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Selective extraction of colorful proteins using reverse micelles

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SELECTIVE EXTRACTION OF COLORFUL PROTEINS USING REVERSE MICELLES

By
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ABSTRACT

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Selective Extraction of Colorful Proteins Using Reverse Micelles
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Biotechnology is an ever expanding field of science. As the field has expanded, research into bioseparation processes has also expanded. The need for more cost effective ways to deliver products that are in high demand is greater now than ever. Bioseparation processes are a very costly part of the overall process of product development. While traditional separation process are effective, there is promise in many new techniques being developed which have the potential to lower the costs of bioseparations.

The continuous separation method of liquid-liquid extraction using reverse micelles is one such technique. While this technique has been researched in the past, previous studies lack information about realistic complex protein mixtures.

Through this study optimum conditions were found for the individual extraction of horse heart cytochrome *c*, EGFP, *Cyanobacterium anabaena* flavodoxin, and DsRed2 from reverse micellar systems. Experiments were also conducted using crude lysate and protein mixtures. Ultimately more research needs to be conducted with reverse micelles for it to become an accepted bioseparation technique, but this research aims to add to the information available on reverse micelle systems.

Acknowledgements

I would like to thank my family and friends for supporting me though this challenging period of my life. I want to thank my Aunt Loretta for encouraging me to go to graduate school in the first place. Whether you realized it or not, it was really your kind words that pushed me to go ahead with that choice. Thanks also to my godfather Ted Salkowski for his continued support throughout my education and for always being interested in my pursuits, especially when no one else found them particularly interesting. I would also like to thank Joe Keenan for sticking with me through my ups and downs along the way, and for helping me to keep a positive attitude. Thanks also to Lisa Scodari, Kyle Smith, Ted Cohen, Eric Skibbe, and Frank Romanski, who all helped me in the laboratory in one way or another. Finally many thanks to Dr. Lefebvre, for his advice along the way.

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Chapter 1: Introduction

1.1 Biotechnology

Biotechnology can be defined as "the application of scientific and engineering principles to the processing of materials by biological agents."[1] The field of biotechnology emerged in the mid-1970s when recombinant DNA technology and hybridoma technology were developed.[2] Since that time the field has made leaps and bounds in scientific advances.

Biotechnology has forever changed the health care industry. The development of biopharmaceuticals is one of the largest contributions of biotechnology. Biopharmaceuticals that have been developed include insulin for diabetes, erythropoietin for anemia, blood clotting factors for hemophilia, as well as various vaccines and cancer drugs. The first biopharmaceutical product to be approved in the US was the recombinant human insulin, Humulin, by Eli Lilly in 1982. By 1993 the global sales of biopharmaceuticals were around US $5 billion.[2] In 2005, the global sales of biopharmaceuticals were over US $40 billion, and this market continues to grow.[1]

While the development of biopharmaceuticals is a fantastic advance, it is by no means the only part of biotechnology to affect the healthcare industry. Advances in biotechnology have also changed the way traditional pharmaceuticals are made, creating

more efficient processes. New methods of medical testing have resulted from biotechnology as well as the exciting avenues of tissue and organ engineering.

The advent of biotechnology has also challenged us to find ways in which traditional processes can be changed and improved. The chemical industry is looking toward using greener methods of production. Biotechnology is being used to create more environmentally sound fuels, agricultural chemicals, and biodegradable materials. Alternatives for environmental bioremediation have also become available. Biotechnology has also significantly impacted food processing, criminal investigation, and even art preseravation.[1, 3]

Biotechnology is currently changing the world for the better with advances leading to environmental and economic benefits.

1.2 Bioseparations

Undoubtedly biotechnology plays a large role in making established processes more efficient. There is always room for improvement on any process. Bioseparation processes and downstream processing equipment represent a major cost in the biotechnology industry. This step alone accounts for about 50% of the production cost of a biological product.[4] In any process, separations represent a large part of the overall product cost, but in biological separations there is added difficulty.

In the area of biopharmaceuticals, products are typically produced by recombinant DNA technology within *Escherichia coli* or Chinese Hamster Ovary (CHO) cells. This type of fermentation technology is used because the protocols are well established, but the resulting products have to be extensively purified to meet the required standards.[2]

Biological products necessitate unusual requirements. These products are fragile and are sensitive to stresses like shearing forces, vibration, heat, and electromagnetic fields. Also, typically in biological processes there is a high throughput of material to deal with which contains very little of the desired product.[5] The product is also often mixed with contaminants that have similar properties.[6] Purification of such products is an expensive and time consuming ordeal that frequently requires many batch separation steps.

Separation processes for biotechnology exist to process the outputs of bioreactors or fermentation products. These processes can be divided into several steps for which different techniques are typically used. All processes start with primary recovery, where suspensions of cells are separated through the use of techniques such as filtration and centrifugation. Primary recovery is an initial phase, which is less complicated and eventually leads to final purification of products, which can become very complex. Final purification of products can involve a large number of unit operations before the final product is obtained.

Techniques currently used for final purification processes include chromatography, membrane filtrations, and liquid extraction. Final purification can also be thought of as consisting of three different phases. These are capture (or concentration), separation (or fractionation), and polishing.[6]

Traditional bioseparations are often very dependent on packed-bed chromatography, which is an expensive operation. Chromatography is also a batch operation, and is time consuming as well. However, chromatography is used because it offers high purity of the product.[7]

There are currently several variations of chromatography that are used for the separation of biological products. These include adsorption chromatography, ion-exchange chromatography, size-exclusion chromatography, hydrophobic interaction chromatography, affinity chromatography, normal phase chromatography, and reversed phase chromatography.[8]

In order to reduce the overall cost of bioseparations, traditional processes like chromatography need to re-evaluated. While the traditional bioseparations are not likely to be completely phased out and replaced by alternative bioseparations, changes need to be made to limit costs.

Since the product in question is often contained in a very dilute feed, the volume of material to process greatly affects the cost of the product recovery.[6] Reduction of this overall volume at an early stage in processing can help to limit this burden.

While chromatography may still be used at the end of a process, there are many bulk separations that can be utilized before the use of chromatography to lessen the load. Some examples of such bulk separations include aqueous two-phase extraction, three-phase partitioning, precipitation, and crystallization techniques.[7] There are also various membrane filtration techniques, such as ultrafiltration, microfiltration, normal flow clarification, virus filtration, and high performance tangential flow filtration.[9] Adsorptive separations, such as monoliths, can also be utilized prior to chromatography.[7] Membrane chromatography is another alternative with advantages over packed bed chromatography. However, more research needs to be conducted before this technique can be fully utilized.[10]

Each bioseparation process is unique, and needs to be considered as such when looking at alternatives to traditionally used methods. Liquid-liquid extraction with reverse micelles is a bioseparation technique with potential for commercial use. This process could be utilized before or in place of chromatography steps to limit the overall cost of bioseparation processes. The equipment necessary is typical of liquid-liquid extraction processes, which is less costly than equipment used for chromatography. It also has the benefit of being a continuous process, so it is not as time consuming as traditional bioseparations.

Investigation into reverse micelle systems began in the early 1980's, and since that time interest in these systems has grown increasingly.[11, 12] As with any new process there are complications and limitations that need to be overcome before the technique can be used commercially. This research study hopes to add to the information currently available on reverse micelle systems and to further develop this process as an accepted bioseparation.

Chapter 2: Background

2.1 Reverse Micelle Technology

Reverse micelles are nanometer sized water droplets surrounded by surfactant molecules solubilized within an organic solvent.[13] They have the ability to solubilize organics like amino acids and proteins.

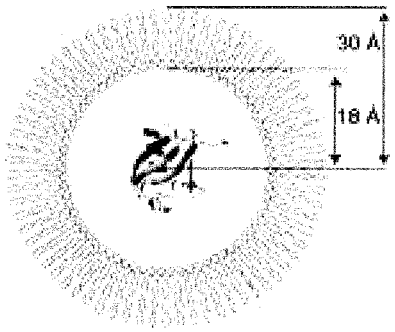

Figure 2.1: Protein in AOT Reverse Micelle System,
Figure Adapted from Babu et al.[14]

Since the biomolecule of concern is contained within an aqueous environment throughout the process, the biological activity is usually preserved.[13] In the liquid-liquid extraction process using reverse micelles an aqueous phase and an organic phase are contacted. The aqueous phase typically consists of a buffer solution with proteins, and some salt content. The organic phase is usually composed of a surfactant in an organic solvent, though sometimes a co-surfactant is used in the system as well. Through manipulation of experimental conditions within the system, biomolecules can be selectively extracted from the aqueous phase to the organic phase. After this initial

transfer, the biomolecules extracted into the organic phase can be back-extracted into a fresh aqueous phase.

