents in ultrasound sonography and more recently have shown great potential as theranostic agents that enable both diagnostics and therapy. Conventional production methods lead to highly polydisperse microbubbles, which compromise the effectiveness of ultrasound imaging as well as of novel therapeutic approaches such as antivascular ultrasound therapy (AVUST). The inefficacy of polydisperse microbubbles in these applications is due to their inability to uniformly resonate under a given ultrasound frequency. In addition to the polydispersity of microbubbles, surfactants that are used to stabilize microbubbles have been limited to
biological molecules or synthetic materials that offer limited possibilities in modifying the shell properties of microbubbles and functionalizing microbubbles for therapeutic applications. In this work, we generate monodisperse gas microbubbles by using a microfluidic flow focusing method. This microfluidic device uses an air-actuated membrane valve that enables production of highly monodisperse sub–10 µm microbubbles with narrow size distribution. The size of microbubbles can be precisely tuned by dynamically changing the dimension of the channel using the valve. The microbubbles are stabilized by an amphiphilic protein, oleosin, which provides versatility in controlling the mechanical properties of the microbubble shell and adding specific ligands for targeted drug delivery applications via recombinant protein technology. We show that it is critical to control the composition of the stabilizing agents to enable formation of highly stable and monodisperse microbubbles that are echogenic under ultrasound insonation. Our protein-shelled microbubbles based on the combination of microfluidic generation and recombinant protein technology provide a promising platform for ultrasound theranostic applications.
5.1 Introduction

Ultrasound imaging is inexpensive, safe, and commonly used for diagnosis in soft tissue and vasculature.\textsuperscript{1} Microbubbles have been used as ultrasound contrast agents; their gaseous core is highly compressible, which greatly enhances the scattering of ultrasound increasing the signal.\textsuperscript{2} Commercially available microbubble contrast agents have a broad size distribution. Tuning the size distribution to a specific imaging application can enhance the effectiveness of these bubbles. Previous methods have been developed to created monodisperse microbubbles but these techniques lead to significant loss of bubbles limiting their commercial relevance.\textsuperscript{3,4} In addition to having broad size distributions, most microbubbles that are currently being developed use phospholipids, proteins, or polymers. Due to the synthesis methods, these materials are not easily modified to have targeting ligands on the microbubble surface or to enable the modulation of the rheological properties of the stabilizing shells. The current materials compromise the effectiveness of microbubbles in ultrasound imaging and novel theranostic approaches such as targeted drug delivery and antivascular ultrasound therapy (AVUST).\textsuperscript{5-11}

In this work, we present a method to create stable protein-shelled microbubbles using a microfluidic device. The Lee lab at the University of Pennsylvania has fabricated a flow focusing device that uses an air-actuated membrane valve. This valve enables the production of highly monodisperse sub–10 µm microbubbles. Although other studies have shown that monodispserse bubbles can be generated based on microfluidic
techniques, the size range of microbubbles that can be generated from such devices is somewhat limited. The air-actuated membrane valve enables precise control over the size of microbubbles while producing highly monodisperse microbubbles. To stabilize the microbubbles generated by the microfluidic technique, we developed a novel soluble mutant of the amphiphilic protein oleosin, oleosin-30G, discussed in Chapter 4. Unlike common proteins that have been used to stabilize microbubbles, oleosin provides versatility in controlling the mechanical properties of the microbubble shell and adding specific ligands for targeted drug delivery applications via recombinant protein technology. We demonstrate an example of such modularity by expressing and incorporating a eGFP-oleosin fusion into the microbubble shell. The Lee lab has demonstrated that careful tuning of the composition of the stabilizing agents is critical in the formation of highly stable and monodisperse microbubbles that are echogenic under ultrasound insonation.

The bubble work has been completed in collaboration with Francesco E. Angliè in Daeyeon Lee’s research group in the Department of Chemical and Biomolecular Engineering, University of Pennsylvania and Chandra M. Sehgal in the Department of Radiology, University of Pennsylvania School of Medicine. This work was a true collaboration where my contributions included engineering oleosin to stabilize the bubbles, creating fluorescent oleosin mutants, and imaging the bubbles under confocal microscopy. The Lee lab created the microfluidic devices, manufactured the bubbles, and characterized the bubbles over time. Dr. Sehgal was instrumental in measuring the echogenicity of the bubbles. The entire work will be presented for clarity and to dictate
the wide variety of applications of oleosin mutants but it should be clearly noted the contribution related to this thesis was the design, production, purification, and characterization of the oleosin surfactants used to stabilize the microbubbles.

5.2 Materials and Methods

Microfluidic Device Fabrication

Microfluidic flow focusing devices with expanding nozzle design (Figure 5.1) are fabricated using single layer soft lithography in poly(dimethylsiloxane) (PDMS).\textsuperscript{17,18} Negative photoresist SU-8 2010 (Microchem, Newton, MA), thinned to a 3:1 ratio with SU-8 developer, is spin coated onto a clean silicon wafers to a thickness of 5 µm and patterned to UV light through a transparency photomask (CAD/Art Service, Bandon, OR) using a Karl Suss MA4 Mask Aligner (SUSS MicroTec Inc., Sunnyvale, CA). To incorporate an air-actuated valve, we use single-layer membrane valves,\textsuperscript{19} which exist in the same plane as the microfluidic channel, allowing us to fabricate the entire microfluidic device in a single layer mold. Sylgard 184 poly(dimethylsiloxane) (Dow Corning, Midland, MI) is mixed with crosslinker (ratio 12:1), degassed thoroughly and poured onto the photoresist pattern, and cured for 1 hr at 65 °C to make the membrane highly compliant. The PDMS replica are peeled off the wafer and bonded to a PDMS membrane fabricated by spin coating PDMS on a glass slide after oxygen-plasma activation of both surfaces. Having a microchannel fully-enclosed in PDMS allows for more efficient use of the valve-membrane.
Gene creation and protein expression

The sunflower seed oleosin gene is provided as a gift from Dr. Beaudoin at Rothamsted Research, Hampshire, England. Multiple rounds of PCR are used to create the Oleosin-30G and eGFP-oleosin-30G. The following PCR primers were used to create the three domains, which were combined in a single PCR step: N-terminal hydrophilic S 5’ – AAGGAGATAGGATCCACCACAACCTACGACC – 3’, N-terminal hydrophilic AS 5’ – GCACCGAGAGCGAGCTTGCCGGGTGAGG – 3’, hydrophobic S 5’ – CCTCAACCGGCAAG CTGCTCTCGGTGC – 3’, hydrophobic AS 5’ – CCTTCACATAATCCCTCTGAAACCCGGTAACACC – 3’, C-terminal hydrophilic S 5’ – GGTGTTACCGGGTTTCAG AGGGATTATGTGAAGG – 3’, C-terminal hydrophilic AS 5’ – TATATGAATCTCGAGTTTCCCCCTTTTTTCG – 3’. The PCRs to create the hydrophilic portions were run with oleosin-30 as the template and the hydrophobic domain PRC was run with the following oligo as the template: 5’ – CTGCTCTCGGTGCACTCCGCTTGGTGTGTATAGGTTTCAGGCCCTGTATTGTCCAGGATGGTATAGGGCTTGCGGTTACCCCGGTTTCAG – 3’. PCR was used to create the eGFP mutants using the following primers: eGFP S 5’ – ATCGGTATACATATGGTGAGCAAGGGCGAGG – 3’ and eGFP AS 5’ – ATCTAAAATGGATCCCTTGTACAGCTCG – 3’ with pBamUK-eGFP as a template (see SI for sequences). The genes are inserted into the expression vector pBamUK, a pET series derivative constructed by the Duyne Laboratory (SOM, Penn). Mutants are confirmed through DNA sequencing prior to protein expression. pBamUK adds a 6-Histidine tag to the C-terminus of the protein for IMAC purification. Protein is expressed in the E. Coli strain BL21 DE3 (Stratagene) controlled by the lac promoter. Cultures are
grown at 37 °C in Luria Bertani (LB) with kanamycin (50 µg ml⁻¹) until OD₆₀₀ ≈ 0.7 – 0.9.

Protein expression is induced with isopropyl-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. Cells are harvested by centrifugation and cell pellets are frozen at −20 °C prior to purification.

Protein Purification and Characterization

B-PER protein extraction agent (Fisher Scientific) is used for protein purification.

Oleosin-30G is expressed in inclusion bodies whereas eGFP-oleosin-30G is expressed in the soluble fraction of the cell. Oleosin-30G is purified according to the B-PER protocol for inclusion bodies and eGFP-oleosin-30G is purified according to the protocol for soluble proteins. Briefly, pellets were resuspended in B-PER (30 ml B-PER per liter of culture) and DNAse was added to a final concentration of 1 µg/ml. The resuspended pellets were centrifuged at 15,000 g for 15 minutes. The oleosin-30G supernatant was discarded and the eGFP-oleosin-30G supernatant was applied to an equilibrated column and allowed to bind for >1 hour. The remaining inclusion body pellet of oleosin-30G was suspended in denaturing buffer (8M urea, 50 mM phosphate buffer, 300 mM NaCl). The solution was centrifuged at 15,000 g for 15 minutes and the supernatant was added to an equilibrated Ni-NTA column (Hispur Ni-NTA resin, Thermo Scientific). The denatured oleosin-30G was allowed to bind to the column for >1 hours and washed three times with denaturing wash buffer (denaturing buffer with 20 mM imidazole). Oleosin-30G refolding was accomplished by diluting the column 50 times with refolding buffer (50 mM phosphate buffer, 300 mM NaCl, 5% by volume glycerol, 4°C) and rocked at 4°C for >1 hr. Both mutants was washed extensively with wash buffer (50 mM phosphate
buffer, 300 mM NaCl, 20 mM imidazole) and eluted in fractions with elution buffer (50 mM phosphate buffer, 300 mM NaCl, 300 mM imidazole). The concentration of purified protein is measured with a Nano-Drop 1000 (Thermo Scientific). Buffer exchange is completed with dialysis. All analysis is completed in PBS unless otherwise noted.

To establish the purity of the proteins, SDS/PAGE gels are run on NuPAGE Novex 4–12 % Bis-Tris mini gels (Invitrogen) in MES buffer. The gel is stained with SimplyBlue SafeStain (Invitrogen) following electrophoresis. The gel is destained overnight in water and imaged with a Kodak Gel Logic 100 Imaging System. Protein molecular weight is confirmed with MALDI-TOF. Sample spots are created with 0.5 µl protein in 1x PBS and 0.5 µl saturated sinapinic acid solution (50/50 acetonitrile/water + 0.1 % TFE). Spectra are collected on an Ultraflextreme MALDI-TOF (Bruker, Billerica, MA) (see Figure S4 for eGFP spectra). To confirm the proteins structural composition, far-UV CD spectra are collected at 25 °C on an AVIV 410 spectrometer (AVIV Biomedical Inc.) using a 1 mm quartz cell. Protein concentration is 15 µM in 50 mM phosphate, 140 mM NaF. NaF is used to replace NaCl due to the strong absorbance of the Cl⁻ ion.

Microbubbles production and characterization

The liquid phase containing the shell material consists of oleosin-30G or a solution containing oleosin-30G and (PEO)$_{78}$–(PPO)$_{30}$–(PEO)$_{78}$ or (PEO)$_{100}$–(PPO)$_{65}$–(PEO)$_{100}$ diluted in phosphate buffered saline (PBS) (pH 7.2, Sigma-Aldrich, St Luis, MO, USA). The components were mixed together to the desired concentration. Microbubbles were
generated using liquid phases containing different combinations of the three components. The liquid phase consisting of oleosin and (PEO)$_m$-(PPO)$_m$-(PEO)$_n$ triblock copolymers, at the optimal concentration dispersed in PBS was supplied to the device using a syringe pump (Harvard Apparatus PHD Ultra) at flow rates between 500 µL h$^{-1}$ to 1000 µL h$^{-1}$. To connect the channels to syringes, polyethylene tubing with an inner diameter of 0.38 mm and an outer diameter of 1.09 mm (BB31695-PE/2, Scientific Commodities Inc, Lake Havasu City, AZ) was used. The gas phase consists of 99.999 % pure nitrogen gas (N$_2$, GTS Welco, Richmond, VA) or octafluorocyclobutane (C$_4$F$_8$) (SynQuest Laboratories, Alachua, FL) supplied to the device using a pressure regulator (Type 700, ControlAir Inc., Amhrest, NH) at pressures between 15 and 20 psi. Polyethylene tubing with an inner diameter of 0.86 mm and an outer diameter of 1.32 mm (BB31695-PE/5, Scientific Commodities Inc, Lake Havasu City, AZ) was used connect the channel to the pressure regulator. The membrane valve was actuated using a dual-valve pressure controller (PCD-100PSIG-D-PCV10, Alicat Scientific, Tucson, AZ) at pressure between 0 and 40 psi.

Microbubbles were produced by first applying a small pressure to the gas inlet (2–4 psi) immediately followed by injecting the liquid phase at the desired flow rate (500-1000 µl h$^{-1}$). The gas phase was increased slowly until steady state of bubble generation is reached. Images of microbubbles production were captured using an inverted microscope (Nikon Diaphot 300) connected to a high speed Phantom V7 camera. For microbubbles that remain stable during generation and collection, long term stability was characterized by collecting microbubbles at the air-water interface in 35 mm petri dishes, acquiring
images under an upright microscope (Carl Zeiss Axio Plan II) connected to a QImaging Retiga 2000R camera. Microbubbles diameter variation over time was measured and images are analyzed using ImageJ (v 1.47v, NIH, USA).

**Ultrasound Imaging**

Microbubbles for ultrasonic imaging were collected and imaged directly in 16 mm membrane dialysis bag, which is pre–filled with buffer solution and sealed at one end. After a desired amount of bubbles is collected, the tube was sealed at the other end carefully avoiding formations of air pockets. The collected microbubbles were imaged using a clinical ultrasound scanner HDI 5000 (Phillips/ATL, Bothell, WA, USA) which is equipped with a broadband high-frequency ultrasound transducer at 7–15 MHz. Gray scale B-mode images are acquired with a mechanical index (MI) of 0.37 and 0.47 with focus between 0.5–1.5 cm and 1–2 cm, respectively. Time gain compensation (TGC) was fixed throughout the experiments.

### 5.3 Results and Discussion

#### 5.3.1 Creation and optimization of the microfluidic device

For a variety of applications that involve microbubbles and ultrasound, the size distribution of microbubble agents drastically influences the efficacy of the image contrast enhancement and therapeutic methods. To enable formation of microbubbles with highly monodisperse and tunable size, the Lee lab developed an expanding nozzle flow-focusing microfluidic device with a single-layer membrane valve at the orifice as
schematically illustrated in Figure 5.1. This design provides the flexibility to tune the size of the microbubbles in the same chip without changing the continuous phase or gas flow rates, by only changing the size of the orifice through the application of pressure to the valve. Furthermore, the use of the single-layer membrane valve overcomes the low resolution that is typically achieved by using polymeric photomasks\(^{20}\) (smallest feature ~ 10 \(\mu\)m).

Figure 5.1: a) Schematic illustration of a PDMS microfluidic device used to generate monodisperse microbubbles of different sizes. b) Schematic of a microbubble stabilized with a mixture of oleosin-30G and (PEO)\(_n\)-(PPO)\(_m\)-(PEO)\(_n\) triblock copolymer.

Nitrogen gas and a common surfactant, sodium dodecyl sulfate (SDS, Sigma-Aldrich, St Luis, MO, USA), were used for the initial testing of the microfluidic device. Monodisperse microbubbles with radius ranging from approximately 2 to 10 \(\mu\)m are produced for several hours without changes in the bubble size. The advantage of this microfluidic device over previous flow-focusing devices is that a wide size distribution of
microbubbles can be generated from a single device. Increasing the applied pressure to the single-layer value restricts the area of the valve opening reducing the resulting bubble size. By altering the pressure, the size of the microbubbles can be controlled (Figure 5.2). It was found that the bubble size is linearly correlated to the width of the nozzle. Although SDS enables the investigation of microfluidic device performance, microbubbles formed using SDS are not stable upon collection.

Figure 5.2: (a1-a9) Series of images of the microfluidic device during the generation of microbubbles. By changing the size of the nozzle, which is controlled by an air-actuated valve placed at the orifice, it is possible to generate uniform microbubbles of different sizes. (b) Effect of orifice width on the size of microbubbles.

5.3.2 Oleosin stabilized bubbles

To produce stable microbubbles with high monodispersity, size tunability and structural
modularity, we turn our attention to oleosin as the bubble shell material. Oleosin is a plant protein that stabilizes oil bodies in seeds.\textsuperscript{22} The protein has a natural amphiphilic structure with N- and C- terminal hydrophilic arms and a central hydrophobic core containing a proline knot forcing the protein into a hairpin structure.\textsuperscript{22-27} Oleosin has been used in various biotechnology and biomedical applications exploiting its amphiphilic properties.\textsuperscript{28-32} Eliminating a large portion of the hydrophobic domain and removing the majority of the secondary structure in the protein backbone has been shown to yield a soluble oleosin mutant that naturally self-assembles into micelles\textsuperscript{19}. The soluble oleosin mutant is named oleosin-30 defining the number of amino acids in the hydrophobic block. The oleosin-30 mutant was modified by inserting five glycines into the hydrophobic core (see Chapter 4 for addition information) creating oleosin-30G. The addition of the five glycines increased the protein expression, stability, and solubility while completely abolishing the secondary structure as shown by circular dichroism (Figure 5.3). Protein is expressed in the \textit{Escherichia coli} strain BL21 (DE3) with isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) induction. Protein is purified using immobilized metal affinity chromatography through a 6-histidine tag on the C-terminus of the protein leading to highly purified products (Figure 5.3). Protein molecular weight is confirmed with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopy (Figure 5.3).
Figure 5.3: A and B) SDS-PAGE gel showing >95% purity for oleosin-30G and >90% purity for eGFP-oleosin-30G. C) MADLI-TOP spectra confirming the molecular weight for oleosin-30G (Expected: 15027, measured: 15025) D) Far UV CD spectra of oleosin-30G indicates a random coil structure. The addition of the five glycines in the hydrophobic block significantly increases the unstructured nature of the protein compared to the previously reported oleosin-30 (Chapter 4).

When microbubbles are produced with only oleosin at concentrations between 1–2 mg mL\(^{-1}\), we can only stabilize bubbles with radius above 10 µm. During the generation of microbubbles with radii smaller than 10 µm, bubbles are observed to coalesce. In addition, the relatively high surface tension between the liquid and the gas phases makes the generations of such microbubbles challenging, often resulting in unsteady formation of microbubbles in the microfluidic device.
A number of microbubble systems that are currently being investigated (e.g., phospholipid-stabilized microbubbles) often have an extra component such as the amphiphilic triblock copolymer Pluronic to provide stability to the microbubbles and to aid in the bubble generation process. We tested two different types of Pluronic triblock copolymers that have been studied: \((\text{PEO})_{100}-(\text{PPO})_{65}-(\text{PEO})_{100}\) and \((\text{PEO})_{78}-(\text{PPO})_{30}-(\text{PEO})_{78}\). When a mixture containing 1–2 mg mL\(^{-1}\) oleosin and 5–20 mg mL\(^{-1}\) \((\text{PEO})_{100}-(\text{PPO})_{65}-(\text{PEO})_{100}\) (average molecular weight 12600) was used, a consistent stream of monodisperse microbubbles were formed at the nozzle; however, these microbubbles undergo coalescence upon collection. In contrast, when \((\text{PEO})_{78}-(\text{PPO})_{30}-(\text{PEO})_{78}\) (average molecular weight 8400) is added to oleosin solutions, monodisperse microbubbles were generated at the nozzle and very limited coalescence was observed upon collection. The optimal concentration for stable microbubble formation was found to be an aqueous phase containing 1 mg mL\(^{-1}\) of oleosin and 10 mg mL\(^{-1}\) of \((\text{PEO})_{78}-(\text{PPO})_{30}-(\text{PEO})_{78}\) (Figure 5.4).
5.3.3 Microbubble stability

Microbubbles generated using the mixture of oleosin and \((\text{PEO})_{78}-(\text{PPO})_{30}-(\text{PEO})_{78}\) (molar ratio of oleosin:triblock copolymer = 1:18) were stable once collected. Microbubbles were collected and stored in water (microbubbles reside at the air-water interface due to their buoyancy). Microbubble radius decreases by about 13% during the first four days and eventually ceases to shrink further. These microbubbles remained stable for at least for 4 weeks (Figure 5.5). The stability of these microbubbles did not depend on whether \(\text{N}_2\) or \(\text{C}_4\text{F}_8\) is used as the gas phase. In contrast, microbubbles generated solely with \((\text{PEO})_{78}-(\text{PPO})_{30}-(\text{PEO})_{78}\) did not exhibit such excellent stability. These results indicate that oleosin plays a critical role in stabilizing the shell of microbubbles, which likely consists of a mixture of oleosin and \((\text{PEO})_{78}-(\text{PPO})_{30}-(\text{PEO})_{78}\), to prevent complete dissolution or coalescence of microbubbles upon their
collection. Similar examples, in which shells suppresses the dissolution of microbubbles, have been observed in microbubbles that have been stabilized with other types of proteins, nanoparticles or synthetic polymers\textsuperscript{18,33-52}.