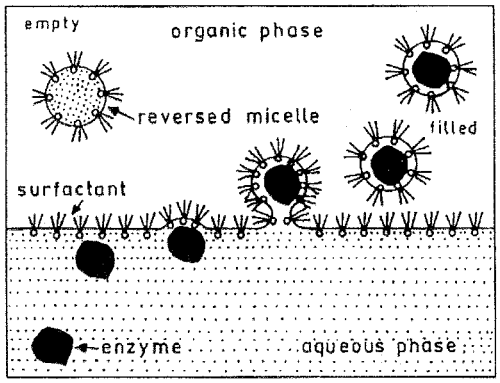

Figure 2.2: Protein Solubilization Mechanism,
Figure Adapted from Krishna et al.[13]

Investigation into reverse micelle systems began in the early 1980's.[11, 12] Since that time further research into these systems has been conducted. Several reviews of reverse micelle systems are currently available. [13, 15-18]

Many factors directly affect the transfer of biomolecules into and out of reverse micelles. In the aqueous phase the pH, ionic strength, and type of electrolyte play an important role. In the organic phase the type of surfactant used and the surfactant concentration in the system are primary factors affecting transfer. There are also many other factors that can affect transfer including the charge distribution of the protein, size of the protein, temperature, and the mixing time allowed for the process.[15]

In reverse micelle systems the forward transfer of proteins or other biomolecules is primarily due to electrostatic interactions. In most cases the protein desired for transfer and the surfactant used in the system need to bear opposite charges in order for transfer to

take place.[17, 19, 20] Hydrophobic interactions within the system, however, also play a role in forward transfer of proteins.[15, 21]

Electrostatic interactions in the system can be affected by the ionic strength of the aqueous phase. As ionic strength increases, the electrostatic interactions decrease and smaller micelles are created. This hinders the solubilization capacity of the micelles; however this capacity is different for individual proteins.[20]

The type of electrolyte affects transfer not only because of the ionic strength it brings to the solution, but also due to interactions of various ions with the other constituents of the micelles. Studies have been conducted on the effects of various ions on protein extraction into reverse micelle solutions, and it was found that extraction was greatly affected by the specific species of cation.[22]

Hydrophobic interactions dominate when the pH of the system is close to the isoelectric point (pI) of the protein of concern. The pI is the pH at which a protein has no net charge. When the protein does not have a net charge, there is no charge based interaction, so the driving force is most likely a hydrophobic interaction between the protein and surfactant used.[21]

Protein size also plays a role in the effect of electrostatic interactions. A larger protein will require more charge for transfer in order to adapt the micelle to the required size. Hence larger proteins require a pH further from their pI's due to this effect. In contrast, smaller proteins can be extracted into reverse micelles at pH's much closer to their pI's.[19, 23]

Similar to forward transfer of biomolecules, back-extraction is also affected by various influences of the system. In simple situations, the protein can be back-extracted

through altering conditions in the fresh aqueous phase. For example, by increasing the ionic strength of the aqueous phase, the proteins contained in the reverse micelles can be expelled due to electrostatic repulsion.[15] However, the back extraction of proteins from reverse micelle systems can in some cases become very complicated, as will be discussed further later.

Regardless of the exact conditions of the solutions used in the system, there are three techniques which are commonly utilized for creating reverse micelle solutions. This can be accomplished through the mixing of a dry, lyophilized protein with an organic surfactant solution, or through the injection of a concentrated protein containing aqueous solution into an organic surfactant solution. Lastly, this can also occur through the bulk phase transfer between an aqueous, protein containing solution, and an organic surfactant solution, which this particular study is concerned with.[4, 13]

2.2 Experimental Studies with Reverse Micelle Systems

The first suggestion for the use of reverse micelle systems for protein extraction was by Luisi, et al., in 1979. These early studies used the cationic surfactant methyl-trioctylammonium chloride (TOMAC) in cyclohexane for the transfer of α-chymotrypsin, trypsin, pepsin, and glucagone into reverse micelles.[11]

In the 1980's further studies were conducted with reverse micelles using the anionic surfactant sodium bis(2-ethylhexyl) sulfosuccinate (AOT) in isooctane to solubilize horse heart cytochrome *c* and other proteins.[12, 20, 23] Cytochrome *c* has been used in many studies with reverse micelles, and successfully transferred into reverse micelles through liquid-liquid extraction on numerous occasions.[12, 20, 23-25]

In 1985, Goklen and Hatton, demonstrated the forward and backward extraction of cytochrome *c* in AOT/isooctane reverse micelle systems. They observed the effects of ionic strength of solution, and the time needed for transfer of the proteins in and out of the system. They found that ionic strength dominated protein transfer and that the back-extraction of proteins was much slower due to weak driving forces.[12]

In 1987, Goklen and Hatton, looked into the effect of pH as well as salt on the transfer of cytochrome *c*, as well as several other proteins.[20] Later studies have looked into other system factors influencing cytochrome *c* transfer including surfactant concentration, and mixing conditions.[24, 25]

Many other individual proteins have also been encapsulated in reverse micelle systems.[21, 23, 26 - 30] In 1996, the liquid-liquid extraction of 30 proteins had been studied in reverse micelles with various surfactants, mainly AOT and TOMAC.[15] It has also been shown that cytochrome *c* is unfolded when encapsulated in AOT reverse micelles. Using an AOT/$C_{12}E_4$ surfactant system has been found to help with this problem.[31, 32]

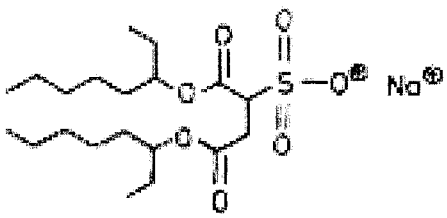

Figure 2.3: Surfactant AOT (bis(2-ethylhexyl) sodium succinate)[33]

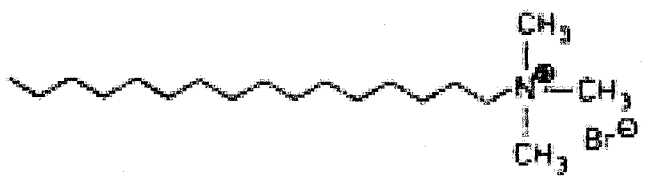

Figure 2.4: Surfactant CTAB (cetyltrimethylammonium bromide)[34]

While extraction studies with individual proteins are very useful in adding to the understanding of protein and system behavior in reverse micelle processes, the process has no benefit for use on already purified proteins. Few studies have looked at how fermentation broth constituents complicate the extraction of proteins.[35]

Similarly, after fermentation the desired protein product is contaminated with not only fermentation broth constituents, but also with other proteins. In recombinant protein expression with bacteria, and with human blood plasma, there are a wide range of proteins present at various concentrations which can make the purification of a single protein from that mix very complicated. [36-38]

Relatively few studies have investigated protein mixtures. In 1987, Goklen and Hatton, conducted studies on the selective separation of cytochrome *c*, lysozyme, and ribonuclease A from an AOT/isooctane reverse micelle system. They were able to successfully extract each individual protein from the reverse micelles using a single extraction step and two stripping steps.[20] In 2000, Stuckey and coworkers also used this mixture of proteins to conduct studies.[24, 25] They also looked at this mixture of proteins with filtered fermentation broth, and found that some of the broth components acted as cosurfactants in the system, but a high recovery yield of protein was still obtained.[24]

2.3 Recent Developments and Complications with Reverse Micelle Systems

Some studies with reverse micelles have revealed complications. In some cases, back-extraction of proteins out of reverse micelle systems can be very difficult. It is believed that problems with back-extraction arise from protein-micellar interactions.

Several solutions to this problem have been investigated, such as the addition of alcohols, adjustment of temperature conditions, and addition of carboxylic acids.[39]

Back-extraction is also a more time consuming process than forward extraction. The addition of alcohols has been shown to improve the rate of back extraction of proteins.[21, 40, 41]

However, in some cases alcohol addition alone does not solve back-extraction problems. Mathew et al., attempted the addition of various alcohols to a reverse micelle system with papain and AOT, and the alcohol addition caused a loss in activity of the papain. The problem was alleviated by the addition of TOMAC to the system, which resulted in high recovery yields and activity. This improvement was believed to be caused because of electrostatic interactions between the AOT and TOMAC molecules.[42]

Another problem that can arise with reverse micelle systems is the denaturation of proteins. This occasionally occurs in reverse micelle processes as a result of the strong electrostatic interactions between protein and surfactants. One solution that has arisen to this problem is the use of non-ionic surfactants for protein extraction in reverse micelle systems.[43, 44]

It has also been discovered that reverse micelles can be used as a protein activation medium. In one recent study, solid denatured RNase A was refolded in a reverse micellar solution.[45] A similar experiment was performed with denatured bovine heart cytochrome *c*. The denatured cytochrome *c* was solubilized in AOT reverse micelles and renatured cytochrome *c* was recovered through back extraction.[41]

Bioaffinity ligands have also been recently investigated for use in reverse micelle systems. It has been found that the incorporation of bioaffinity ligands to reverse micelle systems can increase the selectivity and protein transfer in some cases. [44, 46]

There has also been some recent research involved with the extraction of enzymes from whole cells using reverse micelle systems. Here the focus is the permeabilization of the outer membrane of *E. coli* cells for the selective release of enzymes from the periplasm using AOT/hydrocarbon reverse micelle systems.[47-50]

While most research has focused on protein extraction with reverse micelle systems, other applications have also arisen. One such example is the use of liquid-liquid extraction using reverse micelles for the removal of dyes in the textile and paper industries.[51]

Recently a different approach was taken on the typical liquid-liquid extraction with reverse micelle process. While typically the process requires two steps, the forward and backward extraction of the protein of concern, a study has focused on performing a single step extraction with reverse micelles. In this case lysozyme was selectively extracted from reconstituted freeze-dried egg white using cetyldimethylammonium bromide (CDAB). In this case the lysozyme stayed in the aqueous phase, while the egg white proteins were transferred into the organic phase.[52]

The liquid-liquid extraction process with reverse micelles has been classified as easy to scale-up, however some problems with emulsion formation have been observed and could potentially complicate the scale-up of such a process.[53, 54]

2.4 The Goals of This Reverse Micelle Study

As previously mentioned, few studies have been conducted on reverse micelle systems using protein mixtures. Of the studies that have been done with mixtures, all have focused on lysozyme (pI =11), cytochrome *c* (pI =10.6), and ribonuclease A (pI = 7.8).[20, 24, 25] This mixture, however, is not necessarily a good representation of protein mixtures typically found in nature and has proven easy to separate. A study with 103 proteomes showed that a typical distribution shows "butterfly" patterns on theoretical 2-D gels.[55] Typical proteome distributions have a wide array of both acidic and basic proteins, as well as many proteins that are close in pI. This study seeks to discover if reverse micelle extraction would work with such a protein mixture, which by its nature is more difficult to separate.

In this project the proteins used are believed to be more representative of a typical protein mixture and will be cytochrome *c* from horse heart (pI = 10.6), EGFP from the jellyfish *Aequorea victoria* (pI = 5.6), DsRed2 from a *Discosoma* genus of coral (pI = 6.3), and flavodoxin from *Cyanobacterium anabaena* (pI = 4.2).[20, 56–59]

These proteins were chosen based on physical properties and for their unique ability to be individually monitored through the use of UV/vis spectroscopy.

Table 2-1: Physical properties of the colorful proteins used in this study

Protein Name	Color (λ_{max})	Molecular Weight	Isoelectric Point[59]
Cytochrome *c*	Red (410 nm)	12 kDa[20]	10.6[20]
EGFP	Green (488 nm)	27 kDa[64]	5.6
Flavodoxin	Orange (464 nm)	19 kDa	4.2
DsRed2	Pink (561 nm)	103 kDa[56]	6.3

As previously mentioned, cytochrome *c* has been widely studied and encapsulated in reverse micelles through liquid-liquid extraction on many occasions. DsRed, EGFP, and flavodoxin have been encapsulated in reverse micelles previously through the injection method.[60 - 62]

This project will use UV/vis spectroscopy to monitor the presence of proteins within reverse micellar solutions. It has been shown that encapsulation in AOT reverse micelle systems does not affect the optical properties of EGFP, DsRed2, horse heart cytochrome *c*, or a flavodoxin from another species.[24, 60, 61, 63]

This project seeks to add to the available information on this bioseparation process by investigating the forward transfer of individual proteins into reverse micellar systems as well as selective extraction of the proteins from a complex protein mixture.

Chapter 3: Cytochrome *c* Experiments

3.1 Experimental Methods

The extraction of horse heart cytochrome *c* using reverse micelles was the first system investigated for this project. These experiments were conducted in order to replicate previous results obtained with cytochrome *c*.[12, 24] The replication of these results was performed in order to ensure that the experimental procedures used were adequate.

Horse heart cytochrome *c* was purchased commercially for use in these experiments from Sigma-Aldrich (Product # C 7752, Lot # 102K7053).

The first experiments preformed using cytochrome *c* were experiments in which surfactant concentration was varied in the system. Following the success of these experiments, salt concentration and pH were then varied.

For the experiments with varying surfactant concentration the organic phase consisted of the surfactant AOT (Sigma-Aldrich, Product # D 4422) in isooctane (Sigma-Aldrich, Product # 258776). AOT concentration ranged from 5 to 50 mM. The aqueous phase consisted of 1 g/L cytochrome *c* dissolved in a mixture of 80 % 0.1 M NaCl and 20 % 0.1 M Tris solution. The pH of the aqueous phase was about 9.7 after cytochrome *c* was added.

Equal volumes (750 μL) of the aqueous and organic phases were contacted in a 2 mL glass vial (Fisher Scientific, Cat. No. 03-338AA). The vials were placed in a Barnstead/Thermolyne Labquake® Rotisserie at 8 rpm for 1 hour in order to obtain equilibrium. This time was thought to be more than adequate for the forward transfer of

the proteins. Previous studies have noted that forward transfer can take place on the order of seconds, and that forward transfer is usually complete within 5 minutes.[12, 35] Afterwards the samples were centrifuged in a Forma Scientific, Inc. Refrigerated Centrifuge for 5 minutes at 3000 rpm in order to separate the phases.

Samples were analyzed with a Hewlett Packard 8453 Spectrophotometer at 280 and 410 nm. The organic phase of each sample was analyzed first. Using a Gilson Pipetman micropipettor some of the organic phase was pulled off and diluted with isooctane as needed to obtain a reading. Once a reading for the organic phase was obtained, the remaining organic phase was pulled off and discarded. It was found that the organic phase should be pulled off before attempting to read the aqueous phase, so that the aqueous phase would not be contaminated. The aqueous phase was then sampled and diluted with DI water as needed.

A cleaning procedure was also developed for cleaning the quartz cuvettes used for sampling the phases. When the quartz cuvettes were used, the cuvettes used for the organic phase were rinsed with isooctane, then acetone, and then isooctane. The quartz cuvettes used for aqueous phase were rinsed with DI water, then acetone, and then DI water again. In between experimental runs the quartz cuvettes were soaked in a nitric acid bath.

Following the success of the surfactant variation experiments it was found that using less protein in the samples would aid in the ease of sampling as well as decreasing error associated with the dilution of the samples. It was also decided that a slightly larger sample volume would aid in sampling ease. In order to make sure that these changes

would not affect the results, the surfactant variation experiments were performed again, this time with 900 μL per phase, and less cytochrome *c* (~ 0.22 g/L).

The surfactant variation tests were also performed again with 900 μL per phase and with the original amount of cytochrome *c* (1 g/L), to ensure that the volume of the phases did not affect the results.

For the experiments in which salt concentration was varied the organic phase consisted of the surfactant AOT in isooctane. AOT concentration was held at 50 mM. Aqueous phase consisted of ~ 0.26 g/L cytochrome *c* dissolved in 0.02 M Tris solution at pH ~ 9.7. The salt concentration was varied from 0 to 0.8 M NaCl in the aqueous phase.

Equal volumes (900 μL) of the aqueous and organic phases were contacted in a 2 mL glass vial, and the experimental procedure followed that of the surfactant variation with cytochrome *c*.

Following the experiments with salt concentration variation, the pH in the system was investigated. For these tests the pH of the aqueous phase was varied from ~ 3 to 11 using phosphate, acetate, and carbonate buffers, while the salt concentration in the system was held at 0.08 M NaCl. Acetate buffer was used for the 3-5 pH range, phosphate buffer was used for the 6-8 pH range, and carbonate buffer was used for the 9-12 pH range. There was ~ 0.22 g/L cytochrome *c* in the aqueous phase. The organic phase consisted of 50 mM AOT in isooctane.

The experimental procedure was the same as for both the surfactant and salt variation experiments.

3.2 Results

In order to interpret the results for the experiments using cytochrome *c*, a calibration curve was developed for cytochrome *c* using the spectrophotometer at 410 nm. This curve was used to find the concentration of cytochrome *c* in each sample.