![Micrographs showing microbubbles stability over time.](image)

Figure 5.5: Micrographs showing microbubbles stability over time. (a) Size of microbubbles over 7 days. Microscope images of microbubbles (b) upon collection, (c) 7 days after collection and (d) 24 days after collection

5.3.4 Functional microbubbles

As discussed briefly above, one of the unique aspects of oleosin is that the molecular structure and thus the properties of the monolayer that contains this molecule can be engineered using recombinant protein technology. Recombinant protein technology
allows for precise molecular engineering of proteins generated from microorganisms such as bacteria and thus can be used to generate oleosin species with different functionality and properties. To demonstrate proof-of-principle that this molecule has such modularity, we expressed a green fluorescent protein mutant oleosin by fusing enhanced green fluorescent protein (eGFP) to the N-terminus of the oleosin-30G. The modified oleosin genes were constructed using standard molecular biology techniques and cloned into the expression vector pBamUK. eGFP-functionalized oleosin was added to the aqueous phase during microbubble generation. It is evident that the microbubbles produced with the blend of the two oleosin species (pure at 1 mg mL$^{-1}$, mutant at 0.05 mg mL$^{-1}$) along with 10 mg mL$^{-1}$ (PEO)$_{78}$–(PPO)$_{30}$–(PEO)$_{78}$ had the eGFP mutant species incorporated in the bubble shell, whereas the microbubbles generated without the eGFP mutant species did not show any fluorescence (Figure 5.6). Fluorescence intensity was observed to be fairly uniform on the surface of the bubbles with no signs of phase separation, which had been observed on microbubbles that were stabilized with mixture of phospholipids. Our results clearly indicate that that oleosin with different functionalities can be generated and incorporated into the microbubble shell and that oleosin distributes uniformly on the surface of microbubbles.
5.3.5 Bubble Echogenicity

Echogenicity measurements were carried out using microbubbles generated with a solution containing 1 mg mL$^{-1}$ oleosin and 10 mg mL$^{-1}$ (PEO)$_{78}$–(PPO)$_{30}$–(PEO)$_{78}$. We collected microbubbles directly in a $\sim$3 cm long dialysis tubing with a diameter of 16 mm, which is sealed at one end and pre-filled with PBS solution containing 10 mg mL$^{-1}$ (PEO)$_{78}$–(PPO)$_{30}$–(PEO)$_{78}$. Microbubbles were flown directly into the dialysis tube from the PDMS device outlet using polyethylene tubing, which was submerged in the PBS.
solution. After collecting a desired amount of microbubbles, the tube was sealed on the other end to avoid introducing any air pockets and is stored in 50 mL centrifuge tubes filled with PBS solution containing 10 mg mL$^{-1}$ (PEO)$_{78}$–(PPO)$_{30}$–(PEO)$_{78}$. The tube was rotated at 60 rpm to induce continuous motions of the microbubbles and more importantly to remove large bubbles that may have been collected. The echogenicity of these microbubbles was tested using a broadband high-frequency ultrasound transducer at 7–15 MHz in brightness mode (B-mode). The microbubbles, with a radius of about 4 µm are acoustically active along the entire length of the dialysis tube as shown in Figure 5.7. In contrast, a PBS solution containing 10 mg mL$^{-1}$ (PEO)$_{78}$–(PPO)$_{30}$–(PEO)$_{78}$ without any microbubbles did not show any acoustic activity, indicating that the oleosin-stabilized microbubbles are echogenic. Microbubbles remain acoustically responsive 30 min after the initial measurement and even one week after the first measurement, showing non-detectable changes in the signal brightness. These results clearly indicate that these microbubbles stabilized with oleosin are highly stable and echogenic and have large potential for theranostic applications.
Figure 5.7: Ultrasound sonography images of C₄F₈ microbubbles generated with a solution containing 1 mg mL⁻¹ and 10 mg mL⁻¹ (PEO)₇₈–(PPO)₃₀–(PEO)₇₈. Ultrasound images of microbubbles (a and b) 1-2 hours after generation, (c and d) 30 minutes and (e and f) 7 days after initial imaging. Ultrasound images of control samples are reported in panels g and h.
5.4 Conclusions

We have shown that a recombinant mutant oleosin in combination with a triblock copolymer, \((\text{PEO})_{78}-(\text{PPO})_{30}-(\text{PEO})_{78}\), can be used to successfully produce stable and monodisperse microbubbles with high echogenicity. The Lee lab demonstrated that the use of a PDMS microfluidic device with an air-actuated valve is an effective method to control the size of microbubbles while maintaining narrow size distribution.

Microbubbles incorporating oleosin show high stability and can be further functionalized using recombinant protein technology, which we demonstrated by the incorporation of an eGFP-oleosin fusion mutant into microbubbles. We envisage that the combination of microfluidic generation and oleosin-based stabilization of microbubbles will represent a promising platform for ultrasound theranostic applications. In particular, by functionalizing oleosin with specific targeting ligands via recombinant protein techniques,\textsuperscript{31,32} it will be possible to enable localized antivascular ultrasound therapy. Also by varying the molecular structure of oleosin (e.g., controlling the structure of hydrophobic domain), microbubble shells with different rheological properties could be generated.
5.5 References


Chapter 6

Oleosin stabilized and targeted superparamagnetic iron oxide nanoparticles

The work presented in this chapter is submitted for publication with the following reference: K. B. Vargo, A. Al Zaki, R. Warden-Rothman, A. Tsourkas, & D. A. Hammer, Superparamagnetic Iron Oxide Nanoparticle-Loaded Micelles Stabilized by Recombinant Oleosin for Targeted Magnetic Resonance Imaging Submitted for Publication 2014. The technology has been disclosed to the University of Pennsylvania Center for Technology Transfer and is being evaluated for a patent application.

Abstract

Superparamagnetic iron oxide (SPIO) nanoparticles are used as magnetic resonance contrast agents in many research applications providing strong T\textsubscript{2} weighted contrast. The nanoparticles are typically clustered and stabilized by a polymer or lipid membrane to
increase circulation time. These systems offer little flexibility when engineering the particle surface for biological targeting or controlled degradation. In this work, we engineered the naturally occurring plant protein oleosin to stabilize SPIO nanoparticle emulsions, which eventually dry to packed nanocluster micelles. Oleosin was engineered to be soluble and highly anionic providing electrostatic repulsion between the particles and limited non-specific cellular uptake. The particles are monodisperse after purification, display high relaxivity, and show no toxicity to cells after 4 hours of incubation. The Her2/neu affibody was fused to the N-terminus of the oleosin variant and blended into the structures to provide cell specific targeting. Targeted micelles show increased uptake in Her2/neu+ cells and can be blocked through competitive inhibition with free affibody indicating targeting through the Her2/neu receptor. Including functionality into the shell occurs during particle formation negating the need for additional functionalization steps. The use of recombinant oleosin as a stabilizing shell for SPIO is a encouraging platform for targeted magnetic resonance imaging.
6.1 Introduction

Superparamagnetic iron oxide (SPIO) nanoparticles have gained interest for use as magnetic resonance contrast agents, with the ability to provide T$_2$ weighted contrast enhancement on MR imaging applications.$^{1-4}$ Their strong contrast enhancing capabilities have rendered them useful for molecular imaging applications with various targeting molecules being conjugated to the surfaces of SPIO nanoparticles.$^{5-8}$ These strategies have the potential to increase tumor accumulation, specificity, and therapeutic efficacy. The prerequisite for any targeted nanoparticle is the successful bioconjugation of ligands onto the nanoparticle surface. Many techniques to do so have low reaction efficiencies, require multiple conjugation steps, and often create products with poorly oriented antibodies. Developing recombinant proteins that can stabilize SPIO nanoparticles would allow for the functionalization of particles in the formulation step by directly modifying the protein through molecular biology.

We chose to engineer the naturally occurring surfactant protein oleosin.$^9$ Oleosin is expressed in plant seeds with the native function of stabilizing fat reservoirs called oil bodies. The protein consists of three domains, a central hydrophobic domain flanked by two hydrophilic arms on the C- and N- termini.$^{9,10}$ The protein resembles a hairpin structure with a proline knot embedded in the central hydrophobic domain that forces a 180° turn.$^{11}$ Recombinant oleosin has been exploited for its surfactant nature in many biotechnology applications.$^{12-17}$
We have previously engineered oleosin to self-assembly into vesicles, fibers, or sheets by creating a family of truncation mutants thereby varying the hydrophilic/hydrophobic ratio of the surfactant protein.\textsuperscript{18} Further truncations of the hydrophobic block have led to soluble oleosin mutants that spontaneously self-assemble in aqueous solution as a function of concentration.\textsuperscript{19} These proteins can be highly engineered for specific applications. We present here the engineering of oleosin mutants to stabilize and target iron oxide protein micelles for enhanced magnetic resonance imaging (Figure 6.1 A).

This work was in collaboration with Ajlan Al Zaki and Robert Warden-Rothman in Dr. Andrew Tsourkas’ lab. Ajlan synthesized the SPIO nanoparticles and completed the targeting studies. Rob helped with all the cell culture work and plating for all experiments. This has been a fascinating collaboration with both sides equally contributing to the final result.

6.2 Materials and Methods

\textit{Gene synthesis}

Oleosin-30G(-) was created from the template Oleosin-30G\textsuperscript{20} using sequential PCR steps with the following primers: 1S 5' –

\begin{verbatim}
GATCAGCATGATCAACACACCGGTGACCAGCTCACCCACCCACAGGACCAGCAACAAGGCCCCTCAACCGGCGAACTCGCTCTCGGTGCGACTCC -3', 2S 5' –
\end{verbatim}

\begin{verbatim}
AATTCAATAGGATCCGAAGCCACCACAACCAACCGACCAGCACCACCATGTCACCA
\end{verbatim}
CCACCCCAAACCCAAGATCAGCATGATCAACACACC – 3’, 1AS 5’ –
TATCTGCTGGCCAAAGTCTCGTTCTGTTCTGGCCCGTCTGCTCCCCACATCCT
GCAATTCCCCGTTACGTTTACCTGCACTTGGCCACTGAAACCCGGTAACACC – 3’, 2AS
5’ –
TTCTGCCCTTCTCCACCACCCCTGACACCTGACCTGGCCCTGGTCCTGCTCCCCACATCCT
TTCATGGGGCGTATGCTGTATCTGCTGGCCCTGCCAAGCTG – 3’, 3AS 5’ –
TTTATGAATTCGAGTGATCGATGCTGGTGGTGGTGGTGGTGGTGGTGGCTCCCTCTT
CGTTCTGCCCTTCTCGTTCCCACC – 3’. The Oleosin-30G(-) PCR product was cloned in the expression vector pBamUK. The Her2 affibody was amplified using the primers Her2 1S 5’ – GATGCGCAGGCGCCGAAAGGCGCGTGCCGCGTAGC – 3’, and
Her2 fusion AS 5’ – GTTTGTTGTTGATCTTTTTCGCCCTGC – 3’ and cloned into the vector pBamUK-Oleosin-30G to create pBamUK-Her2-Oleosin-30G. The gene for the expression of the Her2 affibody alone was created using the following primers: Her2
1S 5’ - GATGCGCAGGCGCCGAAAGGCGCGTGCCGCGTAGC – 3’, and Her2 AS
5’ – TAGATAATTCTCGAGTTTTCGCCCTGCGCATCG – 3’ and cloned into
pBamUK. pBamUK adds a 6-histidine tag onto the C-terminus of the protein to allow for immobilized metal affinity chromatography (IMAC).

Protein production and purification
Mutants were expressed under the control of the lac promoter in E. Coli (BL21 DE3, Stratagene). Cultures were grown until OD₆₀₀~0.7 and induced with isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1.0 mM (Fisher Scientific). Cells were pelleted at 5,000 rpm and frozen at -20 °C prior to purification. Oleosin-30G(-) and the
Her2 affibody were expressed solubly, whereas the fusion Her2-Oleosin-30G was expressed in inclusion bodies. The protein mutants were solubilized according to the B-PER protocol for soluble or insoluble proteins respectively. Unpurified protein solutions were added to Ni-NTA beds (Hispur Ni-NTA resin, Thermo Scientific) and allowed to bind to the column for 1 hour at room temperature. Protein was washed and eluted in fractions according to the Hispur protocol. Protein concentration was measured using a Nanodrop-1000 (Fisher Scientific). Buffer exchanges were completed with dialysis or with centrifugal filters (Amicon Ultra, 3 kDa, Millipore).

**SDS-PAGE**

SDS-PAGE gels were run in MES buffer with NuPAGE Novex 4–12% Bis-Tris mini gels (Invitrogen). After electrophoresis, the gels were stained with SimplyBlue Safestain (Invitrogen) and destained in water overnight. The resulting gel was imaged with a Kodak Gel Logic 100 Imaging station.

*Matrix Assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF)*

MALDI-TOF spectra were used to confirm the molecular weights of the mutants. Sample spots were created with 0.5 µl protein in 1x PBS and 0.5 µl saturated sinapinic acid solution (50/50 acetonitrile/water + 0.1% TFE). Spectra were collected on an Ultraflextreme MALDI-TOF (Bruker, Billerica, MA).

*Circular Dichroism (CD)*
Far-UV CD spectra were collected on an AVIV 410 spectrometer (AVIV Biomedical Inc.) at 25 °C in 1 mm quartz cuvettes. Protein concentration was 10 µM in 10 mM phosphate, 140 mM NaF due to the high signal from the Cl⁻ ion in PBS.

**SPIO synthesis**

Briefly, 0.6 mmol of Fe(CO)₅ dissolved in 0.3 mL of ortho-dichlorobenzene (ODCB) was rapidly injected into a hot solution containing 1.2 mL of ODCB and 0.6 mmol of dodecylamine (DDA). The resulting mixture was maintained at 180°C under aerobic conditions. During this process, the initial orange color of the solution gradually changes to slightly brownish black. After 9 hr., the resulting solution was cooled to room temperature and an approximately 3-fold volume excess of toluene was added to adjust the solubility of the nanocrystals. The nanoparticle solution was then centrifuged to remove nanoparticle aggregates. After adding ethanol into the remaining solution, resulting black flocculates were isolated by centrifugation.

**Nanoparticle assembly and purification**

FeO-oleosin micelles were synthesized using an oil-in-water emulsion and stabilized with oleosin-30G(-). FeO nanoparticles were dissolved in toluene at a concentration of 80 mg/ml. Protein stocks were diluted into sterile PBS to a concentration of 2 mg/ml. The FeO nanoparticles in toluene (50 µL) were directly injected to the protein solution and sonicated until a uniform emulsion was created and no visible iron aggregates existed. The emulsion was allowed to dry overnight at room temperature. The particles were purified using sequential centrifugation²¹. The solution was centrifuged at 380 RCF for

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10 minutes and large aggregates were removed in the pellet. The supernatant was centrifuged at 4646 RFC for 30 minutes and the resulting supernatant was removed. Two pellets exist from this spin, a soft soluble pellet, and a hard, insoluble pellet of aggregates. The soft pellet was removed and used for further studies. The nanoparticles were concentrated and solution exchanges were completed using centrifugal filters (Amicon Ultra, 50 kDa, Millipore).

Dynamic light scattering (DLS)
Dynamic light scattering of nanoparticle solutions was performed on samples in PBS using a Malvern Zetasizer Nano ZS (Westborough, Massachusetts). Each sample was run in triplicate.

Cryogenic transmission electron microscopy (Cryo-TEM)
Cryogenic transmission electron microscopy was performed at the University of Pennsylvania in the Nanoscale Characterization Facility (Philadelphia, PA). Lacey formvar/carbon grids (Ted Pella) were rinsed in chloroform to remove the formvar template. The resulting grids were carbon coated with a Quorum Q150T ES carbon coater (Quorum Technologies, United Kingdom). Grids were cleaned with hydrogen/oxygen plasma for 15 seconds using the Solarus Advanced Plasma System 950 (Gatan, Pleasanton, CA). A 2 µl drop of nanoparticles in PBS was deposited onto the grid and added to a Gatan Cp3 cryoplunger (Gatan, Pleasanton, CA). The samples were blotted by hand and plunged into liquid ethane. Grids were transferred to a Gatan CT3500TR cryoholder (Gatan, Pleasanton, CA) and immediately inserted into a JEOL
2100 HRTEM (JEOL, Tokyo, Japan) operating at 200 keV. Micrographs were imaged with an Orius SC200 digital camera.

*Stability*

Particles were incubated at 37°C for 5 days in either PBS or Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. DLS measurements were taken daily to monitor for particle degradation or aggregation.

*Cell Viability Assay*

The viability and proliferation of cells in the presence of Fe-Oleosin nanoparticles were evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. The assay was performed in triplicate in the following manner. NIH3T3 cells were seeded into 96-well plates at a density of $1 \times 10^4$ cells per well in 200 μL of media and grown overnight. The cells were then incubated with various concentrations of Fe-Oleosin (0, 0.025, 0.05, 0.075, 0.1, and 0.15 of Fe/mL) for 4 h. Following incubation, cells were incubated in media containing 0.1 mg/mL of MTT for 1 h. Thereafter, MTT solution was removed, and precipitated violet crystals were dissolved in 200 μL of DMSO. The absorbance was measured at 560 nm.

*Cell lines*

NIH/3T3 and T6-17 cells (i.e., NIH/3T3 cells engineered to stably express the Her2/neu receptor, kindly provided by Dr. Mark Greene, University of Pennsylvania) were
maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin at 37 °C, and 5% CO₂.

**Her2/neu targeting**

T6-17 and NIH/3T3 cells were incubated with 100 µg Fe/mL of Her2/neu-targeted SPIO micelles for 45 min in full media in triplicate. The media was removed and the cells were washed with PBS two times to remove any unbound micelles. Cells were trypsinized and counted. Cell suspensions were diluted to $0.4 \times 10^6$ cells/ml and $T_2$ relaxation times were measured using a bench top relaxometer (Bruker mq60).

### 6.3 Results and Discussion

#### 6.3.1 Protein design, expression, and characterization

Two oleosin genes were engineered, one to stabilize the FeO micelles and a second to target the resulting clusters to Her2/neu+ cells. Previously it has been shown that oleosin can be engineered to stabilize various interfaces such as emulsion droplets and bubbles.\(^{18,20}\) In order to provide adequate repulsion between the micelles, we mutated the hydrophilic arms of oleosin-30G to be negatively charged. Negative nanoparticles have also been shown to limit nonspecific cell targeting.\(^{22-24}\) Specifically, all positive amino acids as well as any tyrosine residues in the hydrophilic arms were mutated to Q, N, D, or E depending on the location and local charge. The negative charge was spread evenly across the hydrophilic arms with an average negative amino acid every six residues. This
mutant is called Oleosin-30G(-). To directly target Her2/neu+ cancer cells, we have fused a Her2/neu affibody onto the N-terminus of the oleosin mutant Oleosin-30G.\textsuperscript{25} This targeted mutant is named Her2/neu-Oleosin-30G. The Her2/neu affibody was expressed independently as a competitive inhibitor for cell studies. Mutants were made using standard molecular biology techniques and cloned into the expression vector pBamUK, which adds a 6-histine tag on the C-terminus of the protein for immobilized metal affinity chromatography (IMAC). Oleosin variants were confirmed through DNA sequencing. Vectors were transformed into the Escherichia coli strain BL21 (DE3) for expression. Her2/neu-Oleosin-30G was insoluble and expressed in inclusion bodies whereas Oleosin-30G(-) was soluble. Mutants were purified using IMAC.

Protein yields were \(~24\) mg, \(~80\) mg, and \(~65\) mg of purified protein per liter of culture for Her2/neu-Oleosin-30G, Oleosin-30G(-), and Her2/neu respectively. SDS-PAGE indicates highly purified products after IMAC (Figure 6.1 B). The band for Oleosin-30G(-) runs much higher than expected on the gel, likely due to its highly negative charge. Molecular weights were confirmed with MALDI-TOF (Figure 6.2) (Oleosin-30G(-) expected: 14956, measured: 14958, Her2/neu-Oleosin-30G: expected: 21714, measured: 21713, Her2/neu expected: 7771, measured: 7773).
Figure 6.1: A) Cartoon depiction of Her2/neu targeted iron oxide nanoparticle micelles stabilized by oleosin. B) Protein purity is accessed to be >95% pure by SDS-PAGE (lane 1: Oleosin-30G(-), lane 2: Her2/neu-Oleosin-30G, lane 3: Her2/neu affibody). C) Circular dichroism indicates an unordered structure for the charged mutant Oleosin-30G(-). D) CD spectra for the fusion Her2/neu-Oleosin-30G show contributions from the helical Her2/neu affibody and the unordered Oleosin-30G. E) CDSSTR analysis of CD spectra shows increased helical structure in the fusion compared to Oleosin-30G indicating that the affibody is likely folded on the N-terminus of the oleosin mutant.

Protein secondary structure was elucidated through circular dichroism. The parent molecule Oleosin-30G is a highly unordered protein. CD indicates that Oleosin-30G(-) remains unordered after the various mutations to they hydrophilic arms (Figure 6.1 C). The secondary structure of Her2/neu-Oleosin-30G was investigated to ensure correct affibody folding as a fusion partner. The Her2/neu affibody is a highly helical protein (Figure 6.1 D) and when fused to oleosin, the Her2/neu-Oleosin fusion displays structure from the helical affibody and the unordered oleosin backbone (Figure 6.1 D). The spectra were fit with the CDSSTR analysis method using Dichroweb (Figure 6.1 E). The analysis shows clear helical structure in the fusion protein indicating that the affibody is likely folded in the fusion.

6.3.2 SPIO-oleosin assembly and characterization

SPIO-oleosin micelles were assembled through an emulsion method. SPIO nanoparticles solubilized in toluene were injected into solutions of protein in PBS. The emulsion was
soincated and the toluene was allowed to evaporate overnight at room temperature. This led to a heterogeneous mixture of micelles. SPIO-Oleosin micelles were purified using stepwise centrifugation.\textsuperscript{21} Cryo-TEM of the various separation fractions indicates large aggregated particles are removed in pellet after low RCF spins and excess protein and small particles in the supernatant of the high RCF spins (Figure 6.3).

Figure 6.3: Cryo-TEM micrographs of the various fractions during purification. A) The hard, insoluble pellet after the high RCF spin shows large aggregates of particles stuck together. B) The soft, soluble pellet that is extracted and used for further studies shows individual nanoclusters. C and D) The supernatant after the high RCF contains excess protein (C) and small nanocluster or individual particles (D). All scale bars are 200 nm.

The mass ratio of the particles to the protein, the oil volume fraction, and the particle stabilization coat all play an important role in the formation of packed nanoclusters. The oil volume fraction and mass ratio of protein to iron was optimized. Previous studies used an oil volume fraction of 4.8\% for particle formation and a 4:4 ratio of nanoparticle to surfactant (mg:mg).\textsuperscript{21} We found that decreasing the volume fraction of
toluene in the emulsion to 1.2% and increasing the protein concentration greatly affected the resulting structures. The optimal particles were created by injecting 50 µl of toluene containing 4 mg of SPIO-DDA coated nanoparticle into a 4 ml solution of protein in PBS at a concentration of 2 mg/ml (Figure 6.4).

![Iron:Protein (mg:mg)](4:4 8:4)

**Figure 6.4:** Optimization of iron-to-protein ratio and oil volume fraction. Increasing the amount of surfactant and decreasing the volume of toluene used in the emulsification led to highly packed particles with little to no aggregates present after purification. Scale bars are all 200 nm.

Dynamic light scattering of the purified particles show a monodisperse population with an average hydrodynamic diameter of 113 nm (peak: 127 nm, PDI=0.104) (Figure 6.5 A). Purified particles were imaged using cryogenic transmission electron microscopy (Cryo-TEM) (Figure 6.5 B). The micrograph displays tightly packed iron oxide nanoparticles and no visible excess protein on the particles. Particles from three
independent batches were directly measured from micrographs and found to have an average diameter of 74 ± 33 nm (N=660 particles) (Figure 6.5 C). As expected, the average diameter measured in micrographs is less than the hydrodynamic diameter measured by DLS. The DLS data are skewed to higher diameters due to increased intensity of scattering from larger particles.

Figure 6.5: A) Dynamic light scattering reveals a monodisperse population of micelles with an average diameter of 113 nm (PDI=.104). B) Cryo-TEM micrograph of FeO micelles stabilized by Oleosin-30G(-) in PBS (pH 7.4). C) Particle size distribution measured directly from cryo-TEM images. The average particle size was found to be 74 ± 33 nm (Standard deviation of the mean, N=660 particles). This diameter is significantly lower than the hydrodynamic diameter from DLS due to the increased scattering from larger particles. D) Protein stabilized particles are stable over 5 days in buffer (1xPBS) and serum at 37°C as measured by DLS. E) Particles show high relaxivity with an $R_2$ value of 407.2 ± 4.0 mM$^{-1}$ s$^{-1}$. F) The $R_1$ value was found to be 4.47 ± 0.46 mM$^{-1}$ s$^{-1}$.

The surface charge of SPIO particles has been shown to have significant impact in the uptake by cells. Zeta potential measurements indicated a negative surface charge at of -
12.5 ± 1.7 mV. The negative charge is needed to provide repulsive electrostatic interactions between the emulsion droplets during particle formation reducing aggregation. The particles show long-term stability in buffer (1 x PBS) and serum with no significant change in the hydrodynamic diameter over 5 days at 37°C (Figure 6.5 D). The particles display extremely high relaxivity with an $R_2$ value of $407.2 \pm 4.0 \text{ s}^{-1} \text{mM}^{-1}$ and an $R_1$ value of $4.47 \pm 0.46 \text{ s}^{-1} \text{mM}^{-1}$ (Figure 6.5 E-F). The potential cytotoxicity of the nanoparticles was assessed using an MTT assay. Over all concentrations, cell viability remained above 97% for the 4-hour incubation with particles (Figure 6.6). The physical and magnetic properties of the oleosin-stabilized nanoparticles are summarized in Table 6.1.

![Bar chart showing cell viability over different iron concentrations](image)

**Figure 6.6:** Particles show no toxicity between 25 and 150 µM after 4 hours of incubation at 37°C with NIH/3T3 cells.
6.3.3 Her2 targeting

Her2/neu+ targeted micelles were created by blending Her2/neu-Oleosin with Oleosin-30G(-) at 10% by weight in the PBS solutions (0.8 mg Her2/neu-Oleosin-30G: 7.2 mg Oleosin-30G(-)). The micelles were prepared and purified in the same manor. The blending of the targeted mutant into the micelles did not change the size of the micelles as measured by DLS (Figure 6.7 A) or the stability of the particles over time (Figure 6.7 B). The surface charge of the particles remains negative but slightly increased to \(-10.7 \pm 0.8\) mV.

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<th>Table 6.1: Physical and magnetic properties of oleosin stabilized nanoparticles</th>
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Figure 6.7: Characterization of Her2+ functionalized nanoparticles. A) DLS spectra shows monodisperse particles with a peak at 131 nm (PDI=0.11) indicating that the Her2-Oleosin-30G blending into the micelles does not affect the overall size. B) Functionalized particles are stable in PBS and serum for up to 5 days at 37°C.
FeO micelles were incubated with Her2/neu- (NIH/3T3) and Her2/neu+ (T6-17) cells at a concentration of 100 µg/ml for 45 minutes. The T2 relaxation time for the NIH/3T3 cells showed no difference between negative control particles, targeted particles, or cells incubated without particles indicating little to no nonspecific binding. In the Her2/neu+ cell line, the cells incubated with the targeted particles show a significantly lower T2 relaxation time, consistent with the presence of SPIO, compared to cells with the negative control particles or cells incubated without particles. A competitive binding study was completed by adding excess Her2/neu affibody to the T6-17 cells before and during the incubation with the targeted particles. The affibody competition led to a significant increase in the T2 time (Figure 6.8). Therefore, these results provide clear evidence that Her2/neu oleosin micelles provide cell specific targeting.
Figure 6.8: Functional evaluation of the Her2/neu SPIO-oleosin micelles conjugates. SPIO-oleosin and Her2/neu-SPIO-oleosin were incubated with either Her2/neu-positive and Her2/neu-negative cells in the presence and absence of excess free affibody. Free affibody served as a competitive inhibitor to confirm specific binding of the Her2/neu receptor. Relaxivity measurements of cells incubated with SPIO-oleosin micelles or Her2/neu-SPIO-oleosin micelles were acquired.

6.4 Conclusions

This work demonstrates the engineering of the naturally occurring surfactant protein oleosin to stabilize and target FeO nanoparticle micelles to Her2/neu+ cells. The functionalization of these particles is trivial due to the ease of incorporating biologically relevant motifs into the coat protein through molecular biology. These particles are extremely stable and display high relaxivity. We envision oleosin stabilized nanoparticle
micelles will represent a promising platform for targeted enhanced imaging applications. Specifically, varying the surface charge and appending specific stealth ligands\textsuperscript{29} to the particles could engineer nanoparticle shells to be nontoxic and maintain long circulation times.
6.5 References


Chapter 7

Conclusions and Future Directions

7.1 Specific Aims

The research presented within this thesis shows that we are able to successfully engineering the surfactant protein oleosin to self-assemble into desired higher order structures and exploit its ability to stabilize interfaces to create novel biomaterials. The specific aims of this work were as follows:

1. Develop a recombinant protein system that self-assembles into bilayer vesicles
2. Engineer soluble recombinant surfactants from the base protein oleosin
3. Develop oleosin mutants to stabilize and target clinically relevant biomaterials
7.2 Specific Findings

7.2.1 Oleosin self-assembly through emulsion templating

Chapter 3 focuses on creating oleosin mutants that naturally self assemble into higher order structures, specifically bilayer vesicles. To achieve this, families of truncation mutants were created and tested for assembly. A family of truncation mutations where the hydrophobic domain was shorted by 22 amino acids and the hydrophilic armed were symmetrically truncated created four mutants with varying hydrophilic fractions and molecular weights that were found to assemble. Assembly was mediated through an emulsion template. The morphology of the self-assembled structures was a function of the protein geometry and the solution ionic strength. The four mutants in the -65- family form vesicles at physiological ionic strength and the membrane thickness of the vesicles was found to be a function of the overall molecular weight. Micron sized vesicles can be created using double emulsions as a template and the resulting structures can be duel labeled to prove the presence of a hydrophobic bilayer membrane. Although the role of secondary structure in the assembly is unclear, there were no changes in secondary structure across the mutants, indicating that it was not a factor in various morphologies observed. This work completes Aim 1 of the thesis.
7.2.2 Creation of soluble oleosin mutants

A major challenged faced in Chapter 3 of this work was the insoluble nature of many of the oleosin mutants. Thus, this work set out to engineer the molecule into a soluble form while maintaining the surfactant character needed for assembly. This was accomplished by removing 65% of the hydrophobic domain creating the oleosin-30 family. This protein was found to self-assemble into spherical micelles above a critical micelle concentration. The micelles were monodisperse with a diameter of about 20 nm. Oleosin-30 micelles were trivially targeted to Ovcar-5 cells displaying active integrins using the cell binding motif RGDS. Micelles with the RGDS peptide showed a 2.9 fold increase in internalization compared to the control micelles. This family of surfactants has been shown to be highly modular. For instance, mutants were created with additional flexibility in the hydrophobic backbone increasing the expression and stability of the molecule. Ionic surfactant were developed by point mutating specific charged amino acids into the hydrophilic arms. These mutants also self-assemble into spherical micelles. It was found that the assembly is a function of secondary structure and therefore could be modulated based on the structure of the protein backbone. This work, described in detail in Chapter 4, accomplishes the goal set out in Aim 2.

7.2.3 Applications of soluble protein mutants

Beyond self-assembly, the oleosin mutants developed in Chapter 4 can be used to stabilize various interfaces to create new functional biomaterials. In Chapters 5 and
6, we exploit the surfactant characteristics of the soluble mutants to create stable microbubbles and targeted iron oxide nanoparticle respectively. In the microbubble work, we collaborated with Daeyeon Lee’s lab at Penn to create monodisperse, stable microbubbles with diameters <10 μm. The bubbles were generated using an air actuated microfluidic device and stabilized with a mixture of oleosin-30G and pluronic. In this work, oleosins ability to stabilize the air-water interface was needed to provide long-term stability to the bubbles. A demonstration of functionalization was completed by fusing enhanced green fluorescent protein to the oleosin mutant and using this molecule to label the bubbles. Bubbles were shown to be echogenic and remained echogenic for a week after the initial measurement.

Chapter 6 of this work used oleosin to stabilize emulsions, which eventually dry to packed iron oxide nanoparticles. This work was completed in collaboration with Dr. Andrew Tsourkas’ lab. The emulsion based technique for creating these particles is suited perfectly for oleosin. A negative mutant was created to provide the need electrostatic repulsion to mitigate coalescence of the emulsion. The size, stability, charge, and magnetic properties of the particles were characterized. The particles show no toxicity in cells after 4 hour of incubation. A mutant was created to target cells that overexpress the Her2 receptor, a common overexpression in various cancers. Particles targeted with the Her2-Oleosin mutant showed significant uptake in Her2+ cells and the targeting could be blocked through competitive inhibition with the free affibody indicating that the targeting is through
the Her2 receptor. Chapters 5 and 6 show just two examples of the many applications that oleosin surfactants can be used for. This work completes Aim 3.

7.3 Future Work

Oleosin’s unique architecture and robust ability to express in bacteria allows for extensive future work to be conducted on the molecule. The number of mutations that can be imagined and created is almost endless. This section will outline specific ideas for future work with oleosin and other proteins.

7.3.1 Self-Assembly

In chapters 3 and 4 oleosin self-assembly was discussed. The insoluble mutants of oleosin hold promise for surfactant work due to their large hydrophobic domains. The full-length oleosin hydrophobic block (87 amino acids) was found to be too hydrophobic limiting expression in bacteria and making handling of the surfactant difficult. Future work on this family of mutants would first need to focus on increasing expression of the surfactant therefore the shorter -65- family of mutants should be explored. The -65- family discussed in Chapter 3 expresses extremely well and we have shown that it is amenable to mutations. In Chapter 4, we explored removing the secondary structure in oleosin and found that it aided in assembly. Removing the secondary structure in the hydrophobic block of the -65- family would allow for increased flexibility in the protein backbone, possibly increasing solubility and allowing for solution self-assembly. Replacing helix forming amino
acids with helix breaking amino acids such as glycine or alanine can complete this. Recent work has created soluble mutants from these large hydrophobic blocks by negatively charging the hydrophilic arms, which will be discussed later in this section.

The soluble mutants created (hydrophobic block 45 and 30) are currently the most promising mutants for future engineering. The 45 mutant expressed in lower quantities and is not as stable as the 30 mutant therefore it was not extensively studied in comparison to the oleosin-30 assembly work shown in Chapter 4. A family of mutants from the Oleosin-30 molecule was created by truncating the hydrophobic arms symmetrically in the hopes of decreasing the head group size allowing for the assembly of lower curvature structures. In all cases, the protein mutants were not stable over time. The shorter truncations (37-30-37 and 27-30-27) led to potentially promising structures as visualized by light microscopy but the structures would quickly aggregate. To mitigate this, one could add a few charged residues to the N- and C-termini to provide electrostatic repulsion between the structures.

Another interesting area to explore is controlling the CMC of the micelles assembled in Chapter 4. Controlling the CMC of these surfactants can be extremely useful for many applications. For drug delivery and imaging, lower CMCs are advantageous for stability when diluted into blood. Adding large hydrophobic amino acids (Phe or Trp) into the central core could lower the CMC of the oleosin-30
families. It was found that the CMC of the micelles directly correlated to the proteins ability to adopt a specific secondary structure therefore changes in the hydrophobic domain secondary structure could lead to control over the CMC. For interfacial work, it would be advantageous to design molecules with high CMCs. This would allow for quick adsorption to an interface without the need to deplete monomers from a micellar assembly. Increasing the disorder in the protein backbone or decreasing the hydrophobic nature of the central domain could increase the CMC.

Micelle forming oleosin mutants can be created with protease sites within. These sites will cleave the protein when the specific protease is present. This would provide the ability to control release in the presence of a specific protease. Mutants have been created where the N-terminal arm of oleosin-30 was truncated from 42 amino acids to 32, 22, or 11 amino acids with the protease site enterokinase (Figure 7.1). These mutants were used to stabilize emulsions and emulsion stability was monitored after protease addition. It was found that the emulsions remained stable after the protein was cleaved. Addition of protease sites on the C-terminal arm and in the hydrophobic block could help control the stability of the emulsions in the future. Also, binding domains could be placed directly after the protease site. The ligand site would be shielded from binding until reaction with a protease, which would expose the site and allow the micelle to bind. This could be useful for targeted delivery in the presence of a specific protease such as thrombin, which is present at the sites of clot formation.
Figure 7.1: A) Cartoon depiction of Oleosin-30 mutants with enterokinase domains added into the N-terminal hydrophilic arm. The enterokinase sites were added at different points in the arm to test if steric hindrances affect enzyme activity. B) SDS-PAGE gel showing the three mutants before and after cleavage. All mutants show almost 100% cleavage after reacting with the protease.

A recent goal of this work was to create helical hydrophobic blocks with highly charged hydrophilic head groups mimicking work from the Deming lab at UCLA\textsuperscript{2-5}. Deming and coworkers created polypeptide vesicles from diblock amphiphilic copolypeptides where the hydrophobic poly-leucine block was alpha helical and the hydrophilic poly-lysine was a random coil.\textsuperscript{4} To accomplish this, a family of negatively charged mutants was created with where both hydrophilic arms were highly charged and the hydrophobic blocks were either 87, 65, 45, or 30 amino
acids long. In all cases, the mutants were soluble. This family is the first example of mutants with an 87 or 65 hydrophobic block that is soluble. These mutants were expressed and purified and preliminary cryo-TEM studies show that they remain micellar. Additional work including CD should be conducted to ensure the helical nature of the 87 and 65 blocks remains and CMC calculations should be completed.

Blending of surfactants has lead to rich phase behavior in previous work.\textsuperscript{6-8} With the large families of oleosin mutants created, blending mutants with different hydrophobic fractions could lead to interesting self-assembled structures. Specifically, blending micelles forming mutants with highly charged soluble mutants could lead to various interesting assemblies.

A key feature of oleosin is the proline knot. The three conserved prolines are thought to allow the 180° turn in the peptide chain creating the hairpin like structure.\textsuperscript{9,10} Previous work has looked at removing the proline knot and focused on the ability of the protein to enter the endoplasmic reticulum and target oil bodies after the mutation.\textsuperscript{11} Preliminary work on mutation the proline knot has consisted of creating an oleosin-30 hydrophobic block with the prolines mutated to alanines (P2A mutants). The P2A mutant showed increased helical structure compared to the native 30 mutant (Figure 7.2). It is hypothesized that the mutations of the prolines will eliminate the kink in the hydrophobic block creating a linear triblock surfactant. Future work could focus on replacing the prolines with various
hydrophobic amino acids specifically glycine for flexibility or more hydrophobic amino acids (Leu, Phe, or Trp) to increase the nonpolar characteristics of the blocks.

Figure 7.2: CD spectra of Oleosin-30 and Oleosin-30 P2A. The mutation of the three prolines to alanines in the proline knot increases in helical and sheet structure compared to the Oleosin-30 mutant. This mutant could lead to interesting linear triblock surfactants.

7.3.2 Stabilizing interfaces

We have exploited oleosin for its ability to stabilize interfaces in the microbubble and SPIO micelle work discussed earlier. The robust nature of soluble oleosin mutants to stabilize interfaces leaves much future work to be explored. We have
been able to engineer an oleosin mutant to stabilize almost any interface. In many cases, oleosin not only stabilizes the interface but it can impart specific functionality to the surface such as charge or the presence of a ligand as we have demonstrated. I will specifically talk about future work related to oleosin-stabilized microbubbles and SPIO micelles but the real limitation in this section arising from the lack of applications. Future collaborations with surface scientists will greatly move this work forward to find novel applications for oleosin mutants.

The oleosin-stabilized microbubbles in Chapter 5 were stabilized with a mixture of pluronic and Oleosin-30G. It is hypothesized that the pluronic was needed to initially stabilize the bubbles generated in the microfluidic device and oleosin was needed for long-term stability. The concentration of oleosin used in the microfluidic device was much greater than the CMC of the mutant meaning the majority of the protein molecules were clustered into spherical micelles. The soluble monomers would quickly be depleted and the micelles would begin to disassemble in order maintain the equilibrium between the monomers in solution and those residing in micelles. It is imaged that this micelle disassembly is relatively slow leading to the inability to quickly stabilize the air water interface. This could be improved by designing oleosin mutants with higher CMCs to stabilize interfaces under flow such as the Oleosin-30G(+) mutant discussed in Chapter 4.

The obvious next step is to replace the eGFP-oleosin fusion with a targeted mutant and test if localized microbubble accumulation can be achieved. This is a
simple next step and can be completed with the cloning of one fusion. Another direction for this project is the addition of switches into the microbubble shell, specifically for degradation. To accomplish this, one could add protease sites throughout the protein backbone. It is hypothesized that the bubbles could be designed to degrade at a controlled rate depending on the frequency of the protease sites. Additionally, bubbles have been seen to stick to each at the air/water interface other implying some interaction between the shells. The addition of charge to the surface of the bubbles could help prevent aggregation.

It is desirable to control the mechanical properties of the bubbles to optimize the ultrasound response. This can be achieved through many of the same mutations discussed earlier. The addition of hydrogen bonding amino acids or cysteine to cross-link can be used to strengthen the membrane. Crosslinking can also be completed through lysines by adding amine-to-amine cross linkers post bubble formation. Another option is to use protein hydrophobic blocks with secondary structure. The block of the -65- family displays strong helical character, which has been known to have strong lateral packing. A recent mutant where the hydrophilic arms have been negatively charged, Oleosin-65(-), would be a good starting point to test this theory.

Current microbubbles are created in the clinic by mechanical shaking. Although this technique produces a polydisperse population of microbubbles, the bubbles perform well enough in the clinic to remain the standard of care. It could be
desired to make targeted oleosin bubbles through mechanical shaking. This would allow for quicker entry into commercialization due to the simple production. Many oleosin mutants will form stable bubbles upon shaking. The size and long-term stability of these mixtures has not been explored but remains an open area for future work. I hypothesize that a mixture of oleosins would work best regardless of the generation method used. There is a need for the development of mutants that quickly stabilize the interface to use in conjunction with long-term stability mutants.

SPIO-oleosin stabilized micelles discussed in Chapter 6 are promising for targeting imaging agents. Many oleosin variants were cloned before Oleosin-30G(-) was found to stabilize the interface of the particles. Three major improvements can be made on this system. The first is the stability of the particles during purification. The use of sequential centrifugation as a purification method led to significant loss of particles. A mechanically sensitive purification method should be developed or the protein shells should be strengthened through crosslinking. Second, the oleosin mutants were not able to stabilize oleic acid coated SPIO particles. This is a major disadvantage because the oleic acid coated particles are highly monodisperse and much larger, which would likely producing a micelle with higher relaxivity. A longer hydrophobic block might interact better with the oleic acid coated particles to provide stable micelles. Finally, we showed targeting but felt that the particle targeting could be enhanced. This is likely due to protein adsorption to the micelle shell.\textsuperscript{12,13} The accessibility and orientation of the Her2 affibody could be improved by adding a peptide linker in between the fusion. This would provide additional
freedom for the affibody on the shell and shield it from non-specific adsorption of serum proteins.

Initial tests were completed to investigate mutant oleosin’s ability to stabilize emulsions. Some interesting results were found for the hydrophobic families as well as the -30- family. The -87- and the -65- families were only soluble in an organic solvent mixture as discussed in Chapter 3. When protein solutions in organic solutions were injected in aqueous solution interesting multilayer emulsions formed (Figure 7.3 A-C). These emulsions were stable for short periods of time due to the evaporation of chloroform but in some cases, vesicle structures were formed in low yields after drying (Figure 7.3 D-E). Many of the emulsions buckled upon drying indicating that the protein layer was likely solid. High yield vesicles could be created with this method if the protein layer was engineered to be fluid allowing for the desorption of molecules upon drying. It is hypothesized that if these protein mixtures were added to a non-volatile oil in water emulsion, say silicone oil in water, the protein would stabilize the chloroform emulsion drops but eventually transfer to the silicone oil as the chloroform evaporated. This could be a reasonable means to use these highly hydrophobic, insoluble proteins to stabilize emulsions created from non-volatile oils in aqueous solutions.
Figure 7.3: Solutions of the -87- and -65- family in 1:2:7 ($\text{Na}_2\text{CO}_3$: chloroform: methanol) into PBS lead to multicompartement emulsions. A) 7-87-7. B) 17-65-17. C) 17-65-17 with Nile red in the injection solution. The dye partitions to the hydrophobic fractions of the emulsions indicating an aqueous core. Once dried, a small number of the emulsions dry to become protein shells. Confocal microscopy shows a clear shell structure in DIC (D) and the segregation of the hydrophobic dye Nile red into the shell in the fluorescence image (E).

The Oleosin-30 family has shown interested emulsion behavior as well.

When Oleosin-30 is added into a mixture of silicone oil and water and emulsified using a high sheer emulsifier, greater than 50% of the emulsion drops are water-in-oil-in-water emulsions ($W/O/W$) (Figure 7.4). The emulsions are stable for hours. In this configuration, Oleosin-30 must stabilize surfaces with inverse curvature. This type of work could be expanded to look at protein mixtures, possibly creating more stable double emulsions by including a smaller head group mutant to stabilize the interior and a larger head group mutant to stabilize the exterior.
Another interesting field is that of nanoemulsions. These could be developed for drug delivery or imaging or used in various material applications. Nanoemulsions are typically created from high-pressure emulsifiers. Future work could focus on creating nanoemulsions stabilized by oleosin mutants for targeted therapies or imaging.