Each experiment was performed in triplicate to ensure the accuracy of the results. To observe the general trend for each experiment the average result of the three replicates was found and is shown in each representative graph of results. The percent of cytochrome *c* that was extracted into the organic phase was determined through comparison with the original amount of cytochrome *c* in the system. For example, the extraction yield was defined as in Equation 3.1 below:

$$\left(\frac{\text{Protein Conc. In Organic Phase}}{\text{Original Protein Conc. In System}}\right) * 100 = \text{Extraction Yield \%} \qquad \text{(Equation 3.1)}$$

These values were based on spectrophotometric readings at the appropriate wavelength. These values were believed to be accurate estimates of the concentration of protein in the sample of concern as reverse micelles are optically transparent.[17, 65] These readings were in the visible range of the spectrophotometer, and were taken using a quartz cuvette with a 0.5 cm path length.

In each of the representative graphs of results, the standard deviation between the three replicates is shown as error bars. Detailed sample compositions and experimental data can be found in Appendix A.

3.2.1 Surfactant Variation

Three different experiments were conducted with cytochrome *c* where surfactant (AOT) concentration was varied in the system. In the first experiment, the volume of each phase was 750 μL, and ~ 1 g/L of cytochrome *c* was present in the system. Following the success of this experiment, the conditions were slightly altered for ease in sampling. The second experiment used a volume of 900 μL per phase, and ~ 0.22 g/L cytochrome *c*. Finally, in order to ensure that the amount of protein in the system did not affect the results a third experiment varying surfactant concentration was conducted. In this experiment, there was again 900 μL of volume per phase, and ~ 1 g/L cytochrome *c*.

Figure 3.1 below shows the results of the surfactant variation experiments where there was 750 μL per phase and ~ 1 g/L cytochrome *c* in the system.

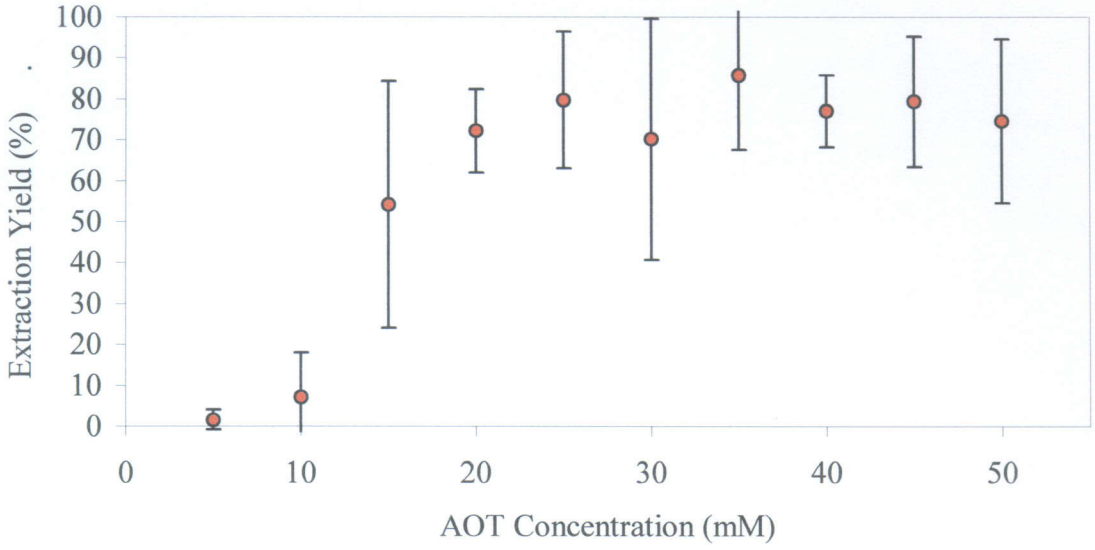

Figure 3.1: Effect of Surfactant Variation on Cytochrome *c* Extraction

Readings of Organic Phase at 410 nm

750 µL per phase

Organic Phase: 5 - 50 mM AOT in isooctane

Aqueous Phase: 80 % 0.1 M NaCl and 20 % 0.1 M Tris solution at pH ~ 9.7, and ~ 1 g/L cytochrome *c*

Following the success of this experiment, the experimental conditions were altered slightly in order to aid in the ease of sampling. The surfactant concentration was varied again, but this time the amount of liquid in each phase was increased to 900 µL. The amount of cytochrome *c* in the system was reduced as well to ~ 0.22 g/L. The results of this experiment are shown in Figure 3.2 below.

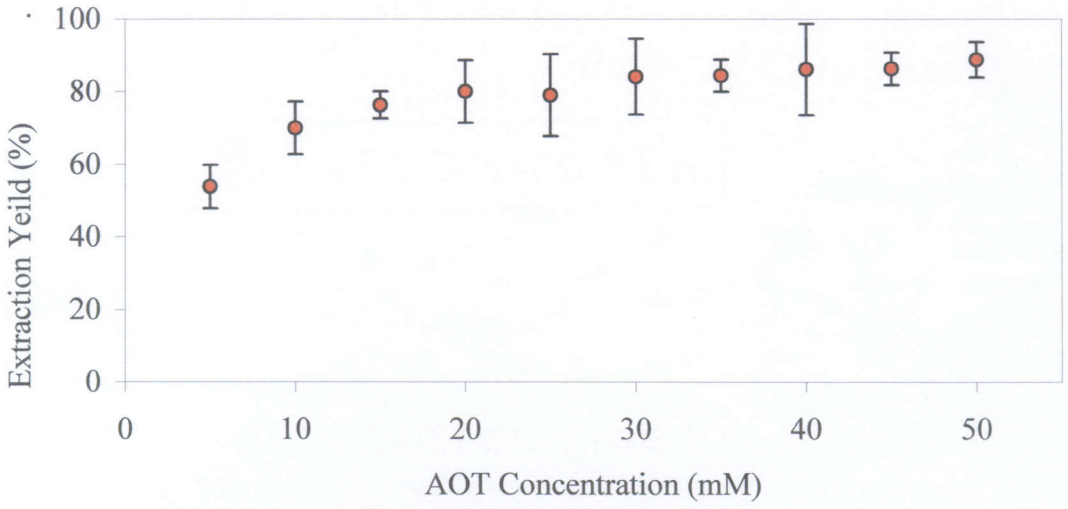

Figure 3.2: Effect of Surfactant Variation on Cytochrome *c* Extraction

Readings of Organic Phase at 410 nm

900 μL per phase

Organic Phase: 5 - 50 mM AOT in isooctane

Aqueous Phase: 80 % 0.1 M NaCl and 20 % 0.1 M Tris solution at pH ~ 9.7, and ~ 0.22 g/L cytochrome *c*

Another surfactant variation experiment was performed to prove that the same results would be obtained with a greater amount of protein. This time the volume of each phase was at 900 μL, but the cytochrome *c* concentration was at ~ 1 g/L as in the original experiment. The results are shown in Figure 3.3 below.

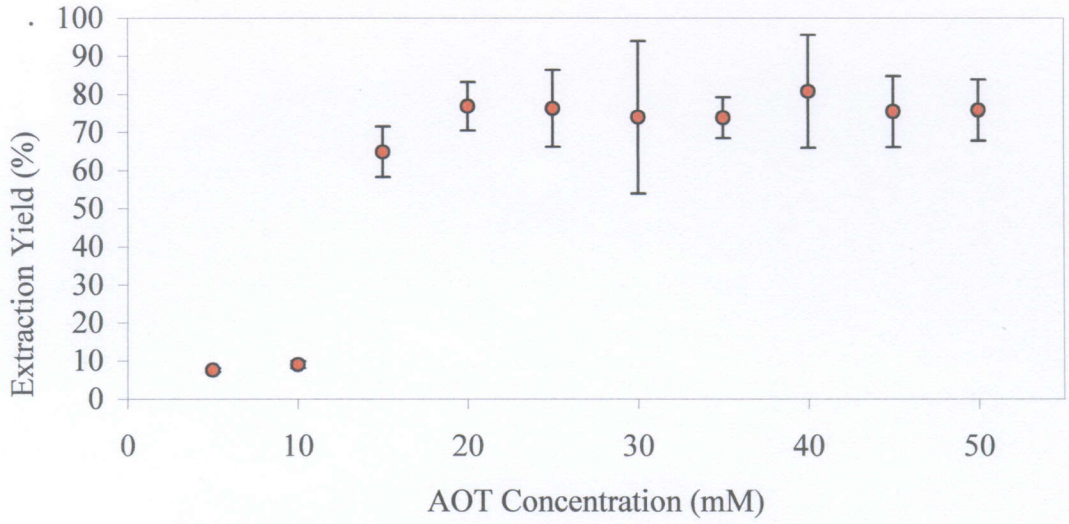

Figure 3.3: Effect of Surfactant Variation on Cytochrome *c* Extraction

Readings of Organic Phase at 410 nm

900 μL per phase

Organic Phase: 5 - 50 mM AOT in isooctane

Aqueous Phase: 80 % 0.1 M NaCl and 20 % 0.1 M Tris solution at pH ~ 9.7, and ~ 1 g/L cytochrome *c*

Overall the results of the cytochrome *c* surfactant variation experiment showed that there was a minimum surfactant concentration needed for an extraction of over 60 % into the organic phase. The increase in the volume of the phases and decrease of the amount of cytochrome *c* in the system did not cause any differences in these results. It should be noted that with less surfactant, a limited number of micelles can be formed and therefore, a limited amount of cytochrome *c* could be solubilized into those micelles. So, it appears that there is a certain capacity due to the amount of surfactant present in the system. This is clear from the comparison of the experiment with ~ 0.2 g/L cytochrome *c* and ~ 1 g/L cytochrome *c*. In all three experiments at the low surfactant concentration of 5 mM AOT, only ~ 0.1 g/L cytochrome *c* was extracted, and in all of the experiments the

overall amount of protein extracted significantly increases at surfactant concentrations of 15 mM AOT and above.

3.2.2 Salt Variation

Following the success of the surfactant variation experiments, salt concentration was then varied with cytochrome *c*. Figure 3.4 below shows the results of the salt variation experiments where there was 900 µL per phase and ~ 0.26 g/L cytochrome *c* in the system.

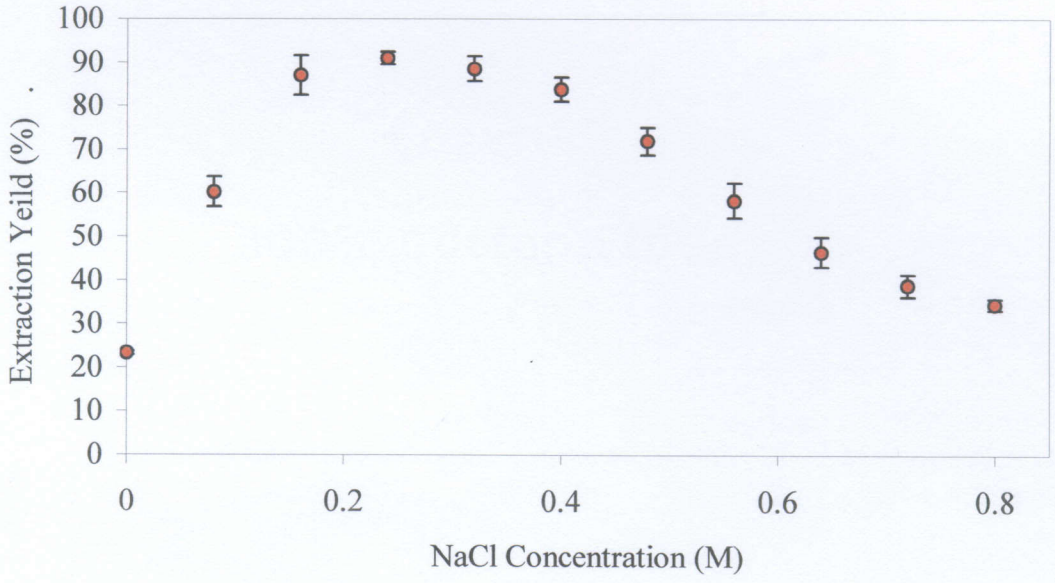

Figure 3.4: Effect of NaCl Variation on Cytochrome *c* Extraction

Readings of Organic Phase at 410 nm

900 µL per phase

Organic Phase: 50 mM AOT in isooctane

Aqueous Phase: 0.02 M Tris solution at pH ~ 9.7 with NaCl varied from 0 to 0.8 M, and ~ 0.26 g/L cytochrome *c*

The results of the salt variation experiments show that there is a minimum amount of salt needed for a cytochrome *c* extraction into the organic phase greater than 80 %.

The amount of salt needed for this extraction yield is ~ 0.16 M. Amounts of salt greater than ~ 0.4 M was shown to decrease the extraction yield to below 80 %.

3.2.3 pH Variation

The last experiments to be performed using cytochrome *c* were experiments where the pH was varied. Figure 3.5 below shows the results of the pH variation experiments where there was 900 μL per phase and ~ 0.22 g/L cytochrome *c* in the system.

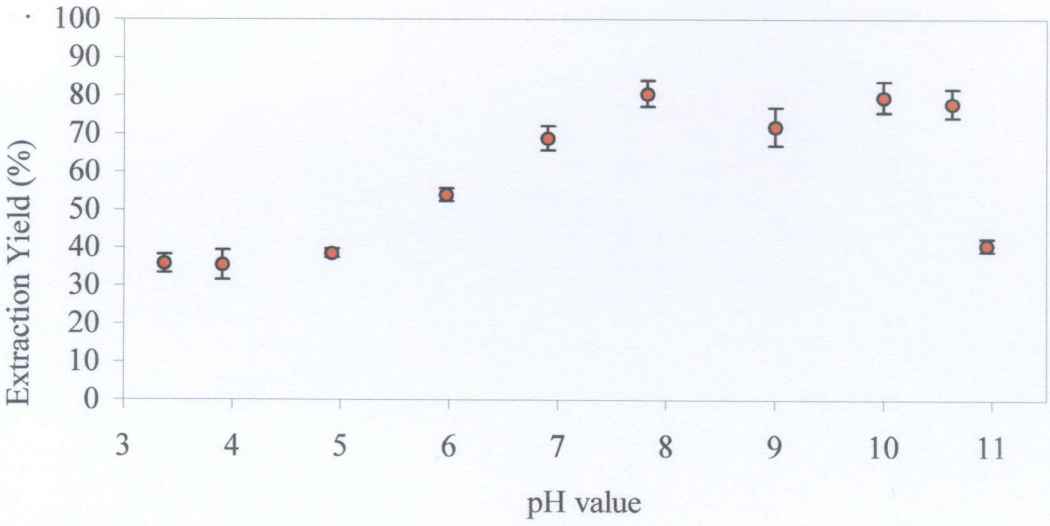

Figure 3.5: Effect of pH Variation on Cytochrome *c* Extraction

Readings of Organic Phase at 410 nm

900 μL per phase

Organic Phase: 50 mM AOT in isooctane

Aqueous Phase: Buffer solution at pH varied from ~ 3 to 11, with NaCl at 0.08 M, and ~ 0.22 g/L cytochrome *c*

The results of the pH experiments showed that at a pH between ~ 8 and 10.5, greater than 70% extraction into the organic phase could be expected. At a pH above the pI of cytochrome *c* (10.6), the extraction yield greatly decreased.[20]

3.3 Discussion

The results of the experiments using cytochrome *c* were important to confirm that the experimental procedures used for these experiments, and for experiments with other proteins would be acceptable. It was found that the experiments were successful, as previous results were found to be similar to the results obtained.

Surfactant variation results were consistent with those obtained by Jarudilokkul, Poppenborg, and Stuckey, in which it was found that a minimum AOT concentration of 16 mM exists for ≥ 90% extraction from a buffer solution at pH 10.[24] The results obtained in Figures 3.1 – 3.3 show that a minimum AOT concentration of ~ 20 mM was found necessary in order to have ≥ 70% extraction from a buffer solution at pH 9.7.

Salt variation results were consistent with those obtained by Golken and Hatton, in which it was found that at low ionic strengths, cytochrome *c* would be completely solubilized in the micellar phase, and at high ionic strengths, this did not occur.[12] A summary of the results obtained can be viewed in Figure 3.4.

The results of the pH experiments showed there was an optimum pH range for effective extraction of cytochrome *c*. Figure 3.5 shows that a pH range of 8 to 10.5 was found to result in greater than 70 % extraction into the organic phase. It was also observed that a pH above the pI of cytochrome *c* resulted in poor extraction, which was expected.

The overall best conditions for extraction into the organic phase were found to be at a pH between 8 and 10.5, a salt concentration of about 0.25 M NaCl, and a surfactant concentration of 20 mM AOT or greater.

Chapter 4: EGFP Experiments

4.1 Experimental Methods

EGFP was produced recombinantly in *E.coli* for use in the reverse micelle experiments. This protein was collected and purified through various methods including anion exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatography, and ultrafiltration. Details on the production and purification processes can be found in Appendix B. The final concentration of the purified EGFP used in the experiments was ~ 3.6 g/L.

For the reverse micelle experiments using EGFP 900 μL of the organic phase and 900 μL of the aqueous phase were contacted in a 2 mL glass vial (Fisher Scientific, Cat. No. 03-338AA). Three different experiments were conducted using EGFP. The experiments performed varied pH, salt concentration, and surfactant concentration in the system. Each experiment was performed in triplicate to ensure the repeatability of the results.