7.3.3 Interfacial measurements

Seeing as oleosin is a surfactant, a wealth of information can be collected on its surface behavior. Initial experiments on oleosin-30 were conducted. Long-term Wilhelmy plate experiments produced results showing that as the protein bulk concentration increased, the surface pressure dropped until the surface was saturated. This occurred very close to the CMC measured with the pyrene assay.
Pendant drop measurements were conducted on oleosin-30 but issues with the drop volume over time precluded the collection of useful data. Kinetic and equilibrium surface pressure measurements should be calculated for oleosin-30 and the various mutants in the family. Modifications to these mutants can be studied to look at how they affect the ability for the surfactant proteins to adsorb to the interface and lower the surface pressure. Specifically, this work would be useful with microfluidic applications where it is unclear if the protein is diffusion limited or adsorption limited when stabilizing interfaces. This understanding could help design optimal protein mutants for different interfacial applications.

Figure 7.5: A) Kinetic surface pressure measurements of Oleosin-30 as a function of bulk concentration. Increasing the bulk concentration of protein increases the rate at which the surface pressure drops. B) Equilibrium surface pressure as a function of concentration. The equilibrium surface pressure drops as concentration is increased until the surface is saturated above 4 μM. This work was completed in collaboration with Dr. Marcello Cavallaro in Dr. Kathleen Stebe’s lab.
7.3.4 Diblock surfactants

Creating protein diblocks is a promising design for self-assembly due to the vast literature available for diblock copolymer assembly. Using simple molecular biology techniques, oleosin triblock structures can be truncated into a diblock structure by splitting the molecule in two. Every hydrophobic block created (87, 65, 45, and 30) has been further engineered into half-oleosins. Unfortunately, almost all mutants do not express in bacteria and the reason for limited expression is unclear. A single half-oleosin created from the full-length molecule, designated half-oleo3 (see Appendix 2 for sequence), expressed but was minimally soluble in aqueous solutions. Finding a family of half-oleosin molecules that expresses well could lead to very promising self-assembling protein structures and assembly could be controlled using the same variables as diblock copolymer assembly. A few ideas include increasing the length of the hydrophilic block past the native length, creating half-oleosin mutants from the proline mutations discussed above, and minimizing the length of the hydrophobic block but maximizing the hydrophobicity.

An alternate route is creating fusion proteins where the hydrophilic block is a polypeptide known to express in bacteria and the hydrophobic block is taken from the hydrophobic domain of oleosin. We have explored two hydrophilic blocks to fuse to oleosin domains, resilin-like polypeptides (RLP) and elastin-like polypeptides (ELP).\textsuperscript{15-18} RLPs are 15 amino acid repeat unit based on the naturally occurring insect protein resilin.\textsuperscript{19} Resilin is a highly elastic protein found in the
joints of insects, specifically dragonflies and grasshoppers and has been used extensively in biomedical applications.\textsuperscript{20} We have fused a trimer RLP to various oleosin blocks (22 and 34 amino acids long creating RLP\textsubscript{3}-Oleosin\textsubscript{22} and RLP\textsubscript{3}-Oleosin\textsubscript{34} respectively) (Figure 7.6 A). Unlike the half-oleosin mutants, RLP-oleosin diblock express well and are highly soluble. Mutants are purified with IMAC and display >95% purity measured by SDS-PAGE (Figure 7.6 B). Both mutants show similar secondary structure to that of RLP alone, with the longer oleosin mutant displaying more unordered structure (Figure 7.6 C).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image}
\caption{A) Schematic of the diblock protein surfactant family RLP-Oleosin. B) SDS-PAGE showing highly purified fusions of RLP\textsubscript{3}-OLE\textsubscript{22} and RLP\textsubscript{3}-OLE\textsubscript{34}. C) CD spectra indicate relatively unordered structures for the diblock surfactants similar to RLP repeats alone.}
\end{figure}
A pyrene assay indicates that a hydrophobic domain is created as the concentration of the diblock protein surfactants is increased (Figure 7.7 A). RLP$_3$-Oleosin$_{22}$ has a CMC of 27.3 μM and RLP$_3$-Oleosin$_{34}$ has a CMC of 12.5 μM. The CMC decreases as the length of the oleosin block increases due to larger hydrophobic driving forces. Dynamic light scattering indicated the both RLP-oleosin mutants likely assemble into spherical micelles with diameters of 18.4 (PDI=0.037) and 18.8 (PDI=0.016) for RLP$_3$-Oleosin$_{22}$ and RLP$_3$-Oleosin$_{34}$ respectively (Figure 7.7 B). Cryo-TEM was used to directly visualize the spherical micelles as seen in Figure 7.7 C-D.

Figure 7.7: A) Pyrene assay shows both RLP$_3$-Oleosin$_{22}$ and RLP$_3$-Oleosin$_{34}$ self assemble as a function of concentration. As the hydrophobic block is increased, the CMC decreases. B) Both mutants show monodisperse populations of aggregates with diameter of ~18 nm. C and D) Cryo-TEM micrographs of the spherical micelles assembled from the RLP-oleosin fusions.
With the formation of spherical micelles, it is hypothesized that two directions could be taken to obtain lower curvature assemblies based on the packing parameter: 1) shorten the RLP block or 2) lengthen the oleosin hydrophobic domain. We have tested RLP\textsubscript{1} blocks and found that the RLP\textsubscript{1}-oleosin fusions did not express well in bacteria likely due to the large asymmetric hydrophobic domain. RLP\textsubscript{2}-oleosin and RLP\textsubscript{6}-oleosin mutants have been cloned. The RLP\textsubscript{6} mutants have been found to express, but large quantities were not purified yet therefore assembly has not been tested. Initial next steps should include testing the expression of the RLP\textsubscript{2}-oleosin family and testing the assembly of RLP\textsubscript{6}-oleosin mutants. Extended future work should focus on increasing the overall molecular weight of the protein fusions. With such a short RLP domain now, we are limited in the ratios of RLP to oleosin that we can create due to expression issues. The RLP domain is providing solubility to the diblock, therefore increasing the length of the RLP would allow for longer, more hydrophobic oleosin domains to be fused. This could allow for all geometries needed to form various self-assembled structures.

Both blocks can also be engineered to include specific amino acids or peptide sequences to help dictate assembly. I will present a few ideas that could force and control the specific assembly. Adding tyrosines into the junction between the hydrophilic and hydrophobic block could lead to a strong hydrogen bond network at the junction point, possibly lowering the curvature of resulting structures. Adding a single cysteine at the junction point and allowing fusion mutants to crosslink into dimers effectively creating X shaped molecules could achieve a similar result. Many
dendrimer molecules that have been shown to assemble into vesicles have this architecture.\textsuperscript{21-23} The RLP sequence is not as amenable to mutations as the oleosin block. The 15 amino acid repeats are specific providing little control over changes to the chemistry. A specific tyrosine in the native protein found in the repeated sequence YGAP has been mutated.\textsuperscript{24} In our RLP-oleosin mutants, the Try has been mutated to at Lys for potential crosslinking. The added charge of the lysine could cause electrostatic repulsion in the head group limiting the assembly of low curvature structures. Future work should focus on mutating this domain back to Tyr as well as exploring other possible amino acids such as Thr to induce hydrogen bonding in the hydrophilic block. The diblock RLP-oleosin system is a promising family of molecules for the self-assembly of a wide range of suprastructures.

Another hydrophilic polypeptide family that we recently explored as a fusion partner is elastin like polypeptides (ELP). ELPs are repeats of the amino acid sequence VPGXG, where X is any guest residue other than proline.\textsuperscript{17} These polypeptide sequences are discussed in detail in the background of Chapter 4. We have used the ELP sequence (VPGAG)\textsubscript{x} where x = 40, 80, or 160 repeats. We have fused these to three different oleosin hydrophobic domains: a half-oleosin domain, the hydrophobic domain of Oleosin-30G, and the Oleosin-30G domain with a 6-His tag on the end. These mutants were purified using inverse transition cycling and lyophilized.\textsuperscript{17} Interestingly, we found that the ELP\textsubscript{40}-Oleo-his assembled into high aspect ratio worm like micelles (Figure 7.8). The remaining mutants remain to be characterized. These fusions have major impact due to the high expression, simple
purification, and temperature switching. Future work should focus on fusing longer hydrophobic domains to the ELPs and experimenting with secondary structure in the hydrophobic block to impact packing. Adding cross-linking sites or coil-coil domains could be useful in controlling packing. Also, tuning the transition temperature closer to body temperature would be needed to created more biologically relevant constructs.

Figure 7.8: Cryo-TEM micrographs of the worm-like micelle assemblies of the ELP-oleosin fusion (VPGAG)40-Oleo-his. Micrographs show the formation of high aspect ratio worm-like micelles. All samples are in water at 150 μM. Scale bars: 200 nm.

7.3.5 Blending protein with lipids and polymers

An interesting prospect for oleosin mutants is the opportunity to blend with lipids and/or polymers. Proteins have been blended into lipid and polymer membranes to provide biological functionality to the resulting structures.25,26 The rich phase behavior of lipids and polymers is well known but the ability to functionalize these
materials is difficult and usually requires additional reactions post assembly. Therefore, if one could engineering an oleosin mutant to directly interact with these systems, functionality could be easily incorporated. For instance, if a soluble oleosin mutant was created that naturally blended into polymersomes, one could imagine making polymersomes that are targeted to any cell type simply by fusing the target ligand to the oleosin. For blending into membranes created by thin film rehydration, a mutant would need to be expressed that is stable at higher temperatures for the rehydration process. It is unclear which family of mutants would offer the best opportunity for success, therefore pilot experiments should be completed looking at a wide range of mutants before experimenting with a specific family.

Blending into membranes can also be achieved through microfluidics. Polymersomes have been created through microfluidics.\textsuperscript{27} Initial studies have shown that oleosin does interact with the membrane forcing large changes in the curvature of the protein. This membrane interaction is not limited to oleosin. BSA and green fluorescent protein (GFP) have been shown to interact with the membrane as well. BSA has some known surfactant character, but GFP is considered a very hydrophilic protein.\textsuperscript{28,29} It is hypothesized that the protein is driven to the interface and then trapped in the PEG brush of the polymersome as the organic solvent evaporates. Designing an oleosin mutant that will intercalate into the membrane without altering the membrane properties would allow for simple functionalization.
Figure 7.9: A and B) DIC images of microfluidic vesicles with Oleosin-30 encapsulated within the interior. The protein clearly interacts with the polymer to force sharp, high curvature turns in the membrane. C and D) DIC and fluorescent images of the encapsulation of eGFP within a microfluidic vesicle. The inclusion of eGFP does not affect the shape of the membrane as observed with Oleosin-30. E and F) DIC and fluorescent images of microfluidic vesicles created with eGFP in the outer phase. eGFP clearly intercalates into the outside membrane and visibly affects the structure of the shell. The mechanism for eGFP interaction with the membrane is unknown.
7.5 References


## Appendix 1: DNA Primers

<table>
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<tr>
<th>Sequence Name</th>
<th>Purpose</th>
<th>Sequence</th>
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<tr>
<td>HA_OLEO_S_P1</td>
<td>S primer for the N-terminus of WT-oleo</td>
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<tr>
<td>GC_OLEO_AS_P2</td>
<td>AS primer for the C-terminus of WT-oleo</td>
<td>TTTCTCACCTCGAGTTTCCCCCCTTTTTTCGCC</td>
</tr>
<tr>
<td>O phobic S</td>
<td>Equivalent to S25. Cut the hydrophilic N-terminal arm down to 25 amino acids</td>
<td>AAGGAGATACATATGCACATGATCAACAC</td>
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<tr>
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<td>WT-oleosin primer on the end of the N-terminal hydrophilic arm</td>
<td>AGATATACCATATGGCCACCACAACCTACGACC</td>
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<tr>
<td>Ophilic 2 S</td>
<td>2nd sense primer to remove hydrophobic amino acids to make -65. Does not prime</td>
<td>CAATAACCGGAATCTTGT TG</td>
</tr>
<tr>
<td>Ophilic 3 S</td>
<td>3rd sense primer to remove hydrophobic amino acids to make -65. Used with oleophi-1AS</td>
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</tr>
<tr>
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<td>WT-oleosin primer on the end of the C-terminal hydrophilic arm. Used with oleophi-3S</td>
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</tr>
<tr>
<td>Ophilic 3 AS</td>
<td>3rd antisense primer to remove hydrophobic amino acids to make -65. Does not prime</td>
<td>TGTTCTAAGGCCAATACTCCCCG</td>
</tr>
<tr>
<td>Ophilic 2 S New</td>
<td>2nd sense primer to remove hydrophobic amino acids to make -65. Used with oleophi 2AS</td>
<td>AAGATAACCGGAATCTTGGTTG</td>
</tr>
<tr>
<td>Ophilic 2 AS New</td>
<td>2nd antisense primer to remove hydrophobic amino acids to make -65. Used with oleophi 2S</td>
<td>AATCCCTCTGGACTGGAACCGACATTGTGACCC</td>
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<tr>
<td>Ophilic 3 AS New</td>
<td>3rd antisense primer to remove hydrophobic amino acids to make -65.</td>
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191
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<th>Primer</th>
<th>Description</th>
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<td>Oleo-pho2-S</td>
<td>S primer to created shorter version of Oleopho. Does not prime</td>
<td>AAGGAGATACATATGACCGGTGACAGACTCACCC</td>
</tr>
<tr>
<td>Oleo-pho2-AS</td>
<td>AS primer to created shorter version of Oleopho</td>
<td>TATATGAATCTCGAGGCCGTATACTCCCCACATCC</td>
</tr>
<tr>
<td>oleo_pho2_S2</td>
<td>New oleopho2 S primer (first one bad)</td>
<td>TACTGATGCCCATATGACCGGTGACAGACTCACCC</td>
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<td>Oleosin-S7</td>
<td>Cuts the N-terminal domain to 7 amino acids</td>
<td>AAGGAGATAGGATCCCCCTCAACCGGCAAGATAA TGG</td>
</tr>
<tr>
<td>Oleosin-AS7</td>
<td>Cuts the C-terminal domain to 7 amino acids</td>
<td>TATATGAATCTCGAGCACATAATCCCTCTGG</td>
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<tr>
<td>Oleosin-S12</td>
<td>Cuts the N-terminal domain to 12 amino acids</td>
<td>AAGGAGATAGGATCCCCGCCCCAGCAACAAGG</td>
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<tr>
<td>Oleosin-AS12</td>
<td>Cuts the C-terminal domain to 12 amino acids</td>
<td>AAGGAGATAGGATCCCCGCCCCAGCAACAAGG</td>
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<td>Oleosin-S17</td>
<td>Cuts the N-terminal domain to 17 amino acids</td>
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<tr>
<td>Oleosin-AS17</td>
<td>Cuts the C-terminal domain to 17 amino acids</td>
<td>TATATGAATCTCGAGATACTCCCCACCATCC</td>
</tr>
<tr>
<td>Oleosin-S37</td>
<td>Cuts the N-terminal domain to 37 amino acids</td>
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<tr>
<td>Oleosin-AS37</td>
<td>Cuts the C-terminal domain to 37 amino acids</td>
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<td>Cuts the N-terminal domain to 42 amino acids</td>
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<tr>
<td>Oleosin-AS32</td>
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<td>Oleosin-S22</td>
<td>Cuts the N-terminal domain to 22 amino acids</td>
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<td>Oleosin-AS22</td>
<td>Cuts the C-terminal domain to 22 amino acids</td>
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<td>Cuts the N-terminal domain to 27 amino acids</td>
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<td>Oleopho2-4S</td>
<td>4th sense primer for Oleopho. Primers used to create Oleo2. This protein</td>
<td>AAGGAGATACATATGACCGGTGACAGACTCACCC ACCCACAGC GCA AC</td>
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<tr>
<td>Oleo2 - 2S</td>
<td>Used with Oleo2 - 2S</td>
<td>TACCGGTTATGTCATGCGCTTTACCTTCC</td>
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<tr>
<td>Oleo2 - 3S</td>
<td>Used with Oleo2 - 2S</td>
<td>ATCACCCCTCGCCAGAAAGACGAAG</td>
</tr>
<tr>
<td>Oleo2 - 1AS</td>
<td>Used with Oleo2 - 2S</td>
<td>ATGACCATAAACC CGGTAACCCGCAAGC</td>
</tr>
<tr>
<td>Oleo2 - 2AS</td>
<td>Used with Oleo2 - 2S</td>
<td>TTCTGCAGCGGTGATACCGGCTAAACC</td>
</tr>
<tr>
<td>Oleo2 - 3AS</td>
<td>Used with Oleo2 - 2S</td>
<td>TATATGAATCTCGAGTTTCCCCCCTTCTTTTCG</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>0-87-0 S</td>
<td>Cut the N-terminal hydrophilic arm off (only good for -87-)</td>
<td>AAGGAGATAGGATCCGTCATCATGGCCTTACTTC</td>
</tr>
<tr>
<td>0-87-0 AS</td>
<td>AS to cut off the C-terminal arm. No good.</td>
<td>TATATGAATCTCGAGTCGAGACCCGACATTGTCG</td>
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<tr>
<td>0-87-0 AS 2</td>
<td>Replaces previous 0-870AS (only good for -87-)</td>
<td>TATATGAATCTCGAGAAACCCGACATTGTCGACC</td>
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<td>oleo2 2S</td>
<td>Replacements for oleo2 - 2S primer</td>
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<td>oleo2 3S</td>
<td>Replacements for oleo2 - 3S primer</td>
<td>TCGGCCAGAAGACGAAGG</td>
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<td>oleo1 2AS</td>
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<td>ATGATGACCATAAACCAGGTAACCGCAAGC</td>
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<td>S3</td>
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<td>Cuts the N-terminal hydrophobic domain to 11 (-65-)</td>
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<td>Creation of -30-hydrophobic domain</td>
<td>AACCAGGAAGGCTCGCTCAGGACTCC</td>
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<td>30phi-3AS</td>
<td>Used with 30phi-2S to make -30- hydrophobic block</td>
<td>AGAGCGAGCTTGCCGAGGAG</td>
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<tr>
<td>30phi-3S</td>
<td>Used with 30phi-2S to make -30- hydrophobic block</td>
<td>TACCCGGTTTCAGAGGAGATATGTAAGG</td>
</tr>
<tr>
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<td>Used with 30phi-2S to make -30- hydrophobic block. No good</td>
<td>ATCCCTCTGAAACCCGCTAAACC</td>
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<tr>
<td>oleo30 2AS</td>
<td>Replaces 30phi-2AS</td>
<td>CATAATCCCTCAGAAACCCGCTAAACCAGC</td>
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<td>GAGGGTGATCTTGCCGTTGAGG</td>
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<td>CATAATCCCTCAGAAGATCACCCCTCGTCG</td>
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45- hydrophobic block
-45-3S Primers used to create -45- hydrophobic block GGTACGTTCCAGAGGGATTATG
-30-G 1AS Primers to create -30-G GCACCGAGAGCGAGCTTGCCGGTTGAGG
-30-G 2S Primers to create -30-G CCTCAACCAGCAAGCTCGCTCGGTGC
-30-G 2AS Primers to create -30-G CTTTCACATACTCCTGAAAACCGGTAAACCC
-30-G 3S Primers to create -30-G GGTGTACCAGGGTTTCAGAGGGATTATGG
30mutant4S Change restriction site to BamHI for Oleosin-30 TTGTCTTATGGATCCTGGACCACAACCTAG
-30- RGD S Add RGD tag onto the N-terminus of oleo arm ATATGTTCATATGGCCGCAAGGCTCGGTGC
-30- DRG S Add DRG tag onto the N-terminus of oleo arm ATATGTTCATATGGACGCGGCGCTCGGTGC
-30- TAT 1S Add TAT tag onto the N-terminus of oleo arm AACGTCGCAGCTCGCTCGAGCGGCGGTGC
-30- TAT 2S Add TAT tag onto the N-terminus of oleo arm ATATGTTCATATGGTCTCGATCGTACGTCG
-35- 1AS Primers to create -35- GAGCCCGATACCGCTCCGGGTGAGG
-35- 2S Primers to create -35- TCAACCAGCAAGGCTCGCTCGGTGC
-35- 2AS Primers to create -35- TTCACATACTCCCTGACGACCCGCAGGCGGTGC
-35- 3S Primers to create -35- TTGGTGACTCAGAGGGATTATGG
P2A phob S Make -30-G-P2A block for RLP fusion AATATCTGTGGATCCTCGCTCTCGGTGC
P2A phob stop AS Make -30-G-P2A block for RLP fusion ACAGATATTCGCTAGTCAAACCCGTAACAC CC
P2A phob Y S Add a Y at the beginning of P2A block for RLP fusion AATATCTGTGGATCCTCGCTCTCGGTGC
30 P2G S Make -30-G-P2G block GGTGTTATAGGTTTCAGCGGTGGTTATTGTTGAGCGATGCGATGCGA CC
30 P2G AS Make -30-G-P2G block ACCGCTGAAACCTATAACACCAACAGCCCAGTC GCACCGAGGCA
30G2NS Adds 2 negative charges to the N-terminus arm AATATCTGTGGATCCTCTATGCTACGTCGCTCTCGGTGC
30G2NAS Adds 2 negative charges to the C-terminus arm CTCGAGTCAGTCATCGTGGGTTGGTGTTGGTGTTGG
30G4NS Adds 4 negative charges to the N-terminus arm AATATCTGTGGATCCATCGGACGAGTACGACACCAACCAACCCACGCAGC
30G4NAS Adds 4 negative charges to the C-terminus arm CTCGAGTCAGTCATCGTGGGTTGGTGTTGGTGTTGG