The organic phase was different from that used in the cytochrome *c* experiments due to the different properties of EGFP that had to be taken into consideration. Cytochrome *c* has a pI of 10.6, in order for the cytochrome *c* to be extracted the anionic surfactant AOT was used so that the cytochrome *c* could be extracted at pH values below 10.6. For the protein to be extracted into the reverse micelle system, the protein and surfactant must typically bear opposite charges. At a pH below the pI of a protein, the protein carries a net positive charge. When the pH is above the pI, the protein has a net negative charge. EGFP has a pI of 5.6, so a cationic surfactant was chosen for use in the system, so that the protein would be extracted at pH values above 5.6. The surfactant

used in the organic phase was CTAB (Sigma-Aldrich, Product # H9151) in an alkane/alcohol solution. For the pH and salt variation experiments the organic phase consisted of 100 mM CTAB in 90 % isooctane (Sigma-Aldrich, Product # 258776) and 10 % hexanol (Sigma-Aldrich, Product # H13303), with a small amount of water. In the experiment varying surfactant concentration the organic phase consisted of CTAB in amounts varying from 5 to 100 mM, with 90 % isooctane, 10 % hexanol, and a small amount of water.

The aqueous phase consisted of a 50 mM phosphate buffer solution at a pH of ~ 8.3 with no salt and EGFP at ~ 0.36 g/L for the surfactant variation experiment. In the salt variation experiment the aqueous phase was a 50 mM tris buffer solution at a pH of ~ 8.3, EGFP at ~ 0.36 g/L, and varying concentration of NaCl from 0 – 0.95 M. For the experiment varying pH, pH was varied from ~ 5 to ~ 9.5 in the aqueous phase using 50 mM acetate, phosphate, and carbonate buffers. Acetate buffer was used for the 3-5 pH range, phosphate buffer was used for the 6-8 pH range, and carbonate buffer was used for the 9-12 pH range. In the pH experiment no salt was used, and the EGFP concentration in the aqueous phase was again ~ 0.36 g/L.

In each experiment the aqueous phases and organic phases were contacted in glass vials through rotary inversion at 8 rpm for 1 hour to obtain equilibrium. The samples were then centrifuged to separate the phases for 5 minutes at 3000 rpm. Organic phases were then analyzed in an UV/vis Spectrophotometer at 280 and 488 nm.

4.2 Results

The results of the experiments using EGFP were interpreted through use of Beer's Law (Equation 4.1). The pathlength of the quartz cuvettes used was 0.5 cm, and the

molar extinction coefficient for EGFP at 488 nm is 55000 $M^{-1}cm^{-1}$.[66] The molar concentration was converted to concentration in g/L using the molecular weight of EGFP of 27 kDa.

$$A = \varepsilon \ell C \qquad \text{(Equation 4.1)}$$

Where :
A = Absorbance at wavelength used
ε = Molar Extinction Coefficient for protein at wavelength used ($M^{-1}cm^{-1}$)
ℓ = Pathlength of Cuvette (cm)
C = Concentration of Protein (M)

Each experiment was performed in triplicate to ensure the accuracy of the results. To observe the general trend for each experiment the average result of the three replicates was found and is shown in each representative graph of results. The percent of EGFP that was extracted into the organic phase was determined through comparison with the original amount of EGFP in the aqueous phase of each sample (See Equation 3.1). In each of the representative graphs of results, the standard deviation between the three replicates is shown as error bars. Detailed sample compositions and experimental data can be found in Appendix A.

4.2.1 pH Variation

The first experiments performed with EGFP were those where the pH was varied in the system to determine the pH at which the following experiments should be performed. Figure 4.1 below shows the results of the pH variation experiments where there was 900 μL per phase and ~ 0.36 g/L EGFP in the system.

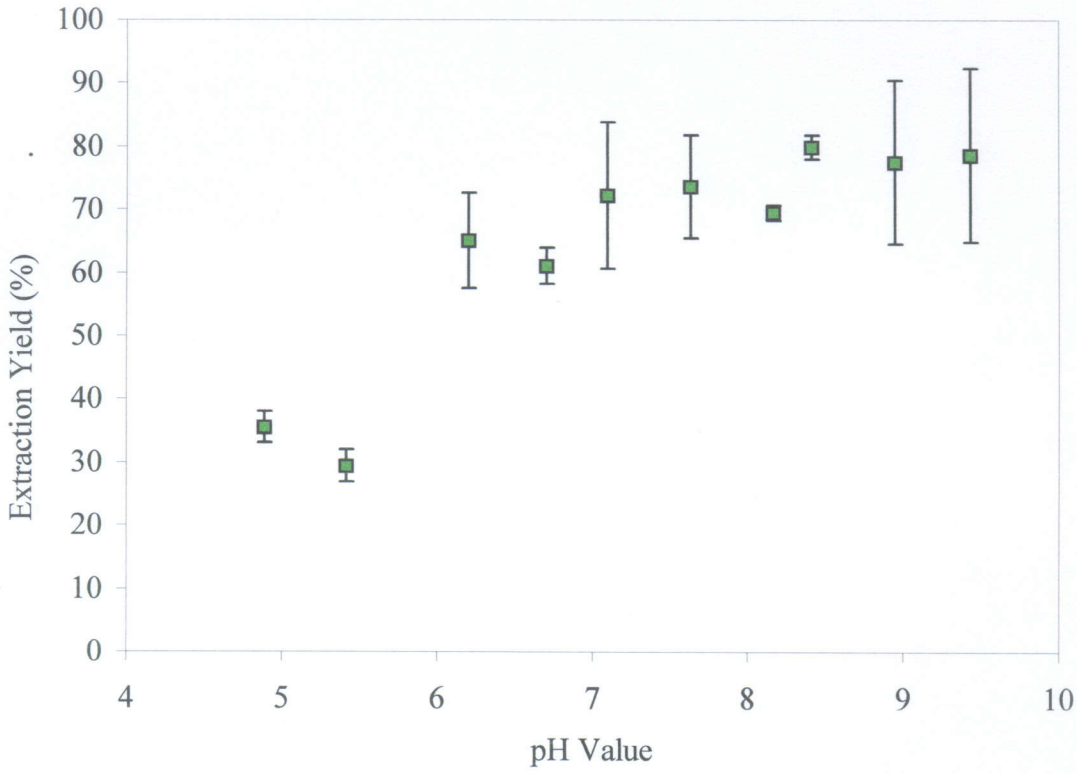

Figure 4.1: Effect of pH Variation on EGFP Extraction

Readings of Organic Phase at 488 nm

Organic Phase: 100 mM CTAB in 90 % isooctane, 10 % hexanol

Aqueous Phase: 50 mM Buffer at pH varied from ~ 5 to 9.5, ~ 0.36 g/L EGFP, No Salt

It was found that for an EGFP extraction into the organic phase greater than 70 %, a pH of 7 or higher was needed. A pH of ~ 8.3 was chosen to use for the remaining experiments using EGFP, as the greatest extraction (~ 80 %) was observed at this pH. This pH is about 2.5 points higher than the pI of EGFP, which is 5.6.[59] At pH values below the pI, very poor extraction was observed.

4.2.2 Salt Variation

Following the pH experiments, salt content was varied in the system. Figure 4.2 below shows the results of the salt variation experiments where there was 900 μL per phase and ~ 0.36 g/L EGFP in the system.

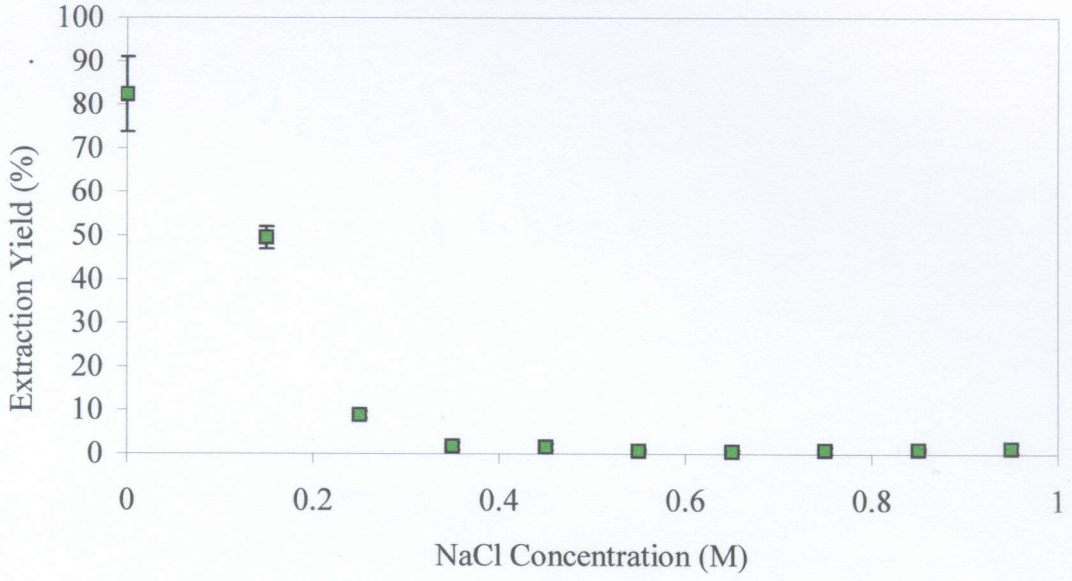

Figure 4.2: Effect of NaCl Variation on EGFP Extraction

Readings of Organic Phase at 488 nm

Organic Phase: 100 mM CTAB in 90 % isooctane, 10 % hexanol

Aqueous Phase: 50 mM Tris Buffer at pH ~8.3, ~ 0.36 g/L EGFP, NaCl Varied from 0 to 0.95 M

In these experiments it was found that the best extraction occurred when no salt was present in the system (~ 82 %). As the salt concentration in the system increased, the extraction yield decreased. After a concentration of 0.35 M NaCl in the system, very little protein was transferred into the organic phase.