**Diblocks and Half Oleosins**

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<th>Purpose</th>
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<td>Primers/Terminator</td>
<td>Description</td>
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<td>HalfoleoCterm AS</td>
<td>Cuts the protein in half (AS primer) (protein didn't express)</td>
<td>TATATGAATCTCGAGCTTTGCGCGGAAGTCC</td>
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<tr>
<td>halfoleo3 - S</td>
<td>S primer for 1/2 oleo3</td>
<td>GATATCCCAACCAACCCACACCCACCACGCCCACCAACACCTACG</td>
</tr>
<tr>
<td>halfoleo3 - AS</td>
<td>AS primer for above</td>
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</tr>
<tr>
<td>HalIO AS W stop</td>
<td>Diblock half oleosin with stop codon</td>
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<td>halfole3S2</td>
<td>S for half oleo with proline knot</td>
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<tr>
<td>halfole3AS2</td>
<td>AS for half oleo with proline knot</td>
<td>AGTCATCTACTCGAGTCACGCTATCATCGTGG</td>
</tr>
<tr>
<td>BU-S1</td>
<td>Build up primer 1 - Prime with BUAS1</td>
<td>CCAACAAGATTCTATGCGCTTGCAGCTG</td>
</tr>
<tr>
<td>BU-AS1</td>
<td>Build up AS primer 1 - prime with BU S1</td>
<td>GGATCCCGACCTATGCGCAGCTTGCAGCTG</td>
</tr>
<tr>
<td>BS-S2</td>
<td>Build up S primer 2 - prime with BU AS2</td>
<td>TTTTATCCATGGATCCTGCTCCTAGCT</td>
</tr>
<tr>
<td>BU-AS2</td>
<td>Build up AS primer 2 - prime with BU S2</td>
<td>TAGATGACTCTCGAGTCAGAAATATAACAAACAGCCGAGCCGAGAGCAGC</td>
</tr>
<tr>
<td>Hblock1S</td>
<td>Oligos for Pho1</td>
<td>TCGAGACCAGCAAAGCCGGCGGAAACCCACCAACCCAGCAGACCCGACCACAACCCGAAAACCCAGCAGACCCGAGCCGAGAGCAGC</td>
</tr>
<tr>
<td>Hblock1AS</td>
<td>Oligos for Pho1</td>
<td>TCGAGACCAGCAAAGCCGGCGGAAACCCACCAACCCAGCAGACCCGACCACAACCCGAAAACCCAGCAGACCCGAGCCGAGAGCAGC</td>
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<tr>
<td>Hblock2S</td>
<td>Oligos for Pho2</td>
<td>TTTTATCCATGGATCCTGCTCCTAGCT</td>
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<td>Hblock2AS</td>
<td>Oligos for Pho2</td>
<td>TTTTATCCATGGATCCTGCTCCTAGCT</td>
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<tr>
<td>Hblock3S</td>
<td>Primers for -30-G hydrophobic block (Pho3)</td>
<td>TTTTATCCATGGATCCTGCTCCTAGCTC</td>
</tr>
<tr>
<td>Hblock3AS</td>
<td>Primers for -30-G hydrophobic block (Pho3)</td>
<td>TTTTATCCATGGATCCTGCTCCTAGCTC</td>
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<tr>
<td>phil1S</td>
<td>Primers for Phi1</td>
<td>AAAATAGCATATAGCCAGCACACACCG</td>
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<td>phil1AS</td>
<td>Primers for Phi1</td>
<td>AAAATAGCATATAGCCAGCACACACCG</td>
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<td>phil2AS</td>
<td>Primers for Phi2</td>
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<tr>
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<td>Primer to add stop codon to Pho1</td>
<td>ATCTAAATTCTCGAGTCAACCCCGGCAAGCC</td>
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<td>pho2 AS stop</td>
<td>Primer to add stop codon to Pho2</td>
<td>ATCTAAATTCTCGAGTCAACCCCGGCAAGCC</td>
</tr>
<tr>
<td>Pho3 AS Stop</td>
<td>Primer to add stop codon to Pho3</td>
<td>ATCTAAATTCTCGAGTCAACCCCGGCAAGCC</td>
</tr>
<tr>
<td>Phi1 6His</td>
<td>Primers to add 6-his tag to Phi1</td>
<td>AAAATAGCATATAGCCAGCACACACCG</td>
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<table>
<thead>
<tr>
<th>Primers/Terminator</th>
<th>Description</th>
<th>Sequence</th>
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<tr>
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<td>Primer to add stop codon to Pho1</td>
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<td>Primer to add stop codon to Pho3</td>
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<tr>
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<tr>
<td>Phi1 6His</td>
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<td>AAAATAGCATATAGCCAGCACACACCG</td>
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<td>ATCTAAATTCTCGAGTCAACCCCGGCAAGCC</td>
</tr>
<tr>
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<td>Primer to add stop codon to Pho3</td>
<td>ATCTAAATTCTCGAGTCAACCCCGGCAAGCC</td>
</tr>
<tr>
<td>Phi1 6His</td>
<td>Primers to add 6-his tag to Phi1</td>
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<td>Primer to add stop codon to Pho1</td>
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</tr>
<tr>
<td>pho2 AS stop</td>
<td>Primer to add stop codon to Pho2</td>
<td>ATCTAAATTCTCGAGTCAACCCCGGCAAGCC</td>
</tr>
<tr>
<td>Pho3 AS Stop</td>
<td>Primer to add stop codon to Pho3</td>
<td>ATCTAAATTCTCGAGTCAACCCCGGCAAGCC</td>
</tr>
<tr>
<td>Phi1 6His</td>
<td>Primers to add 6-his tag to Phi1</td>
<td>AAAATAGCATATAGCCAGCACACACCG</td>
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**Phi2 6His**

Primers to add 6-his tag to Phi1

```
AATTAATGACATATGCACCACACCACACCAC
CACAGGGATTATGTGAAGG
```

**P2A Nhalf S**

Diblock of oleosin-30-G-P2A

```
ATATTACTAGGATCCAAAAACACCACCAC
CACCACACCACACACCTACG
```

**P2A Nhalf AS**

Diblock of oleosin-30-G-P2A

```
ATGATAATTCTGAGTACTGAAACCCGGT
AAACC
```

**P2A Chalf S**

Diblock of oleosin-30-G-P2A

```
ATATTACTAGGATCCAAAAAACACCAC
CACACCACACCACACCACCTACG
```

**P2A Chalf AS**

Diblock of oleosin-30-G-P2A

```
ATAATGTATCTCGAGTCATTTTTTGTGGTG
TTGTGGTTGTTTCCCCCTTTTTCTC
```

---

### Tags, fusions, and sequencing

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Purpose</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| Avitag S      | Oligo for Avitag. Missing site. Use ones labeled New | TACTGATGCCATATGGGTCTGAACGACATC
TTCGAGGTCGAGAAAAATAGC |
| Avitag AS     | Oligo for Avitag. Missing site. Use ones labeled New | TATATGATGATCGATCTTGACCATTACGTTAGCCAGTTCTGAGG |
| avitag 2S     | Sense primer to make avitag BamUK vector | TATGGGTCTGAAAACATCTTTCTCGAGGCTCAAAATAAGAG |
| avitag 2AS    | Antisense of above | GATCTTCTCGCGATAATTTCGAGTTTTTCTGAGGCTCA |
| T7 promoter   | S Primer for vector in colony PCR | TAATACGACTACTATAGGG |
| T7 Reverse    | AS Primer for vector in colony PCR | TATGCTAGTTATTGGCTCAG |
| S_TAT_Linker  | S TAT-linker for TAT-Bamuk | CAAATGATATGGGTCGAAATGACGACATC
TTCGAGGTCGAGAAAAATAGC |
| AS_TAT_Linker | AS tAT-linker for TAT-BamUK | CATTGAGGATCCACAACCAACCACACCAGAAAACATCTTTCTCGAGG |
| -30-W1S1      | Sense primer for Oleosin-30 Trp mutant 1 | GCTGGTGTGTATATGGGACGACCCATATAGGG |
| -30-W1AS2     | Antisense primer for Oleosin-30 Trp mutant 1 | GGAACAATAAACAGGGGCTCCATATAAACAAAGC |
| -30-W2S1      | Sense primer for Oleosin-30 Trp mutant 2 | GCTTGCGGTTACCAGGGGTGGCAGAGGGATATT |
| -30-W2AS2     | Antisense primer for Oleosin-30 Trp mutant 2 | CGCAGAACGACCCACACCAGAAAACATCTTTCTCGAGG |
| -30-W3AS      | Antisense primer for Oleosin-30 Trp mutant 3 | CCTTCACATAATCCCTCTGGCAGCCCGGTACCGACGC |
| S GFP         | Sense primer for eGFP | ATCGGTTACGATATGGGACGCAAGGGGAGC |
| AS GFP        | AS primer for eGFP (prime off RP vector) | TACTAGGATGATCGGATCGCTGACAGT |
| S linker      | Primers with AS linker to make (GGGGS) 3 with a 12 base pair overhang on the S side with eGFP | GGAACAGGCGTACGCGCCGAGGCGGAGGTAGG |
| AS linker     | Primers with S linker to make (GGGGS)3 with BamHI on 3' | TACTAGGATGATGACGCGGAGCAGG |

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<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Cterm E11 S3</td>
<td>Primers to create 47E11-30-62</td>
<td>CTTGCAGGAGATGGACGACGACGACAAAGGGAGTATACG</td>
</tr>
<tr>
<td>Cterm E11 AS2</td>
<td>Primers to create 47E11-30-62</td>
<td>CGTATACTCCCCCTTTGTCGTCGTCCTCACACCTCC</td>
</tr>
<tr>
<td>her2 C2G S</td>
<td>Primers to create mutant C to G in Her2-linker</td>
<td>GGTTGGAAGTATACG</td>
</tr>
<tr>
<td>her2 C2G AS</td>
<td>Primers to create mutant C to G in Her2-linker</td>
<td>GCTACCCGCCACCGCCTTCGCGGCGCTGC GCATC</td>
</tr>
<tr>
<td>Her2AS no linker</td>
<td>Removing the linker attached to Her2</td>
<td>GGTTGTGGATCCTTCGCGGCTGC</td>
</tr>
<tr>
<td>30S her2 no linker</td>
<td>Removing the linker attached to Her2</td>
<td>GCAGGGCGCGAAAGGATCCACCAACCAACCC</td>
</tr>
<tr>
<td>TAT1S</td>
<td>Adding TAT to the N-terminus of oleosin</td>
<td>AACGTCTCGACGCCTCGTCGTCGCTCGCCAGGAT CCACACACAC</td>
</tr>
<tr>
<td>RGDS 1AS</td>
<td>Adding RGD after the 6-his tag on the C-terminus. Mistake: Actually DRG</td>
<td>ATCTGTGATGTTGAGTATCTCCCTCTCCCTTCGT</td>
</tr>
<tr>
<td>RGDS 2AS</td>
<td>Adding RGD after the 6-his tag on the C-terminus. Mistake: Actually DRG</td>
<td>TGAACAGATCTCGAGCTCGCAGGCAGATC CGTGATGGTGGATGGTG</td>
</tr>
<tr>
<td>RGD1ASnew</td>
<td>Adding RGD after the 6-his tag on the C-terminus</td>
<td>GCCGTGATGGTGGATGGTGTTTCCCCCGTC</td>
</tr>
<tr>
<td>RGD2ASnew</td>
<td>Adding RGD after the 6-his tag on the C-terminus</td>
<td>TGAACAGATCTCGAGCTCGCAGGCAGATC CGTGATGGTGGATGGTG</td>
</tr>
<tr>
<td>egfp Bam AS</td>
<td>Adds xho1 site onto C-terminus of eGFP</td>
<td>ATCTAAAAATTGGATCCCTTGTACGCTCG</td>
</tr>
<tr>
<td>Her2-nde1-S</td>
<td>Add nde1 site onto the N-terminus of Her2</td>
<td>TTAGATTCTCATAATGGTAGAATAAACAAATTAC</td>
</tr>
<tr>
<td>Her2-1AS</td>
<td>Add a linker onto the C-terminus of Her2</td>
<td>ACCAGACCGCCCACTCCGCTACGCGCACC GCCGATTTCCGCGCGCTCGC</td>
</tr>
<tr>
<td>Her2-2AS</td>
<td>Add a linker onto the C-terminus of Her2</td>
<td>AATTAGAGGATCCGCTACCGCCACCGCCCG AGCCACCCACACACACCC</td>
</tr>
<tr>
<td>47E32 1S</td>
<td>S primer to make one section of 47E32-30-62. Used with Oleosin Oleo2 3AS</td>
<td>AAATATGAAACATGACGACGACGACAAAGTC ACCACCCAC</td>
</tr>
<tr>
<td>47E32 2S</td>
<td>S primer to make one section of 47E32-30-62. Used with Oleosin Oleo2 3AS</td>
<td>TATATCTAGGATCCACCAACCAACCTACGACC GCCACCATGACGACGACGAC</td>
</tr>
<tr>
<td>47E22-30-62 1AS</td>
<td>AS primer to make one section of 47E22-30-62. Used with Oleosin S42</td>
<td>CCAATACGACGACGACGACGACAAAGGATA TAATTAGGT</td>
</tr>
<tr>
<td>47E22-30-62 S2</td>
<td>S primer to make one section of 47E22-30-62. Used with Oleosin Oleo2 3AS</td>
<td>AAATATCATGACGACGACGACGACAAAGGAT CAACACAC</td>
</tr>
<tr>
<td>47E12-30-62 1AS</td>
<td>AS primer to make one section of 47E12-30-62. Used with Oleosin S42</td>
<td>GGTGACGACGACGACGACGACGACGACGAC</td>
</tr>
<tr>
<td>47E12-30-62 S2</td>
<td>S primer to make one section of 47E12-30-62. Used with Oleosin Oleo2 3AS</td>
<td>AAATACGACGACGACGACGACGACGACGAC</td>
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## RLP fusions

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Purpose</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 phob S</td>
<td>Isolate -45- block for fusion to RLP3</td>
<td>AATATCTGTGGATCCATCACCCCTCGTCG</td>
</tr>
<tr>
<td>45 phob AS</td>
<td>Isolate -45- block for fusion to RLP3</td>
<td>ACAGATATTCTCGAGGAACGTACCCGAAG</td>
</tr>
<tr>
<td>65 phob S</td>
<td>Isolate -65- block for fusion to RLP3</td>
<td>AATATCTGTGGATCCATAACCGGAATC</td>
</tr>
<tr>
<td>65 phob AS</td>
<td>Isolate -65- block for fusion to RLP3</td>
<td>ACAGATATTCTCGAGACTGGAACCGAG</td>
</tr>
<tr>
<td>45phobAS stop</td>
<td>Add stop codon to end of -45- block</td>
<td>ACAGATATTCTCGAGTCAGAACGTACCCGAAG</td>
</tr>
<tr>
<td>65phobAS stop</td>
<td>Add stop codon to end of -65- block</td>
<td>ACAGATATTCTCGAGTCAGACTGGAACCGAG</td>
</tr>
<tr>
<td>RLP3-E-30- G S</td>
<td>Add entrokinase site between RLP3 and -30-G</td>
<td>TTTATCCATGGATCGACGACGACGACAAAA TGGCTCGCTCTCGGTGCT</td>
</tr>
<tr>
<td>-30-GF 1S</td>
<td>Primers to make hydrophobic block rich in G and F</td>
<td>TTCGCGACTCCGCTGTTTGGTGTTTTATAG GTTTCAGC</td>
</tr>
<tr>
<td>-30-GF 1AS</td>
<td>Primers to make hydrophobic block rich in G and F</td>
<td>AATAAACGCTATACCCATCGCGATGGAAC AATAACAGG</td>
</tr>
<tr>
<td>-30-GF 2S</td>
<td>Primers to make hydrophobic block rich in G and F</td>
<td>TTTATCCATGGATCCTTTCTCGCTCTCGGTGTTT CGCGACTCC</td>
</tr>
<tr>
<td>-30-GF 2AS</td>
<td>Primers to make hydrophobic block rich in G and F</td>
<td>TAACGAAACCCCGAAGCCAATAAACGCTA TACC</td>
</tr>
<tr>
<td>-30-GF 3AS</td>
<td>Primers to make hydrophobic block rich in G and F</td>
<td>GTAACTATACTCGAGTCAAACCCCGGTAAAC GAAACC</td>
</tr>
<tr>
<td>-65- phob W S</td>
<td>Add a W at the beginning of the hydrophobic block when fused to RLP</td>
<td>AATATCTGTGGATCCTGGATAACCCGAATCT</td>
</tr>
<tr>
<td>RLP1_S</td>
<td>Oligos to make RLP1 with KGAP</td>
<td>GATCTGGCGGCACCGCGTCTGATAGCAAG GGCACCGGCGGTGGTGTTGACG</td>
</tr>
<tr>
<td>RLP1_AS</td>
<td>Oligos to make RLP1 with KGAP</td>
<td>GATCCGTTACCGACCCCGGTCGGCTCTTTGC TATCAGACCGCGGCGGC</td>
</tr>
<tr>
<td>RLP1F_S</td>
<td>Oligos to make RLP1 with FGAP</td>
<td>GATCTGGCGGCGCCCGGTCGGCTCTTTGC TATCAGACCGCGGCGGCA</td>
</tr>
<tr>
<td>RLP1F_AS</td>
<td>Oligos to make RLP1 with FGAP</td>
<td>GATCCGTTACCGACCCCGGTCGGCTCTTTGC TATCAGACCGCGGCGGCA</td>
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</table>

## Charged Oleosin Mutants

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Purpose</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>30GminusW 1S</td>
<td>Primer to extend arms to make all anionic arms</td>
<td>GATCAGCATGATCAACACACCGGTGACCAG CTCACCCACACAGGACCAGCAACAAAGC CCCCACCCCGGAACCTCGCTCTCGGTGCG ACTCC</td>
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<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Description</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>30GminusW 2S</td>
<td>Primer to extend arms to make all anionic arms</td>
<td>AATTCAATAGGATCCGAAGCCACCACAAACCCACAAAGATCAGCATGATCAACACACCGGTACCTAGCCATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>30GminusW 1AS</td>
<td>Primer to extend arms to make all anionic arms</td>
<td>TATCTGCTGGCCCAAGTCTCGCTGTCTCTGGGCTGCTCAGTCTCCACATCTGCAATCCCCTGTCACGTTTTACCTGCAACCTGCAATGACACCA</td>
</tr>
<tr>
<td>30GminusW 2AS</td>
<td>Primer to extend arms to make all anionic arms</td>
<td>TTCTGGCTCTGCCCCACATCTGCAATCCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>GminusW 35S</td>
<td>Truncation of Oleosin-30-G-W</td>
<td>AATATTCTAGGATCCACACATGACCATGAAACCTGCAATGACACCA</td>
</tr>
<tr>
<td>GminusW 42AS</td>
<td>Truncation of Oleosin-30-G-W</td>
<td>TATCTGCTGGCCCAAGTCTCGCTGTCTCTGGGCTGCTCAGTCTCCACATCTGCAATCCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>GminusW 25S</td>
<td>Truncation of Oleosin-30-G-W</td>
<td>TTCTGGCTCTGCCCCACATCTGCAATCCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>GminusW 30AS</td>
<td>Truncation of Oleosin-30-G-W</td>
<td>TATCTGCTGGCCCAAGTCTCGCTGTCTCTGGGCTGCTCAGTCTCCACATCTGCAATCCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>87-W S</td>
<td>Primer off of the -87- hydrophobic core to make negative arms. Use the Oleosin-30-G-W primers to continue to extend the arms.</td>
<td>GATCAGCATGATCAACACACCGGTACCCACCTGCAATGAAACCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>87-W AS</td>
<td>Primer off of the -87- hydrophobic core to make negative arms. Use the Oleosin-30-G-W primers to continue to extend the arms.</td>
<td>TATCTGCTGGCCCAAGTCTCGCTGTCTCTGGGCTGCTCAGTCTCCACATCTGCAATCCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>65-W S</td>
<td>Primer off of the -65- hydrophobic core to make negative arms. Use the Oleosin-30-G-W primers to continue to extend the arms.</td>
<td>GATCAGCATGATCAACACACCGGTACCCACCTGCAATGAAACCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>65-W AS</td>
<td>Primer off of the -65- hydrophobic core to make negative arms. Use the Oleosin-30-G-W primers to continue to extend the arms.</td>
<td>TATCTGCTGGCCCAAGTCTCGCTGTCTCTGGGCTGCTCAGTCTCCACATCTGCAATCCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>45-W S</td>
<td>Primer off of the -45- hydrophobic core to make negative arms. Use the Oleosin-30-G-W primers to continue to extend the arms.</td>
<td>GATCAGCATGATCAACACACCGGTACCCACCTGCAATGAAACCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>45-W AS</td>
<td>Primer off of the -45- hydrophobic core to make negative arms. Use the Oleosin-30-G-W primers to continue to extend the arms.</td>
<td>TATCTGCTGGCCCAAGTCTCGCTGTCTCTGGGCTGCTCAGTCTCCACATCTGCAATCCCCTGTCACGTTATTCCTGCCACATGAAACCGGTAAACCAC</td>
</tr>
<tr>
<td>Primer Type</td>
<td>Description</td>
<td>Primers</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>30-W S</td>
<td>Primer off of the -30- hydrophobic core to make negative arms. Use the Oleosin-30-G-W primers to continue to extend the arms.</td>
<td>GATCAGCATGATCAACACACCCTGGTGACCAG CTCAACCACACAGGACCAGCAACAAGGC CCCCACACCGCAACTCTCTCGCAGCTC</td>
</tr>
<tr>
<td>30-W AS</td>
<td>Primer off of the -30- hydrophobic core to make negative arms. Use the Oleosin-30-G-W primers to continue to extend the arms.</td>
<td>TATCTGCTGCCCAGTCCTGGTGTGCGTTCG CCCCCTGGCTCACCACATGCAATCTCC CCGTTACGTTATCTGCAAAACCTGGGTAAC CCGCAAGC</td>
</tr>
<tr>
<td>30G+ W S</td>
<td>Primer used to make -30-G+W</td>
<td>GTACGGGTTCATGGAACAAAGTGAAAGG</td>
</tr>
<tr>
<td>30G+ W AS</td>
<td>Primer used to make -30-G+W</td>
<td>CCTCTACTGGTTCCACTAGAACCCGTAAC</td>
</tr>
<tr>
<td>30G+ W 1S</td>
<td>Primer used to make -30-G+W</td>
<td>AAACCCAAAAAAAAACCATCAACACACCACACG TAACAGACTCCACCACCCAA CCCCACACAG</td>
</tr>
<tr>
<td>30G+ W 2S</td>
<td>Primer used to make -30-G+W</td>
<td>AAAACCGGCCACCATGTCACCGTTTACACGTTT</td>
</tr>
<tr>
<td>30G+ W 3S</td>
<td>Primer used to make -30-G+W</td>
<td>ATACCTATGGAATCCACCACAAACCAAACAG</td>
</tr>
<tr>
<td>30G+ W 1AS</td>
<td>Primer used to make -30-G+W</td>
<td>TGGAAACCTATAACACAAACAGCGGAGTCG CACCGAGAGGAGCTATG</td>
</tr>
<tr>
<td>30G+ W 2AS</td>
<td>Primer used to make -30-G+W</td>
<td>ATCGCTATACCACTCGAATAACAAACAG</td>
</tr>
<tr>
<td>30G+ W 3AS</td>
<td>Primer used to make -30-G+W</td>
<td>TCCCACTGAAACCCGTAACACCGCAACAG CCAATCGCTAATCCACCCACTACG</td>
</tr>
<tr>
<td>30G+ W 4AS</td>
<td>Primer used to make -30-G+W</td>
<td>TTCTTGCCCAACGTTCTGCAACTTCCTCTTC ACTTTGTTCCACTGAAACC</td>
</tr>
<tr>
<td>30G+ W 5AS</td>
<td>Primer used to make -30-G+W</td>
<td>TATCTTGCGCACAAGTCTCTTGTCGTTCTTCG CACCCTTCTGGCCACAG</td>
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<tr>
<td>30G+ W 6AS</td>
<td>Primer used to make -30-G+W</td>
<td>TGGCCCTGGTTACCTCTGATGCGCGGTAT ATGCTATCTTCTCGG</td>
</tr>
<tr>
<td>30G+ W 7AS</td>
<td>Primer used to make -30-G+W</td>
<td>TCCGGCTCCTTTTCCACGACCTGACCCTG ACCCTGGCCCTGGTTTACC</td>
</tr>
<tr>
<td>30G+ W 8AS</td>
<td>Primer used to make -30-G+W</td>
<td>ATATAGTATCTCGAGTTTTCTCGCCCTGTTT CGCCCCTTGTTTCC</td>
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## Appendix 2: DNA Sequences

<table>
<thead>
<tr>
<th>Name (s)</th>
<th>Description</th>
<th>DNA sequence</th>
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<tbody>
<tr>
<td><strong>BamUK cloning sites</strong></td>
<td>Cloning sites for BamUK. This is useful for sequencing to make sure that you don't have the original gene.</td>
<td>ATACATATGGGATCCATGGCCGGAATTCGAGCTCCGTCGACAAAGCTTTGCGGCCCCACTCGAGACCAACCCAACCCACCAG</td>
</tr>
<tr>
<td>Avitag</td>
<td>Avi-tag gene (biotin binding site). Can be added to the N-terminus of the protein and reacted with BirA to bind biotin specifically to the K in the sequence.</td>
<td>GGTCTGAACGACATCTTCGAGGCTCAGAAAAATCGAATGGCAGAAGAA</td>
</tr>
<tr>
<td>TAT-linker</td>
<td>TAT ligand can be fused to the N-terminus to provide cell binding. There is a (GGGGS)3 linker between the protein and the tag.</td>
<td>TCAATGCATATGGGTCGTAATGAAAACGTCGTACGCTCGTGCTCCAGCAGGGACGTTATATCGTCGTCGTTCTGGTGGTGGTCTGCTGTTCTGGTGTCTGAGTATC</td>
</tr>
<tr>
<td>Oleosin, 42-87-57</td>
<td>Full length wild type sunflower seed. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
<td>ATGGCCACACACCACTACGACCCAGCACCAGATGCAACAACCCCACTACCGCAGGACTCCACACCAACCCCAACCCAGGAGCAACAGAAGTCGACACTGGCCTTACGTTATATCATCCTCGGTTACGTCGTTCTGGTGGTGGTCTGCTGCTTGTGGTGGT</td>
</tr>
<tr>
<td>7-87-7</td>
<td>Truncation mutant of 42-87-63 where both hydrophilic arms are truncated to 7 amino acids. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
<td>GGATCCCCCCTCAACCCGAGATAATGCTACGACGCTGATGATGAGAATTCGAGTACGACGCTGACGACTCCACCCAGGAGCAACAGAAGTCGACACTGGCCTTACGTTATATCATCCTCGGTTACGTCGTTCTGGTGGTGGTCTGCTGCTTGTGGTGGT GGATCCCCCCTCAACCCGAGATAATGCTACGACGCTGATGATGAGAATTCGAGTACGACGCTGACGACTCCACCCAGGAGCAACAGAAGTCGACACTGGCCTTACGTTATATCATCCTCGGTTACGTCGTTCTGGTGGTGGTCTGCTGCTTGTGGTGGT GGATCCCCCCTCAACCCGAGATAATGCTACGACGCTGATGATGAGAATTCGAGTACGACGCTGACGACTCCACCCAGGAGCAACAGAAGTCGACACTGGCCTTACGTTATATCATCCTCGGTTACGTCGTTCTGGTGGTGGTCTGCTGCTTGTGGTGGT GGATCCCCCCTCAACCCGAGATAATGCTACGACGCTGATGATGAGAATTCGAGTACGACGCTGACGACTCCACCCAGGAGCAACAGAAGTCGACACTGGCCTTACGTTATATCATCCTCGGTTACGTCGTTCTGGTGGTGGTCTGCTGCTTGTGGTGGT</td>
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201
<table>
<thead>
<tr>
<th>Truncation Mutant</th>
<th>Oligo Sequence</th>
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<tr>
<td><strong>7-87-57</strong></td>
<td>Truncation mutant of 42-87-63 where the N-terminal hydrophilic arm is truncated to 7 amino acids and the C-terminal arm remains the full length. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td></td>
<td>GGATCCCCCTCAACCCGGAAGATAATGGTCATCA TTGGCCTTACTTCCAATAACCCGGAATCTTTGGTG TTAGCCGGAATCCCTCCTGGGGACGGTTATCAGG GCTCCCTCTCGCACTCGGCTGTGTATAATCCA GCCCTGTATTTGTACCCGATGATTGCAATGGGGCA CTTGCGGTTACCCGATTTTCGACTCGGTGACAATGT CCGGTTTACGGGTTAAGCTCTTGTTTCGATATTGG TTAATATGCTGAGGCGCTCAGAATATGTCGTTCC AGTCGCAGGGGATTATGTGAAGGGGGAAGTGGGGA AACTCGAG</td>
</tr>
<tr>
<td><strong>42-87-7</strong></td>
<td>Truncation mutant of 42-87-63 where the N-terminal hydrophilic arm remains the native length and the C-terminal arm is truncated to 7 amino acids. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td></td>
<td>GGATCCACCAACACTAGCCGCAACATGTCGCA CACCCACCAACCCCCAACTCCGGAATCATGCAACAAC CACGCTGAGCATACCAACCCACAGCCGCCAG CAACAAGGGCCCCACACCGAAGATAATGTCGCA TCAATGCGCTTCTTCCAAAAAGGAAATTTGTTTC GGTATACCCGGAATCCCTCCTGGGGACGGTTATCGG GCTCGCTCTCGCGACTCCGCTGTTTGTTATATTGG GCCCTGTTATTGTTCCAGCGATGATAGCGATTGGG CTGGGTTACCCGGAATCCCTCCTGGGGACGGTTATCGG</td>
</tr>
<tr>
<td><strong>12-87-27</strong></td>
<td>Truncation mutant of 42-87-63 where the N-terminal hydrophilic arm is truncated to 27 amino acids and the C-terminal arm is truncated to 27 amino acids. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td></td>
<td>GGATCCCCGACGCAAAAGGCCCCCTCAACCGGA CAGATAATGGTCATCATTCCATTCCAAATAACC GGAATCCTTGGTTACCGCTGGATCCCGGTCGACTCGG CCGGAGGGTTAGTCCCGGTTTACCCGGAATCCCTGCG TGTTTTGGTATATACGGCCTTGGTATATGGTTGTCGCA GATGATACGGGATTGCGGTTACCCGGATTGGGTTTTGG TAGCTTGGGTAAGGCCTCCTCGGTTAACGGGGGAAGTTGCAGGATGTGGGAGTATACGGCCTTGGTATATGGTTGTCGCA GAGAACGGAAGGCAAAGAAGGAAAGGCAGAAGAGGACCTGGGACCGCTGAG</td>
</tr>
<tr>
<td><strong>7-87-12</strong></td>
<td>Truncation mutant of 42-87-63 where the N-terminal hydrophilic arm is truncated to 7 amino acids and the C-terminal arm is truncated to 12 amino acids. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td></td>
<td>GGATCCCCCTCAACCCGGAAGATAATGGTCATCA TTGGCCTTACTTCCAATAACCCGGAATCTTTGGTG TTAGCCGGAATCCCTCCTGGGGACGGTTATCAGG GCTCCCTCTCGCACTCGGCTGTGTATAATCCA GCCCTGTATTTGTACCCGATGATTGCAATGGGGCA CTTGCGGTTACCCGATTTTCGACTCGGTGACAATGT CCGGTTTACGGGTTAAGCTCTTGTTTCGATATTGG TTAATATGCTGAGGCGCTCAGAATATGTCGTTCC AGTCGCAGGGGATTATGTGAAGGGGGAAGTGGGGA AACTCGAG</td>
</tr>
<tr>
<td>12-87-12</td>
<td>Truncation mutant of 42-87-63 where the N-terminal hydrophilic arm is truncated to 12 amino acids and the C-terminal arm is truncated to 12 amino acids. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td>---</td>
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</tr>
<tr>
<td></td>
<td>GGATCCGCCAGCAACAAAGGCCCTCAACCGGGCA AGATAATGGTCATCATGGCCTTACTCTCAAATACC GGAATCTTGTTTGGTTTACAGCGGTATACACCACTCGT CCGGACGGTTATCGGGCTCGCTCTCGGAAGCCAGCTCG AGTTTGTTATATCCGGCTTTATATGCTCAGGGAGT GATAGTACGGATTTGCGGTTAACCAGGGTTTT TGACCTCGGTATACGGGTGTTAACGGAAGATTATGT GAAAGGGAAATGGTCAGCTCGAG</td>
</tr>
<tr>
<td>22-87-37</td>
<td>Truncation mutant of 42-87-63 where the N-terminal hydrophilic arm is truncated to 22 amino acids and the C-terminal arm is truncated to 37 amino acids. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td></td>
<td>GGATCCACCACCGGGTGACAGACTCAACCCACCCACAG CGGCAGCAACAAAGGCCCTCAACCGGGCA AGATAATGGTCATCATGGCCTTACTTCCAATAACCGG AGTACGGTTATCGGGCTCGCTCTCGGAAGCCAGCTCG AGTTTGTTATATCCGGCTTTATATGCTCAGGGAGT GATAGTACGGATTTGCGGTTAACCAGGGTTTT TGACCTCGGTATACGGGTGTTAACGGAAGATTATGT GAAAGGGAAATGGTCAGCTCGAG</td>
</tr>
<tr>
<td>42-87-32</td>
<td>Truncation mutant of 42-87-63 where the N-terminal hydrophilic arm remains the native length and the C-terminal arm is truncated to 32 amino acids. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td></td>
<td>GGATCCACCACACCTACGACCGCCACCCATGGCA CCACCACACCACACCACATCCGCACATGACACAC CACCGTGACAGACTACCCACACCCACAGCGGCA CACCGGCCCTCAACCGGCAAGATAATGGTCA TCACTGGCTTACTCTCAATACCCGGAATCTTGGTT GGTAGCGTACGCCGTTAACCAGCGATAGCAGATT GGGACGGTGTAACCGGGTTTTGGAATTCGGGTAC GTTCGGGTAAACGGGTGTAAAGCTCGTGTTCTGAATT TGTATATATATGTGAGCGTGACCAATGGTCTCGG CCACGTGCAAGGAATTATGTGAAGAGGGAATTGGC AAGATGTGGGGGGATAGTACCGAAGACGAAGGACT TGGGCCAGAAGATACAGCATTACGCTCGAG</td>
</tr>
<tr>
<td>17-87-17, Oleo phobic</td>
<td><strong>Truncation mutant of 42-87-63 where the N-terminal hydrophilic arm is truncated to 17 amino acids and the C-terminal arm is truncated to 17 amino acids. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</strong></td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------------------------------------------------------------------------</td>
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<tr>
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<td><strong>CATATGCGCCATGATCAACACACCGGTGACAGAC</strong>&lt;br&gt;<strong>TCACCCACCCACACGCGCCACGAACAAAGGCCCTC</strong>&lt;br&gt;<strong>AACCGGCAAGATATAATGGTCTACATGGCCTTACTT</strong>&lt;br&gt;<strong>CAATAACCCGGAATCTTGTGTTTATAGCGGTAT</strong>&lt;br&gt;<strong>CACCCTTCGAGGACAGGTGTTACTCGGCTCTCGC</strong>&lt;br&gt;<strong>CGATCCCGCTGGTGTATATATGACCCGTTGAA</strong>&lt;br&gt;<strong>GGGTTAAGCTCGTGTGTCGTATTTGTTAATATGGT</strong>&lt;br&gt;<strong>GAGGGCGTCGACAATGTCGTTCCAGTCAGAGG</strong>&lt;br&gt;<strong>GATTATGTGAAGGGGAAGTGCAGGATGTGGGGG</strong>&lt;br&gt;<strong>AGTATACCGGCCCCAGAAAGCAGAAGGACTTGGC</strong>&lt;br&gt;<strong>CAAGAAATCAGCATCGGCCCCATGAAATGGGTGAC</strong>&lt;br&gt;<strong>CAGGGGCACGTCGACCCACACCCACCAAGCA</strong></td>
</tr>
<tr>
<td>27-87-12</td>
<td><strong>Truncation mutant of 42-65-63 where the N-terminal hydrophilic arm is truncated to 27 amino acids and the C-terminal arm is truncated to 12 amino acids. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</strong></td>
</tr>
<tr>
<td></td>
<td><strong>GGATCTACCCGCCATGATCAACACACCGGTGACAG</strong>&lt;br&gt;<strong>GACTCAACCCACCCACAGGCGCCACGAACAAAGGCCCTC</strong>&lt;br&gt;<strong>CTCAAACCGCAAGATAATGGTCTACATGCGCTTA</strong>&lt;br&gt;<strong>CTTCCAATAACCCGGAATCTTGTGTTTATAGCGGTAT</strong>&lt;br&gt;<strong>TATCACCCCTGCGAGGCGTTAATCGGGCTCGCTC</strong>&lt;br&gt;<strong>TTCGCGACTCCCGCTGGTGTATATATGACCCGTTG</strong>&lt;br&gt;<strong>TAACCGGGTTTTGACTCGGTGACGTGTCGTTCCAGT</strong>&lt;br&gt;<strong>GACGGGTAAAGCTCGTGTGTCGTATTTGTTAATATGG</strong>&lt;br&gt;<strong>TGAGGCGCGTCGACAATTGTCGTTCCAGTCAGACA</strong>&lt;br&gt;<strong>GGGATTATGTGAAGGGGAAGTGCAGTGG</strong></td>
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<tr>
<td>22-87-12</td>
<td><strong>. Low expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</strong></td>
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<td><strong>GGATCCCCACACCGGTGACAGACTCAACCCACCCAC</strong>&lt;br&gt;<strong>AGGGCCAGAACAAGGCCGCTCACAACCGGAAGAT</strong>&lt;br&gt;<strong>AATGGTCTACATGCGCTTAATCCCAAAACCCGGA</strong>&lt;br&gt;<strong>ATCTTGTGTGTTACGCGGTATACCCCTGCTCGG</strong>&lt;br&gt;<strong>GACGGGTATCAGGGCTCGCTCGAGTCCCGCTGCTG</strong>&lt;br&gt;<strong>TTGTATATCCAGCCCTGTATTGTCGTTACCGGAT</strong>&lt;br&gt;<strong>AGATCCGATTTGGGCTGTTACGGGTGTTTGGAC</strong>&lt;br&gt;<strong>TTGGTGTACGTTGCCTGGTAAGGGGTATAAGCTCGT</strong>&lt;br&gt;<strong>TGTCGTATTTGTTTATATGTGTGAGGCGCGTCGACA</strong>&lt;br&gt;<strong>ATGTCCGTTCCAGTCAGAGGTAATTGTGAGGA</strong>&lt;br&gt;<strong>GGGAAGTGGCAGTGG</strong></td>
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<tr>
<td>42-65-57, oleo philic</td>
<td><strong>Truncation mutant of 42-87-57 where the hydrophobic domain is truncated at both junction points leaving 65 amino acids in the core. High expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</strong></td>
</tr>
<tr>
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<td><strong>CATATGGCCACCAACCCACTCGACGCGCCACCCATG</strong>&lt;br&gt;<strong>TCACCCACCCACACGCGCCACGAACAAAGGCCCTC</strong>&lt;br&gt;<strong>AACCGGCAAGATATAATGGTCTACATGGCCTTACTT</strong>&lt;br&gt;<strong>CAATAACCCGGAATCTTGTGTTTATAGCGGTAT</strong>&lt;br&gt;<strong>CACCCTTCGAGGACAGGTGTTACTCGGCTCTCGC</strong>&lt;br&gt;<strong>CGATCCCGCTGGTGTATATATGACCGTTGAA</strong>&lt;br&gt;<strong>GGGTTAAGCTCGTGTGTCGTATTTGTTAATATGGT</strong>&lt;br&gt;<strong>GAGGGCGTCGACAATGTCGTTCCAGTCAGAGG</strong>&lt;br&gt;<strong>GATTATGTGAAGGGGAAGTGCAGGATGTGGGGG</strong>&lt;br&gt;<strong>AGTATACCGGCCCCAGAAAGCAGAAGGACTTGGC</strong>&lt;br&gt;<strong>CAAGAAATCAGCATCGGCCCCATGAAATGGGTGAC</strong>&lt;br&gt;<strong>CAGGGGCACGTCGACCCACACCCACCAACGCC</strong></td>
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<tr>
<td>7-65-7</td>
<td>Truncation mutant of 42-65-63 where the N-terminal hydrophilic arm remains the native length and the C-terminal arm is truncated to 32 amino acids. High expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>12-65-12</td>
<td>Truncation mutant of 42-65-63 where the N-terminal hydrophilic arm is truncated to 12 amino acids and the C-terminal arm is truncated to 12 amino acids. High expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td>17-65-17</td>
<td>Truncation mutant of 42-65-63 where the N-terminal hydrophilic arm is truncated to 17 amino acids and the C-terminal arm is truncated to 17 amino acids. High expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td>27-65-27</td>
<td>Truncation mutant of 42-65-63 where the N-terminal hydrophilic arm is truncated to 27 amino acids and the C-terminal arm is truncated to 27 amino acids. High expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td>42-65-7</td>
<td>Truncation mutant of 42-65-63 where the N-terminal hydrophilic arm remains the native length and the C-terminal arm is truncated to 7 amino acids. High expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td>7-65-57</td>
<td>Truncation mutant of 42-65-63 where the N-terminal hydrophilic arm is truncated to 7 amino acids and the C-terminal arm remains the native length. High expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
</tr>
<tr>
<td>17-65-17-tat</td>
<td>Truncation mutant of 42-65-63 where the N-terminal hydrophilic arm is truncated to 17 amino acids and the C-terminal arm is truncated to 17 amino acids. High expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</td>
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### 27-65-27 no avi

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<td>TGGGGGAGTATACCGGCCAGAAGACGAGACTG</td>
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<td>GGGCCAGCTCGAGCACCCACCCACAC</td>
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### Half Oleo 3

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<tr>
<th>A half oleosin mutant of the N-terminal hydrophilic block and half of the 87 hydrophobic block cut after the proline knot. High expression. Inclusion bodies. Low solubility in aqueous solutions. Soluble in organic/aqueous mixtures.</th>
</tr>
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<tbody>
<tr>
<td>GGATCCACACCCACACCACCCACGCGCCACCA</td>
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<td>CTCAACCCACCACGCGACGCCAACAAAGCC</td>
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<td>CATACGGCGAACATGTTGACGACTTGCCT</td>
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<td>TCCACATACCCGAGGACGGGTCGACGACTC</td>
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<tr>
<td>GCCGACTCGGCTGTTTGTATATTCAGCCCTG</td>
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<tr>
<td>TTACCCACCGATGATACGGTGACTCGAG</td>
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### BU

<table>
<thead>
<tr>
<th>Bottom up approach. Taking pieces of oleosin and connecting them to get the optimal 1/2 oleosin. Little to no expression</th>
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<tbody>
<tr>
<td>CATATGCACCCACCCACCCACACCCACACCA</td>
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<td>AACCGCCATGACGCTACACCCACACGCGCAAGA</td>
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<tr>
<td>ACAAGGCGCCTACACCAGGCAAGATAACCGGAATC</td>
</tr>
<tr>
<td>TGTGTTGTTTAAACCGGTATACACTCCCTTGGG</td>
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<tr>
<td>CTGTCTCGGACTCGCCTCCGCACTCCTGCTGTGTT</td>
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<tr>
<td>TTATATTCTGACTCGAG</td>
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### 1/2O-N 24-30

<table>
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<th>Short 1/2 oleosin of the N-terminal domain. 24 hydrophilic amino acids and 30 hydrophobic amino acids. Little to no expression</th>
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<td>GGATCCACACCCACACCCACCCACGCGCCACCA</td>
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<tr>
<td>CCAAATACCCGAGTAGAACACAGCGGTGACAGA</td>
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<td>CTCAACCCACCACGCGACGCCAACAAAGCC</td>
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<td>CATACGGCGAACATGTTGACGACTTGCCT</td>
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<td>TCCACATACCCGAGGACGGGTCGACGACTC</td>
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<td>GCCGACTCGGCTGTTTGTATATTCAGCCCTG</td>
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### 1/2 O 65

<table>
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<tr>
<td>CCAAATACCCGAGTAGAACACAGCGGTGACAGA</td>
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<td>CTCAACCCACCACGCGACGCCAACAAAGCC</td>
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<td>CATACGGCGAACATGTTGACGACTTGCCT</td>
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<td>TCCACATACCCGAGGACGGGTCGACGACTC</td>
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<tr>
<td>GCCGACTCGGCTGTTTGTATATTCAGCCCTG</td>
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<td>TTACCCACCGATGATACGGTGACTCGAG</td>
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### 1/2O 30

<table>
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<tr>
<td>CCAAATACCCGAGTAGAACACAGCGGTGACAGA</td>
</tr>
<tr>
<td>CTCAACCCACCACGCGACGCCAACAAAGCC</td>
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<td>Domain</td>
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<td>half -30-</td>
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<td>G+W</td>
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<tr>
<td>Phi 1</td>
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<tr>
<td>Phi2</td>
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<tr>
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<tr>
<td>Pho1</td>
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<td>Pho2</td>
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<tr>
<td>Pho 3 ,-30-G</td>
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<tr>
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<tr>
<td>Phil-Pho1</td>
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<tr>
<td>Phil-Pho2</td>
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Phi2-Pho1

Fusion of Phi2-Pho1

Phi2-Pho2

Fusion of Phi2-Pho2

42-30-57,
Oleosin-30, 30-, oleo30

Truncation mutant of
42-87-57 where the
hydrophobic domain is
truncated at both
junction points leaving
30 amino acids in the
core. High expression.
Inclusion bodies.
Soluble after refolding.
Sensitive to salt and
buffer concentrations

37-30-37

27-30-27

!