4.2.3 Surfactant Variation

Finally, surfactant concentration was varied. Figure 4.3 below shows the results of the surfactant variation experiments where there was 900 μL per phase and ~ 0.36 g/L EGFP in the system.

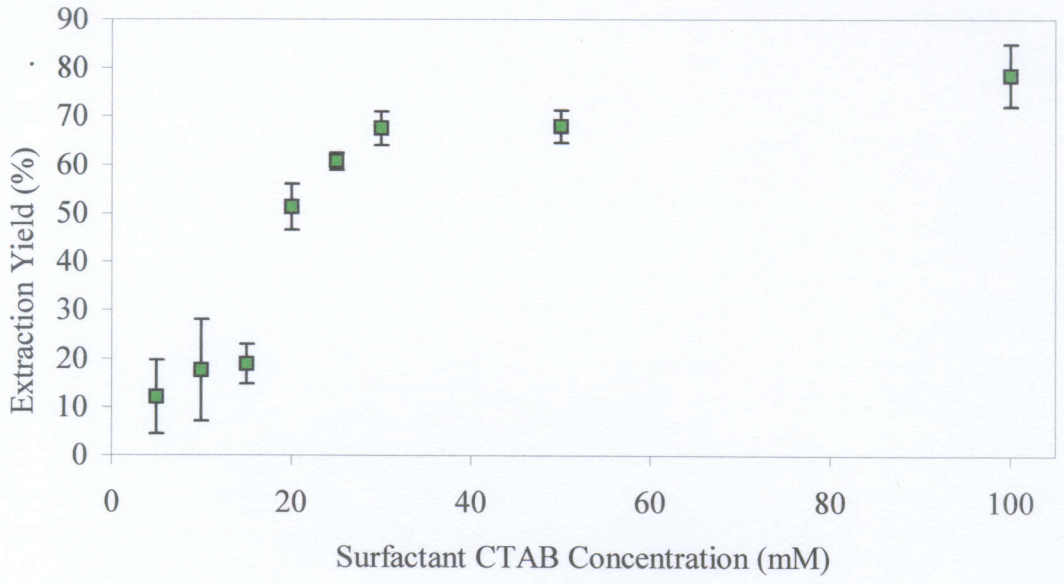

Figure 4.3: Effect of Surfactant Variation on EGFP Extraction

Readings of Organic Phase at 488 nm

Organic Phase: CTAB varied from 5 to 100 mM in 90 % isooctane, 10 % hexanol

Aqueous Phase: 50 mM Phosphate Buffer at pH ~ 8.3, ~ 0.36 g/L EGFP, No Salt

The results show that a minimum surfactant concentration exists of 30 mM CTAB for greater than 65 % extraction into the organic phase. The greatest extraction yield was observed at 100 mM CTAB.

4.3 Discussion

The results of the EGFP experiments show the optimum conditions for the extraction of EGFP into the organic phase in a reverse micelle system.

In the pH variation experiments a pH of 7 or higher was found to result in an extraction into the organic phase greater than 70 %. A pH of ~ 8.3 was found to have the greatest extraction at ~ 80 %. This pH is about 2.5 points higher than the pI of EGFP, at 5.6. At pH values below the pI, very poor extraction was observed.

It was found that the EGFP did not extract well when the salt concentration in the aqueous phase was high. When no salt was present in the system, ~ 80 % of the protein could be extracted. With 0.15 M NaCl present, less than 50 % of the protein was extracted, and at 0.35 M NaCl and higher, less than 2 % of the protein was extracted into the organic phase.

With the surfactant variation a minimum of 30 mM CTAB was found for extraction greater than 65 %. The greatest extraction yield was observed at 100 mM CTAB.

Overall the best conditions for the extraction of EGFP are with no salt in the aqueous phase at a pH of about 8.3, and with 100 mM CTAB in the organic phase.

Chapter 5: Flavodoxin Experiments

5.1 Experimental Methods

Cyanobacterium anabaena flavodoxin was produced recombinantly in *E. coli* for use in the reverse micelle experiments. This protein was collected and purified through various methods including anion exchange chromatography and ultrafiltration. Details on the production and purification processes can be found in Appendix B. The final concentration of the purified flavodoxin used in the experiments was ~ 2.15 g/L.

For the reverse micelle experiments using flavodoxin 900 μL of the organic phase, and 900 μL of the aqueous phase were contacted in a 2 mL glass vial (Fisher Scientific, Cat. No. 03-338AA). Three different experiments were conducted using flavodoxin. The experiments performed varied pH, salt concentration, and surfactant concentration in the system. Each experiment was performed in triplicate to ensure the repeatability of the results.

The organic phase used was the same as with the EGFP experiments. Flavodoxin has a pI of 4.2, so a cationic surfactant was used so that the protein could be extracted at pH values above 4.2, where the protein has a net negative charge. The surfactant used in the organic phase was CTAB (Sigma-Aldrich, Product # H9151) in an alkane/alcohol solution. For the pH and salt variation experiments the organic phase consisted of 100 mM CTAB in 90 % isooctane (Sigma-Aldrich, Product # 258776) and 10 % hexanol (Sigma-Aldrich, Product # H13303), with a small amount of water. In the experiment varying surfactant concentration the organic phase consisted of CTAB in amounts varying from 5 to 100 mM, with 90 % isooctane, 10 % hexanol, and a small amount of water.

The aqueous phase consisted of a 50 mM phosphate buffer solution at a pH of ~ 7 with no salt and flavodoxin at ~ 2.15 g/L for the surfactant variation experiment. In the salt variation experiment the aqueous phase was a 50 mM phosphate buffer solution at a pH of ~ 7, flavodoxin at ~ 2.15 g/L, and varying concentration of NaCl from 0 – 0.95 M. For the experiment varying pH, pH was varied from ~ 4.5 to ~ 8 in the aqueous phase using 50 mM acetate, phosphate, and carbonate buffers. Acetate buffer was used for the 3-5 pH range, phosphate buffer was used for the 6-8 pH range, and carbonate buffer was used for the 9-12 pH range. In the pH experiment no salt was used, and the flavodoxin concentration in the aqueous phase was again ~ 2.15 g/L.

In each experiment the aqueous phases and organic phases were contacted in glass vials through rotary inversion at 8 rpm for 1 hour to obtain equilibrium. The samples were then centrifuged to separate the phases for 5 minutes at 3000 rpm. Organic phases were then analyzed in an UV/vis Spectrophotometer at 280 and 464 nm.

5.2 Results

The results of the experiments using flavodoxin were interpreted through use of Beer's Law (Equation 4.1), as with EGFP. The pathlength of the quartz cuvettes used was 0.5 cm, and the molar extinction coefficient for flavodoxin at 464 nm is 5280 $M^{-1}cm^{-1}$.[67] The molar concentration was converted to concentration in g/L using the molecular weight of flavodoxin of 19 kDa.

Each experiment was performed in triplicate to ensure the accuracy of the results. To observe the general trend for each experiment the average result of the three replicates was found and is shown in each representative graph of results. The percent of flavodoxin that was extracted into the organic phase was determined through comparison

with the original amount of flavodoxin in the aqueous phase of each sample (See Equation 3.1). In each of the representative graphs of results, the standard deviation between the three replicates is shown as error bars. Detailed sample compositions and experimental data can be found in Appendix A.

5.2.1 pH Variation

As with EGFP, the first experiments performed with flavodoxin were those where the pH was varied in the system to determine which pH the following experiments should be operated at. Figure 5.1 below shows the results of the pH variation experiments where there was 900 μL per phase and ~ 2.15 g/L flavodoxin in the system.