Truncation mutant of
42-30-63 where the Nterminal hydrophilic
arm is truncated to 37
amino acids and the Cterminal arm is
truncated to 37 amino
acids. High expression.
Inclusion bodies.
Soluble after refolding.
Sensitive to salt and
buffer concentrations
and will aggregate over
time.
Truncation mutant of
42-30-63 where the Nterminal hydrophilic
arm is truncated to 27
amino acids and the Cterminal arm is
truncated to 27 amino
acids. High expression.
Inclusion bodies.
Soluble after refolding.
Aggregation over time.

ATGCACCACCACCACCACCACAGGGATTATGTGA
AGGGGAAGTTGCAGGATGTGGGGGAGTATACGG
GCCAGAAGACGAAGGACTTGGGCCAGAAGATAC
AGCATACGGCCCATGAAATGGGTGACCAGGGCCA
GGGTCAGGGTCAGGGTGGTGGGAAAGAAGGGCG
AAAAGAAGGGGGGAAAGGATCC
ATGCACCACCACCACCACCACAGGGATTATGTGA
AGGGGAAGTTGCAGGATGTGGGGGAGTATACGG
GCCAGAAGACGAAGGACTTGGGCCAGAAGATAC
AGCATACGGCCCATGAAATGGGTGACCAGGGCCA
GGGTCAGGGTCAGGGTGGTGGGAAAGAAGGGCG
AAAAGAAGGGGGGAAAGGATCCTGGGGTGGCTG
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CTGGGTGGTGTGGGCGGCGCGGGCGGTTGACTCG
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GGCTTTGGCGGTTTTGGTGGTTTCGGCGGCTTTGG
CGGTTGACTCGAG
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CCACCACCCAACCCCAATACCGCCATGATCAACA
CACCGGTGACAGACTCACCCACCCACAGCGCCAG
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CGACTCCGCTGTTTGTTATATTCAGCCCTGTTATT
GTTCCAGCGATGATAGCGATTGGGCTTGCGGTTA
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G

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ATAGCGATTGGGCTTGCGGTTACCGGGTTTCAGA
GGGATTATGTGAAGGGGAAGTTGCAGGATGTGGG
GGAGTATACGGGCCAGAAGACGAAGGACTTGGG
CCAGCTCGAG

209!


Truncation mutant of 42-30-63 where the N-terminal hydrophilic arm is truncated to 17 amino acids and the C-terminal arm is truncated to 17 amino acids. High expression. Inclusion bodies. Low solubility after refolding and will heavily aggregate after elution from the column.

GGATCCCTCACCCACCCACAGCGCCAGCAACAAGAGCTGTTCAACCGGGG
GCCCTCAACCGCAAGCTGGACGCGACGCTCG
GCTGTTTGTATATTAAGCCTGTCGACGCTCG
CGATGATACGGATTTGGGCTTGGTTTTACCGGGT
CAGAGGGATTATGTGAAGGGGAAGTTGCAGGATG
TGGGGGAGTTATCTCGAG

egfp

eGFP with a 6-his tag for purification. Soluble. Expresses well

ATGGTGAGCAAGGCGAGGAGCTGTTCAACCGGGG
TGTTGCGCATCTGTCTGAGCTGGACGCGACGT
AAAAGCCCAAGTTAGCCTGCGGCGAGGCGGC
GAGGGCGATGCCACCTACGCAAGCTGACCCTGA
AGTTCACTTGCACACCACCGGGAAGCTGCCGTCGCC
CTGCCGGCACCCTCTGGAACACCGGACGCTACGG
GCGATGATAGCGATTGGGCTTGCGGTTACCGGGTTT
CAGAGGGATTATGTGAAGGGGAAGTTGCAGGATG
TGGGGGAGTTATCTCGAG

egfp

eGFP with a 6-his tag for purification. Soluble. Expresses well
eGFP fusion to 27-65-27. Does not express well.

```
CATATGGTGAGCAAGGCGAGGAGCTGTTCAACCG
GGGTGGTGCCCATCTGTGTCAGAGCTGAGCGCGA
CGTAAGGGCGCCACAAGTTCAGGTCGTCGGCGAG
GGCGAGGGCCATGCCCACCTAGGCAAGCTGAGCC
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GAAGACGACAGCTTCTTTCAAGTCCAGCTATCGGACGCCC
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GGGTGGTGCCCATCTGTGTCAGAGCTGAGCGCGA
CGTAAGGGCGCCACAAGTTCAGGTCGTCGGCGAG
GGCGAGGGCCATGCCCACCTAGGCAAGCTGAGCC
TGAAGTTCATCTTCAAGTCCAGCTATCGGACGCCC
eGFP fusion to oleosin-30. Expressed but stuck to the column

CATATGGTGAGCAAGGGCGAGGAGCTGTTCACCG
GGGTGGTGCCCATCTCTGCTGAGCCTGGACGGCGGA
CGTAAGCGCACAAGGCTACCCCGCTACGGTTCGAGCGAG
GCGAGCCGGCATGACCTCGAGCAAGCAGGTACCCCGAC
TGAAGGTTCCTCAGGCGACCTCCGGACCTTGAGCGAG
GCGAGGGCGATGCCA
CCTACGGCAAGCTGACCC
TGAAGTTCATCTGCACCACCGGCAAGCTGCCCGT
GCCCTGGCCACCCTCGTGACCACCCTGACCTACG
GCGTGCAGTGCTTCGAGCCGCTACCCCGACCACAT
GAAGCAGCACGACTTCTTCAAGTCCGCCATGCCC
GAAGGCTACGTCCAGGAGCGACCCACCTACCGCGCCGAGGT
GAAGTTCGAGGGCGACACCCTGGTGAACCGCAT
GAGCTGAAGGGCATCGACTTCAAGGAGGACGGCA
ACATCCTGGGGCAAGCTGGAGTACAACATCAAA
CAAGCCACAACGTCTATATCATGCGGCACAAGCAG
AAGAAGCGCATCAAAGTGAACCTCAAATGGCTGACC
ACACTAACAGCAGAAACCCCCCATCGGCCGACGGC
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TGTCACCTCAAATGCCCATGAT
CAACACACCGTGACAGACTCACCCACCAACAGC
GCCGCAACAGGCCGCCCTCAACCCGCAAGCTCCG
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TTATTGCTGGCAGTGAAGCTTGCG
GTTACCGGGTTTCAGAGGGATTATGTGAAGGGGA
AGTTGCAAGATGTGGGGGAGTATACGGGCCAGAA
GACGAAGAAGATGGCCAGAAGATACAAGCATACG
GCCCATGAAATGGGTGACCAGGGCCAGGGTCAGG
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GGGGGAAACTCGAG
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GGGTGGTGCCCATCTGGTCTGAGGCTGACGGCAG
CGTAACACGGCCAACAAGTTCTAGGCTGGTACGAG
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TGAAGCTATCTGCACCAACGGACACGGCAGCTGAG
GCCCTGGGCCCCCTGTTGCAACCCCTGACAGCAC
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CAGCCACAAACGTCTATATCATGGGGCACAGGAC
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CAGCCACCAGCAGAACACCCCCATCGGCGACGGC
CCACTACCGAGGAAACCTACACGCACCCGAGCG
CCGCTGCTGGTCCAGCAACACCTACAGAAGGTG
GCCGCCGGGATCACTTCTGCAGCAGAGCTCT
ACGCTAGGGCCACCACACCTACACGGCAGGCCAC
TTGTCACACCACCAACCCCAATACCCGCCCATAGT
CAACACACGGTGACAGACTCACCCACCCACAGC
GCCAGCACACAAGGGCCCTCAACCGGCAAGCTCGC
TCTCGCGACTCCGGTTGTATATATACGGCCCTG
TTATGCTACAAGGAGATTATGTAAGGGGA
AGTTCGAGGTGTGGGGAGGTATACGGGCCAGAA
GACGAAAGGACTTGGGCCAGCTCGAG

eGFP fusion to 42-30-27. This mutant was created to see if the 6-his tag was not exposed in the egfp-42-30-63 mutant. Expressed but stuck to the column.
eGFP fusion to 42-30-12. This mutant was created to see if the 6-his tag was not exposed in the eGFP-42-30-63 mutant. Expressed but stuck to the column.

DewA
Hydrophobin DewA. Expressed well. Soluble.

DewAW
DewA with W mutated in on the N-terminus to allow for fluor and A280. Expressed well, soluble.
<table>
<thead>
<tr>
<th>GPF-DewA</th>
<th>GFP tagged on the N-terminus of DewA. Expressed well, soluble.</th>
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<td></td>
<td>GTGACGCTGACGGCTGCTGACATGCCCCTTGCC</td>
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GFP-linker-DewAW

DewAW with eGFP-linker on the N-terminus

CATATGCAACCACACCCACCCACAGTGAGCAAGG
GCAGAGAGCTGGTTCACCCGCGGAGGGCGATCCAA
TTCAGGCTGTCCGCGAGGGCGAGGGCGATCCAA
CTACCCGAAGCTGACCCCCCCCTGAGGCTAGGATCTAC
CAACGCGCAAGCTGACCCCCCCCTGAGGCTAGGATCTAC
GATTCACATCCGACACCAGCTGAGCAAGCTGACCCCCCCCTGAGGCTAGGATCTAC
CATATCGGACCAGCTGAGCAAGCTGACCCCCCCCTGAGGCTAGGATCTAC
GGATCCGCCAAGGGCGAAGCTGACCCCCCCCTGAGGCTAGGATCTAC

Point mutations in the hydrophobic block to add Gs. Also remove charge in arms. Expresses well. Inclusion bodies. Soluble after purification

GGATCCGCCAACCACACCCACCCACAGTGAGCAAGG
GCAGAGAGCTGGTTCACCCGCGGAGGGCGATCCAA
TTCAGGCTGTCCGCGAGGGCGAGGGCGATCCAA
CTACCCGAAGCTGACCCCCCCCTGAGGCTAGGATCTAC
CAACGCGCAAGCTGACCCCCCCCTGAGGCTAGGATCTAC
GATTCACATCCGACACCAGCTGAGCAAGCTGACCCCCCCCTGAGGCTAGGATCTAC
CATATCGGACCAGCTGAGCAAGCTGACCCCCCCCTGAGGCTAGGATCTAC
GGATCCGCCAAGGGCGAAGCTGACCCCCCCCTGAGGCTAGGATCTAC

-30- G+

216
Oleosin-30G with all positive hydrophilic arms. A single W is added for A280. Expresses well. Inclusion bodies. Soluble after purification

GATCCGCCACCCAAACCAAAACCGCCACCAGTG
TCACACACACACCAACCCAAACCCGAAAACGCCATAACCA
ACACACCGGTAAACGACTCCCAACCCACAGCGC
CAGCAACAGGCCCCCTCAAACCGGAAGCTCGGCTTC
TCGCTGCGACTCCGCGTTTTGTTTAGTGTTTGC
AGCCCTGTTTATGTTCTACGCGATGGGTATAGGAT
TGCGCTTGCAGGTTATACGCGGTGTTTACAGTGGAAC
AAAGTGAGAGGGAAGTTTCAGGAACGTGGGGAG
AAAAACGGCCAGAAGACGAAGAACTTGGGCCAG
AAAGATACAGCATAAGGCAACAGAAGACACCTTGAGCCAG
AGGCAAGGGCTAGGCTAGGTGACTGTCGAGTTGGAAAC
AGGGGCGAGAACAGGGGAGAGGCTCGGCGT

GFP-linker
eGFP with a linker for fusion to other mutants. Not made for expression

ATGGTGAGCAAGGGCGAGGAGCTGTTGTCACCGGAG
TGCTGCCCATCCTGTGCGACTGAGCAGCCCGACGT
AAAAGGGCGAGAAAGTCACTGAGCTGGCGCCGGCG
GAGGGCGAGAGTGACCTACGGGCAAGCTGACCATGA
AGCCAGACGACTTCTAAGCTGCGGCGACGATCC
AGGCTAGCTTCAAGCCAGCGACATCCCTCTTCAAG
GACGCCAGCAATACAAGGCCAGGGGCGGAGGTT
AGTTGAGGAGGACACCCCTGCTGAACCGCAGATCG
GCTGAAAGGGCTCGACTTCAAGGAGAGCGCCCAAC
ATCCTGGCGACAGTGGAGTACCACTACAAAA
GCCAACAGTCTATATATGCGGCAAGAAGCAAGAA
GAAAGGCACATCAAGGTAAGTCACTAGAGATCCGACAC
AACATCGAGAGCGAGCGTCAGCTCGGCGACC
ACTACCCAGAAGAAGCCCATCCTGCGGACCACCC
CTGGCTGCTGCGGCAACAGCACTACCGTGACACC
CAGTCCGGCCTGACGAAAGACCACCGAACAGAAGC
GCCGATCAGATGGCTCGGTAGTTCTGTCGAGCCGC
CGCGGGGAGTACCTCGGCGATGAGCAGCTGATAC
GGGCTGCGGTGAGCGTAGCGGGTCGCTGGGATCC
-30-G+ with eGFP-linker on the N-terminus. Fusion kills expression. Very little purified as the majority of the protein sticks to the column.

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eGFP fusion to oleosin-30 with the 6-his tag on the N-terminus. Moving the 6-his tag does not help with purification.

6-his-gfp-linker-30-

-30-W M1

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<th>42-45-63</th>
<th>Truncation mutant of 42-87-63 where the hydrophobic block was truncated to 45 amino acids. Expresses well. Inclusion bodies. Soluble after purification.</th>
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Her2-C-linker-30-

Her2-linker fused to oleosin-30. A single C was added for dying. The C caused aggregation unless it was reduced.

-30- W M1-avi

The -30- W M1 mutant with the avi tag on the N-terminus. Expressed well. Inclusion bodies. Soluble after purification.

42-30G-63, -30-G, Oleosin-30G

The oleosin 30 mutant with 5 glycines added into the hydrophobic block. Expressed better than Oleosin-30. Inclusion bodies. Highly soluble after purification. Used in microbubble project.

GTGGATAACAAATTTACAAGAATCGCGCAACGCGTGATTATCGACAGCCTG
CAACACCAGAAACCCCGCTTTATACGGACGACCTG
TATGATGATCCGAGCCAGAGCGCGAACCTGCTGG
CGGAAGCGAAAAACTCGACAGGCGAAAATTGAAGGCGG
CGGATCCACCACAACCTACGAGCGCACCCACAGCGCC
AGCAAAAGCCGGCTAACCGGAGTATGCGATTGGCGTCT
CGCCACTCCTCCGTGTTTGTATATTCAGCCCTGTTA
TGTTTCCAGCGATAGATGCGATTTGCGCTGCGGT
ACC GGCTTTACAGAGGATTATG TAAAGGCAAGAGGCGCAG AAAGCA
CGCATCGACTGCGACTCCGCTGTTTGGTGTTATAC
GGAGCATCGCTGCTGCTTGTTAATAT
GGAAGCTGCTGGTTATACGAGGATGGCT
TGAAGGGGAAATTTGGGATGATGCGATTGGCGTT
GGCCAGAACGGAAGGACTTGGGCCAGAAGACGAT
CATACGGCCCATGAAATGGGTGACCAGGGCC
AGGGTGGTGGGAAAGAAGGGCGAAAAGAAGGGG
GGAAACTCGAGCACCCACACCCACCACCCACTGAA
ATGCCGGCGTGGCGTACCGAGTGGCGGCTGTCT
GGTGGGCGTGGCTCGGCAGTGAGC

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TCGAATGGCCAGAAGATCTACCAACGAACTACGA
GGCCGCCATCTGCAACCACCACCTACCAAATAT
CGCCATGATCAACACACCAGTGACAGACTCAACC
ACCCACAGCGCCAGCACAAGGCCCTACACCAGG
CAAGCTCCTGCTTCGGACTCGCTGTTTGTATAT
GGAGCCTTGGTTATATTCAGCGATGATGCGATTGGC
GGCTGCTGCTGCTGCTGCTGTTTGGTGTTATAC
GGAGCATCGCTGCTGCTTGTTAATAT
GGAAGCTGCTGGTTATACGAGGATGGCT
TGAAGGGGAAATTTGGGATGATGCGATTGGCGTT
GGCCAGAACGGAAGGACTTGGGCCAGAAGACGAT
CATACGGCCCATGAAATGGGTGACCAGGGCC
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GGTGGGCGTGGCTCGGCAGTGAGC

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CCACCACCCACCAACCTACGACCGCCACCATGTCA
CAACCGGTCAGACAGTCCACCCACCCACAGCGCCAG
CAACCAAAGCGCCCTACACCGGCAAGCTCGCTCCTG
GTGGCAGCTCCTGGTTATATACGAGGATGGCT
CCTGTTATATGTTTCCAGCGATGATGCGATTGGC
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GGCCAGAACGGAAGGACTTGGGCCAGAAGACGAT
CATACGGCCCATGAAATGGGTGACCAGGGCC
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221
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<th>eGFP-42-30G-63</th>
<th>eGFP fused to oleosin-30G. Used to label microbubbles. Soluble. Low expression.</th>
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<th>46E32-30-62</th>
<th>Oleosin-30 with an enterokinase site that cuts the N-terminal arm down to 32 amino acids. Expressed well. Inclusion bodies. Soluble after purification.</th>
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<td>47E22-30-62</td>
<td>Oleosin-30 with an enterokinase site that cuts the N-terminal arm down to 22 amino acids. Expressed well. Inclusion bodies. Soluble after purification.</td>
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<td>47E12-30-62</td>
<td>Oleosin-30 with an enterokinase site that cuts the N-terminal arm down to 12 amino acids. Enterokinase didn't cut well due to proline after site. Expressed well. Inclusion bodies. Soluble after purification.</td>
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<td>47E11-30-62</td>
<td>Oleosin-30 with an enterokinase site that cuts the N-terminal arm down to 11 amino acids. Expressed well. Inclusion bodies. Soluble after purification.</td>
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ATTCAGCCCTGTGTATTGTTCACAGTGAAGGAGA
TTGGGCTTCGCGGTTCACCGGACGGATATGATGATG
GTGAAAGGGAAGTTGCAAGATGTGGGAGGATGATA
CGGGCCAGAAGACGAGACTTTGGGACAGAAGA
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CCAGGGTCAAGGTCAGGTTGTGGAAAGAAGAGG
GGCAAAAGAGGGGAGGAAAAGCTCGAGCCACACCA
CCACCAACACTGA

GGATCCACCAACCTACGACCACCACCATAGTCA
CCACCAACCAACCCATACCACCCCATGACGAGCA
CGAGAAAGATCAACACACCCGTTGACAGACTCAC
CCACCCACAGGCAAGCAAACAGGCCCCCTCAACC
GGCAAGCTCAGTCTCGGACTCGCATCCTGGTTTTGTTAT
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GTGAAAGGGAAGTTGCAAGATGTGGGAGGATGATA
CGGGCCAGAAGACGAGACTTTGGGACAGAAGA
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CCAGGGTCAAGGTCAGGTTGTGGAAAGAAGAGG
GGCAAAAGAGGGGAGGAAAAGCTCGAGC

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CGAGAAAGATCAACACACCCGTTGACAGACTCAC
CCACCCACAGGCAAGCAAACAGGCCCCCTCAACC
GGCAAGCTCAGTCTCGGACTCGCATCCTGGTTTTGTTAT
ATTCAGCCCTGTGTATTGTTCACAGTGAAGGAGA
TTGGGCTTCGCGGTTCACCGGACGGATATGATGATG
GTGAAAGGGAAGTTGCAAGATGTGGGAGGATGATA
CGGGCCAGAAGACGAGACTTTGGGACAGAAGA
TACAGCATACGACCACCAATTGAGTGACACAGGA
CCAGGGTCAAGGTCAGGTTGTGGAAAGAAGAGG
GGCAAAAGAGGGGAGGAAAAGCTCGAGC

CGGCAAAAGAGGGGAGGAAAAGCTCGAGC

223
**GFP-linker-42E32-30-63**

42E32-30-63 with eGFP-linker on the N-terminus. Fusion kills expression. Very little purified as the majority of the protein sticks to the column.

**RLP3-Pho1**

RLP3 resilin fusion to Pho1. Little to no expression

**RLP3-pho2**

Trimer of resilin-like-polypeptide fused to Pho2. Expressed well.
**RLP3-pho3 (-30-G)**


CATATGCACCATCACCATCACACAAAGCTTAGAT
CTGGCGGGTCGTCCGTCAGACTCTCTAAAAGGCGCTCC
GGGCGGGCGCAACCGGCGGCCTCGGAGAATTCT
AAAGGGCGGGCGGGGTGGCGCAATGGCGGTCGTC
CGAGTGATTCTAGTTTGCACCCGCTGTATTGTCTCA
CCGATGGGTATAGGATGTTGCCTGGGCTGTATA
CCGGTGGTTTTTGA

**RLP3-half 30**

Trimer of resilin-like-polypeptide fused to half of the oleosin-30 hydrophobic block. Expressed well. Soluble expression.

CATATGCACCATCACCATCACAAAGCTTAGAT
CTGGCGGGTCGTCCGTCAGACTCTCTAAAAGGCGCTCC
GGGCGGGCGCAACCGGCGGCCTCGGAGAATTCT
AAAGGGCGGGCGGGGTGGCGCAATGGCGGTCGTC
CGAGTGATTCTAGTTTGCACCCGCTGTATTGTCTCA
CCGATGGGTATAGGATGTTGCCTGGGCTGTATA
CCGGTGGTTTTTGA

**RLP3-half 45**

Trimer of resilin-like-polypeptide fused to half of the oleosin-45 hydrophobic block. Expressed well. Soluble expression. No A280.

CATATGCACCATCACCATCACAAAGCTTAGAT
CTGGCGGGTCGTCCGTCAGACTCTCTAAAAGGCGCTCC
GGGCGGGCGCAACCGGCGGCCTCGGAGAATTCT
AAAGGGCGGGCGGGGTGGCGCAATGGCGGTCGTC
CGAGTGATTCTAGTTTGCACCCGCTGTATTGTCTCA
CCGATGGGTATAGGATGTTGCCTGGGCTGTATA
CCGGTGGTTTTTGA

**RLP3-65**


CATATGCACCATCACCATCACAAAGCTTAGAT
CTGGCGGGTCGTCCGTCAGACTCTCTAAAAGGCGCTCC
GGGCGGGCGCAACCGGCGGCCTCGGAGAATTCT
AAAGGGCGGGCGGGGTGGCGCAATGGCGGTCGTC
CGAGTGATTCTAGTTTGCACCCGCTGTATTGTCTCA
CCGATGGGTATAGGATGTTGCCTGGGCTGTATA
CCGGTGGTTTTTGA

**RLP3-half 65**

Trimer of resilin-like-polypeptide fused to half of the oleosin-65 hydrophobic block. Expressed well. Soluble expression. No A280.

CATATGCACCATCACCATCACAAAGCTTAGAT
CTGGCGGGTCGTCCGTCAGACTCTCTAAAAGGCGCTCC
GGGCGGGCGCAACCGGCGGCCTCGGAGAATTCT
AAAGGGCGGGCGGGGTGGCGCAATGGCGGTCGTC
CGAGTGATTCTAGTTTGCACCCGCTGTATTGTCTCA
CCGATGGGTATAGGATGTTGCCTGGGCTGTATA
CCGGTGGTTTTTGA
| RLP3-oleo-45 | Trimer of resilin-like-polypeptide fused to the oleosin-45 hydrophobic block. Expressed well. Soluble expression. | CATATGCAACCACCACCACCACCAAGCTTAGATCTGGCGGTCGTCCGTCAGACTCTAAAGGCGCTCGGGCGGCGGCAACGGCGGCCGTCCGAGCTTTCTTCTATTAGGTTTCCAGCGATGATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTTGA |  |
| RLP2-30G- | RLP dimer fused to Pho3 (-30-G). Little to no expression. | CATATGCAACCACCACCACCACCAAGCTTAGATCTGGCGGTCGTCCGTCAGACTCTAAAGGCGCTCCGGCGGCGGCAACGGCGGCCGTCCGAGCTTTCTTCTATTAGGTTTCCAGCGATGATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTTGA |  |
| RLP2-halfO | RLP dimer fused to Pho3 (Half oleo 30). Little to no expression. | CATATGCAACCACCACCACCACCAAGCTTAGATCTGGCGGTCGTCCGTCAGACTCTAAAGGCGCTCCGGCGGCGGCAACGGCGGCCGTCCGAGCTTTCTTCTATTAGGTTTCCAGCGATGATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTTGA |  |
| RLP3-P2A | RLP3 resilin fused to the hydrophobic block of -30-G-P2A. No Y or W | CATATGCAACCACCACCACCAAGCTTAGATCTGGCGGTCGTCCGTCAGACTCTAAAGGCGCTCCGGCGGCGGCAACGGCGGCCGTCCGAGCTTTCTTCTATTAGGTTTCCAGCGATGATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTTGA |  |
| RLP3-30-G P2AY | RLP3 resilin fused to the hydrophobic block of -30-G-P2A. Added a Y at the intersection to give A280 | CATATGCAACCACCACCACCAAGCTTAGATCTGGCGGTCGTCCGTCAGACTCTAAAGGCGCTCCGGCGGCGGCAACGGCGGCCGTCCGAGCTTTCTTCTATTAGGTTTCCAGCGATGATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTTGA |  |
### -30-G-W

Oleosin-30G mutant with anion arms. Expresses very well. Highly soluble.

```
GGATCCGAAGCCACCACAACCAAGGACCAGGCACC
ATGTCAACCACCCACCCAACTTGGCAGTTCTACCACCCACACAG
GACCAGCAAACAGGCCCCCTCAACCCGGCAAGCTCGTCT
CTTCTCCGGTCGACCTCCGCTGTTTGGTGTTATAGGT
TTCAGGCCCCTTTATGTCCACAGGATGGGATATACGC
GATTGGGTGCGGTTATAGGTTCAGCCCTGTTATTGTTCCAGCG
TGGGTATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTCAGTG
AGGACAGACGGCAGGAACCGAAGGGAGGGGAAACCCACAC
CACACCACCACCGATGACTGACTCGAG
```

### 35-30G-42 -W

Truncation mutant of -30-G-W where the N-terminal arm is cut to 35 amino acids and the C-terminal arm is cut to 42 amino acids.

```
GGATCCCTCGCTCGCTGCGACCTCCGCTGTTTGG
TGTATAGGTTTTCAGGCCCCTTTATGTCCACAGGAGATGG
GTGCATGATCGCTTGGCGTGTGTATAGGTTCAGCCCTGTTATTG
TCCAGGCTGTTATTGTTCCAGCGATGGGTATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTCAGTG
AGGACAGACGGCAGGAACCGAAGGGAGGGGAAACCCACAC
CACACCACCACCGATGACTGACTCGAG
```

### 25-30G-30 -W

Truncation mutant of -30-G-W where the N-terminal arm is cut to 25 amino acids and the C-terminal arm is cut to 30 amino acids.

```
GGATCCGACATCGATCAGACCAACGCCTGCTCGCTTGGCGTGT
GTTATAGGGTTTTCAGGCCCCTTTATGTCCACAGGAGATGG
GTGCATGATCGCTTGGCGTGTGTATAGGTTCAGCCCTGTTATTG
TCCAGGCTGTTATTGTTCCAGCGATGGGTATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTCAGTG
AGGACAGACGGCAGGAACCGAAGGGAGGGGAAACCCACAC
CACACCACCACCGATGACTGACTCGAG
```

### -30-G N-C+

Zwitterionic mutant of Oleosin-30G where the N-terminal arm is anionic and the C-terminal arm is cationic.

```
GGATCCGAAGCCACCACAACCAAGGACCAGGCACC
ATGTCAACCACCCACCCAACTTGGCAGTTCTACCACCCACACAG
GACCAGCAAACAGGCCCCCTCAACCCGGCAAGCTCGTCT
CTTCTCCGGTCGACCTCCGCTGTTTGGTGTTATAGGT
TTCAGGCCCCTTTATGTCCACAGGATGGGATATACGC
GATTGGGTGCGGTTATAGGTTCAGCCCTGTTATTGTTCCAGCG
TGGGTATAGCGATTGGGCTTGCGGGTGTTACCGGGTTTCAGTG
AGGACAGACGGCAGGAACCGAAGGGAGGGGAAACCCACAC
CACACCACCACCGATGACTGACTCGAG
```

### Notes
- **Oleosin-30G mutant**: Contains anion arms that express well and are highly soluble.
- **Truncation mutant of -30-G-W**: Cuts the N-terminal arm to 35 amino acids and the C-terminal arm to 42 amino acids.
- **25-30G-30 mutant**: Cuts the N-terminal arm to 25 amino acids and the C-terminal arm to 30 amino acids.
- **Zwitterionic mutant of Oleosin-30G**: Features an anionic N-terminal arm and a cationic C-terminal arm.
<table>
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<tr>
<th>Mutant Type</th>
<th>Description</th>
<th>DNA Sequence</th>
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<td><strong>-30-G N+C-</strong></td>
<td>Zwitterionic mutant of Oleosin-30G where the C-terminal arm is anionic and the N-terminal arm is cationic. Missing the W on the C term, no A280</td>
<td>GGATCCGCCACAAACAAACAAAACACGCCACCATG TCACCAACCACCCACACCCACACCAACCGCTATCAACACACCGGTGTAACAGACTCACCCACCACCCACCGCGTCGACGACAAGGCACCTCAACGCGCAAGCTCGCTGGTTATAGGTTTCACTCTCGCAGTATAAGGGAATTGCAGGATGTGGGGAGTATACGGGCCAACGAAAGACGAGATCTCGGAG</td>
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<tr>
<td><strong>-30-G P2A</strong></td>
<td>Oleosin-30G mutant where the glycines are replaced with alanines</td>
<td>GGATCCACCACCAACATCGACGCGCAACCATGTCA CCACCAACCAACCCAAATAACGCGCATGATCAACAACACCGGTGACAGCTACCCACCACACACCGCGACGACTCTCGAAGTACCCACCACAGGACGAGATCTCGGAG</td>
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<td><strong>37-30G-37 P2A</strong></td>
<td>Truncation mutant of oleosin-30G-P2A where the N-terminal hydrophilic arm is truncated to 37 amino acids and the C-terminal hydrophilic arm is truncated to 37 amino acids</td>
<td>GGATCCACCACCAACATCGACGCGCAACCATGTCA CCACCAACCAACCCAAATAACGCGCATGATCAACAACACCGGTGACAGCTACCCACCACACACCGCGACGACTCTCGAAGTACCCACCACAGGACGAGATCTCGGAG</td>
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<tr>
<td><strong>27-30G-32 P2A</strong></td>
<td>Truncation mutant of oleosin-30G-P2A where the N-terminal hydrophilic arm is truncated to 27 amino acids and the C-terminal hydrophilic arm is truncated to 32 amino acids</td>
<td>GGATCCACCACCAACATCGACGCGCAACCATGTCA CCACCAACCAACCCAAATAACGCGCATGATCAACAACACCGGTGACAGCTACCCACCACACACCGCGACGACTCTCGAAGTACCCACCACAGGACGAGATCTCGGAG</td>
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<td><strong>42-30G-17 P2A</strong></td>
<td>Truncation mutant of oleosin-30G-P2A where the N-terminal hydrophilic arm remains the native length and the C-terminal hydrophilic arm is truncated to 17 amino acids</td>
<td>GGATCCACCACCAACATCGACGCGCAACCATGTCA CCACCAACCAACCCAAATAACGCGCATGATCAACAACACCGGTGACAGCTACCCACCACACACCGCGACGACTCTCGAAGTACCCACCACAGGACGAGATCTCGGAG</td>
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<td>Half P2A N</td>
<td>N-terminal diblock of the Oleosin-30G-P2A mutant. Little to no expression</td>
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<td>Half P2A C</td>
<td>C-terminal diblock of the Oleosin-30G-P2A mutant. Little to no expression</td>
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<td>TGCTGCGGAAAGGGTGAAGGGGCAAAACGTCGATTGC</td>
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<td>Her2</td>
<td>Her 2 affibody. Expresses very well. Highly soluble.</td>
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<td>TGCTGCGGAAAGGGTGAAGGGGCAAAACGTCGATTGC</td>
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<tr>
<td>Her2-30-G</td>
<td>Oleosin 30 with the Her2 affibody fused to the N-terminus. Expressed well. Inclusion bodies. Used to target the microbubbles.</td>
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<td>Her2-30-G-W</td>
<td>Her2 affibody fused to -30-G-W for FeO targeting. Expresses well. Targeting was no successful with this mutant.</td>
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<tr>
<td>-30-G 2N</td>
<td>Oleosin-30-G with 2 negative charges each terminal arm. Expressed well.</td>
<td></td>
</tr>
<tr>
<td>-30-G 4N</td>
<td>Oleosin-30-G with 4 negative charges each terminal arm. Expressed well.</td>
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</table>

| CATATGGTGGATAAACAATTTAACAAGAAATGCGA | CATATGGTGGATAAACAATTTAACAAGAAATGCGA |
| GCAACGCCGATTTGGAATTGCGCTTCGCTCCGAAA | GCAACGCCGATTTGGAATTGCGCTTCGCTCCGAAA |
| CCTGAAACACGCCAGAAGACCGCGTTTATTGGC | CCTGAAACACGCCAGAAGACCGCGTTTATTGGC |
| AGCTGTTATAGTATGACTCGAGCACAGGCGAACC | AGCTGTTATAGTATGACTCGAGCACAGGCGAACC |
| TGCTGGGCGGAAAGCGAAAAAAGCTGAAGCGACGAA | TGCTGGGCGGAAAGCGAAAAAAGCTGAAGCGACGAA |
| GGCGGCAGAAAGATCGCGAGCCACCCAAACCAAC | GGCGGCAGAAAGATCGCGAGCCACCCAAACCAAC |
| GACACCACCACTGGTACCCACCACTGGTACCCACCA | GACACCACCACTGGTACCCACCACTGGTACCCACCA |
| GACACCACCACTGGTACCCACCACTGGTACCCACCA | GACACCACCACTGGTACCCACCACTGGTACCCACCA |
| GACACCACCACTGGTACCCACCACTGGTACCCACCA | GACACCACCACTGGTACCCACCACTGGTACCCACCA |
| ATCAGGTAGTACCTACGCGAAGGCGAGATATGGG | ATCAGGTAGTACCTACGCGAAGGCGAGATATGGG |
| TATGCTGGGCGGAAAGCGAAAAAAGCTGAAGCGACG | TATGCTGGGCGGAAAGCGAAAAAAGCTGAAGCGACG |
| ATGCTGGGCGGAAAGCGAAAAAAGCTGAAGCGACG | ATGCTGGGCGGAAAGCGAAAAAAGCTGAAGCGACG |
| GGCGGCAGAAAGATCGCGAGCCACCCAAACCAAC | GGCGGCAGAAAGATCGCGAGCCACCCAAACCAAC |
| GACACCACCACTGGTACCCACCACTGGTACCCACCA | GACACCACCACTGGTACCCACCACTGGTACCCACCA |
| GACACCACCACTGGTACCCACCACTGGTACCCACCA | GACACCACCACTGGTACCCACCACTGGTACCCACCA |
| GACACCACCACTGGTACCCACCACTGGTACCCACCA | GACACCACCACTGGTACCCACCACTGGTACCCACCA |

| GGATCCGATGACACCACCAACTCTACGCGACCACCACCAC | GGATCCGATGACACCACCAACTCTACGCGACCACCACCAC |
| ATGCTCGACCACCCCACTCACTCACTCACTCACTCACTCA | ATGCTCGACCACCCCACTCACTCACTCACTCACTCACTCA |
| TCAACACACCACGGTGACAGACTCAACCACCCCAAGC | TCAACACACCACGGTGACAGACTCAACCACCCCAAGC |
| GCACCGACAAACGCGGCCCTCAACCAGCCAAACAGTC | GCACCGACAAACGCGGCCCTCAACCAGCCAAACAGTC |
| ATGTCACCACCACCCCAACCCCAATACCCACCAAT | ATGTCACCACCACCCCAACCCCAATACCCACCAAT |
| CGCGAGCGAAAGGCGGCCTCAACCAGCCAAACAGTC | CGCGAGCGAAAGGCGGCCTCAACCAGCCAAACAGTC |
| GGATCCGATGACGATGACACCACCAACTCTACGCGACC | GGATCCGATGACGATGACACCACCAACTCTACGCGACC |
| GGATCCGATGACGATGACACCACCAACTCTACGCGACC | GGATCCGATGACGATGACACCACCAACTCTACGCGACC |
| ATGCTCGACCACCCCACTCACTCACTCACTCACTCACTCA | ATGCTCGACCACCCCACTCACTCACTCACTCACTCACTCA |
RLP6-halfo

RLP6 resilin fused to the hydrophobic domain of half oleosin. Expression tested, soluble.

CATATGCAACCACCATCACCCACCaTCACAAGCTTAGATCTGGCCGTTGTCGCCATCGACTCTAAGGCGGCTCCGGCCGCAGGCATGCTCTAAGGCGGCGGCAACGGCGGCCGTCCGAGCGATTCTAAGGCGCGCCGGGTGGCGGCATGGCGGTCGTCCGAGTGATTCAAAAGGTGCTCCGGGCGGTGGTAACGGGATCTGGCGGTCGTCCGTCAGACTCTAAAGGCGCTCCGGGCGGCGGCAACGGCGGCCGTCCGAGCGATTCTAAGGCGCGCCGGGTGGCGGCATGGCGGTCGTCCGAGTGATTCAAAAGGTGCTCCGGGCGGTGGTAACGGGATCTGGCTCGCTCTCGGGACTCCGCTGTTTGGTGTTATAGGTTTCAGCCCTGTTATTGTTCCAGCGATGATACTGGGCTTGCGGGTTACCGGGTTTTGACTTCGGGACGTTCGGGTTAACGGGGTTAAGCTCGTTGTCGTATTTGTTTAATATGGTGAGGCGGTCGACAATGTCGGTTCCAGTCTGGCAGGATAACGTGAACGGGGAATGTGGGAGCAGACGGGCCAGAACACGAACGACTTGGGCCAGCAGATACAGCATAAACGCCCATGAAATGGGTGACCAGGGCCAGGGTCAGGGTGGTGGGAACGAAGGGCAGAACGAGGGGGGAACCACCACCACCACCACCACGATGACGTACTCGAG

CATATGCACCATCACCCACTACAAAGCTTAGATCTGGCCGTTGTCGCCATCGACTCTAAGGCGGCTCCGGCCGCAGGCATGCTCTAAGGCGGCGGCAACGGCGGCCGTCCGAGCGATTCTAAGGCGCGCCGGGTGGCGGCATGGCGGTCGTCCGAGTGATTCAAAAGGTGCTCCGGGCGGTGGTAACGGGATCTGGCTCGCTCTCGGGACTCCGCTGTTTGGTGTTATAGGTTTCAGCCCTGTTATTGTTCCAGCGATGATACTGGGCTTGCGGGTTACCGGGTTTTGACTTCGGGACGTTCGGGTTAACGGGGTTAAGCTCGTTGTCGTATTTGTTTAATATGGTGAGGCGGTCGACAATGTCGGTTCCAGTCTGGCAGGATAACGTGAACGGGGAATGTGGGAGCAGACGGGCCAGAACACGAACGACTTGGGCCAGCAGATACAGCATAAACGCCCATGAAATGGGTGACCAGGGCCAGGGTCAGGGTGGTGGGAACGAAGGGCAGAACGAGGGGGGAACCACCACCACCACCACCACGATGACGTACTCGAG

CATATGCAACCACCATCACCCACCtaTCACAAGCTTAGATCTGGCCGTTGTCGCCATCGACTCTAAGGCGGCTCCGGCCGCAGGCATGCTCTAAGGCGGCGGCAACGGCGGCCGTCCGAGCGATTCTAAGGCGCGCCGGGTGGCGGCATGGCGGTCGTCCGAGTGATTCAAAAGGTGCTCCGGGCGGTGGTAACGGGATCTGGCTCGCTCTCGGGACTCCGCTGTTTGGTGTTATAGGTTTCAGCCCTGTTATTGTTCCAGCGATGATACTGGGCTTGCGGGTTACCGGGTTTTGACTTCGGGACGTTCGGGTTAACGGGGTTAAGCTCGTTGTCGTATTTGTTTAATATGGTGAGGCGGTCGACAATGTCGGTTCCAGTCTGGCAGGATAACGTGAACGGGGAATGTGGGAGCAGACGGGCCAGAACACGAACGACTTGGGCCAGCAGATACAGCATAAACGCCCATGAAATGGGTGACCAGGGCCAGGGTCAGGGTGGTGGGAACGAAGGGCAGAACGAGGGGGGAACCACCACCACCACCACCACGATGACGTACTCGAG

RLP6-30G

RLP6 resilin fused to the hydrophobic domain of oleosin-30G. Expression tested, soluble.

GGATCCGAAGCCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAACCACCAGTCGACACCACCACCAACAAACGCAGGACCAAC
Oleosin-65 with highly anionic N- and C-terminal arms. High expression, soluble.

GGATCCGAAGCCACCACAACCAAGACCAGACCACC
ATGTCAACCACAACCACAACCCCAAGATCAGCATGA
TCAACACAGCGTGAGAACCTCAACCACCACCAAC
GACCCAGAACGAGGCCCCCTCAACCAGCACCAAAAT
CCGGGAATCTTTTGTTTGTTTACCGGTATCCCTCAT
GTCGCTGTTTATATTCAGCCCATGTTATTGTTCCAG
CGATGATAGCGATTGGGCTTGCGGTTACCGGGTTTT
TTGACTTCGGGTACGTTCGGGTTAACCGCGTACAG
AATGTCGGTTCTACGCTTGAGTTAACGGCGTACAGC
ATACGGCCCATGAAATGCTGACAGGACAGCAGCG
TGACGGTCAAGGTTGCTGGGAAAGGACGACAGA
CAAGGGAACACACCAACCACCACCACCACCACG
ATGACTGACTCGAG

Oleosin-45 with highly anionic N- and C-terminal arms. High expression, soluble.

GGATCCGAAGCCACCACAACCAAGACCAGACCACC
ATGTCAACCACAACCACAACCCCAAGATCAGCATGA
TCAACACAGCGTGAGAACCTCAACCACCACCAAC
GACCCAGAACGAGGCCCCCTCAACCAGCACCAAAAT
CCGGGAATCTTTTGTTTGTTTACCGGTATCCCTCAT
GTCGCTGTTTATATTCAGCCCATGTTATTGTTCCAG
CGATGATAGCGATTGGGCTTGCGGTTACCGGGTTTT
TTGACTTCGGGTACGTTCGGGTTAACCGCGTACAG
AATGTCGGTTCTACGCTTGAGTTAACGGCGTACAGC
ATACGGCCCATGAAATGCTGACAGGACAGCAGCG
TGACGGTCAAGGTTGCTGGGAAAGGACGACAGA
CAAGGGAACACACCAACCACCACCACCACCACG
ATGACTGACTCGAG

Oleosin-30 with highly anionic N- and C-terminal arms. High expression, soluble.

GGATCCGAAGCCACCACAACCAAGACCAGACCACC
ATGTCAACCACAACCACAACCCCAAGATCAGCATGA
TCAACACAGCGTGAGAACCTCAACCACCACCAAC
GACCCAGAACGAGGCCCCCTCAACCAGCACCAAC
GCTCTCGGCAGCTCGTTTGTTATATTCAGCCCATG
GTCGCTGTTTATATTCAGCCCATGTTATTGTTCCAG
CGATGATAGCGATTGGGCTTGCGGTTACCGGGTTTT
TTGACTTCGGGTACGTTCGGGTTAACCGCGTACAG
AATGTCGGTTCTACGCTTGAGTTAACGGCGTACAGC
ATACGGCCCATGAAATGCTGACAGGACAGCAGCG
TGACGGTCAAGGTTGCTGGGAAAGGACGACAGA
CAAGGGAACACACCAACCACCACCACCACCACG
ATGACTGACTCGAG