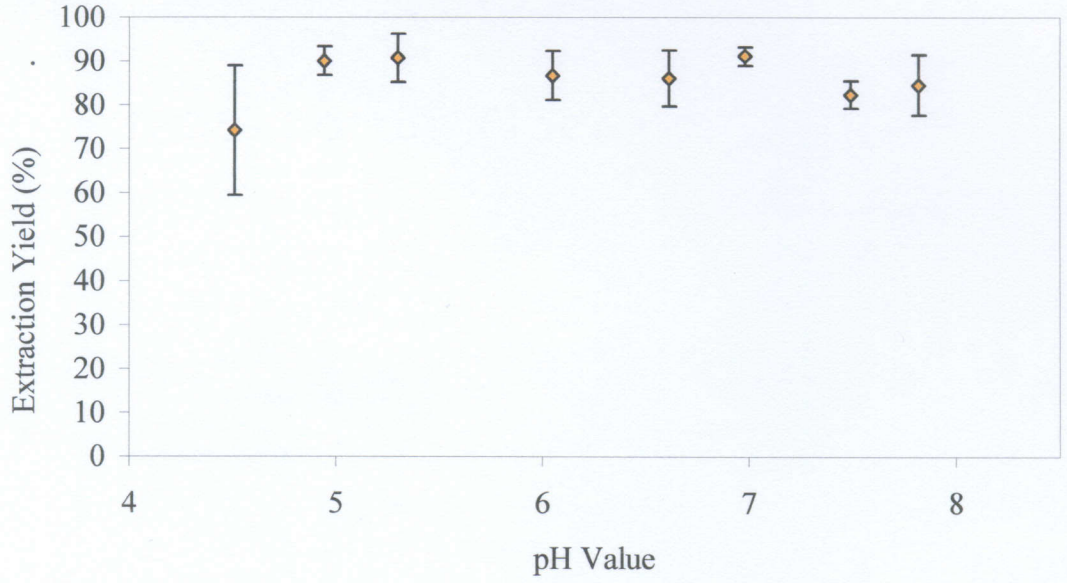

Figure 5.1: Effect of pH Variation on Flavodoxin Extraction
Readings of Organic Phase at 464 nm
Organic Phase: 100 mM CTAB in 90 % isooctane, 10 % hexanol
Aqueous Phase: 50 mM Buffer with pH varied from ~ 4.5 - 8, ~ 2.15 g/L flavodoxin, No Salt

As with EGFP, it was found that a pH about 2.5 - 3 points above the pI of the protein worked best. The pI of flavodoxin is 4.2, and the flavodoxin extracted into the

organic phase best at a pH of about 7.[59] At a pH of 7, ~ 90 % of the protein was extracted. Overall, flavodoxin extracted well in a broad range of pH values. In the range from a pH of 5 to 8, 80% or greater extraction was observed.

5.2.2 Salt Variation

After an acceptable pH was found for the flavodoxin experiments, salt content was varied in the system. Figure 5.2 below shows the results of the salt variation experiments where there was 900 μL per phase and ~ 2.15 g/L flavodoxin in the system.

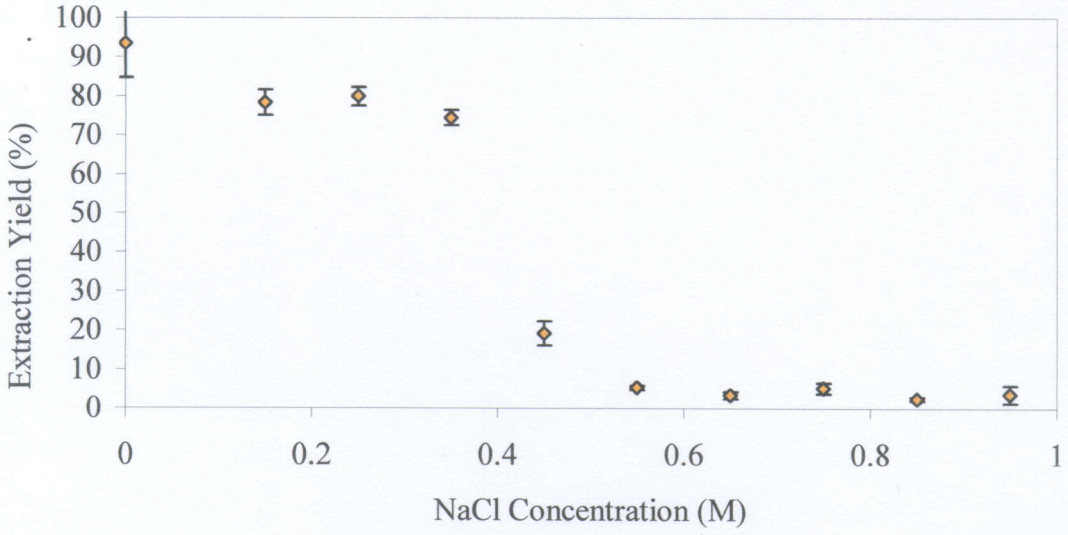

Figure 5.2: Effect of NaCl Variation on Flavodoxin Extraction

Readings of Organic Phase at 464 nm

Organic Phase: 100 mM CTAB in 90 % isooctane, 10 % hexanol

Aqueous Phase: 50 mM Phosphate Buffer at pH ~7, ~ 2.15 g/L flavodoxin, NaCl Varied from 0 to 0.95 M

Flavodoxin was found to be more tolerant of the salt concentration in the aqueous phase than EGFP. The best extraction yield occurred with no salt in the system (~ 90%). However, with low salt concentrations between 0.15 M and 0.35 M NaCl, 70 – 80 % of

the protein was still extracted. At salt concentrations greater than 0.4 M NaCl, the extraction yield was greatly reduced.

5.2.3 Surfactant Variation

Lastly, surfactant concentration was varied. Figure 5.3 below shows the results of the surfactant variation experiments where there was 900 μL per phase and ~ 2.15 g/L flavodoxin in the aqueous phase.

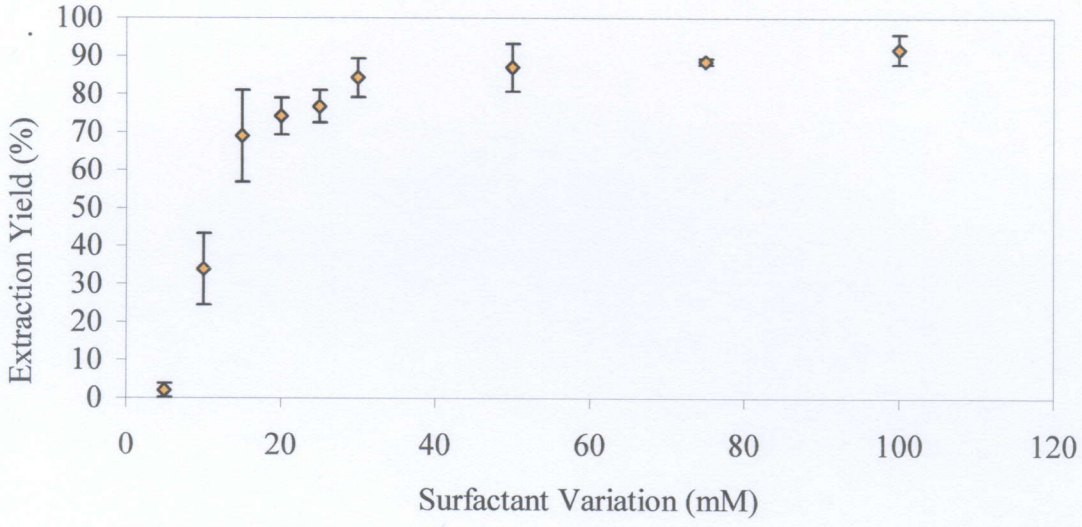

Figure 5.3: Effect of Surfactant Variation on Flavodoxin Extraction
Readings of Organic Phase at 464 nm
Organic Phase: CTAB varied from 5 - 100 mM in 90 % isooctane, 10% hexanol
Aqueous Phase: 50 mM Phosphate Buffer at pH ~ 7, ~ 2.15 g/L flavodoxin, No Salt

A minimum surfactant concentration of 30 mM CTAB was found to exist for extraction yields of 80 % or higher. The optimum surfactant concentration was 100 mM CTAB with extraction yields around 90 %.

5.3 Discussion

The results of the flavodoxin experiments give a profile of the conditions best suited for flavodoxin extraction in reverse micelle systems.

Flavodoxin extraction was not greatly dependent on the pH of the system, and had good extraction (80% or greater) in a pH range of 5 to 8. As seen with EGFP, it was found that a pH about 2.5 - 3 points above the pI of the protein worked best. A pH of 7 was found to have the best extraction with ~ 90 % of the protein being extracted into the organic phase.

With salt concentration variation, the best extraction yield occurred with no salt in the system (~ 90%). However, with low salt concentrations between 0.15 M and 0.35 M NaCl, 70 – 80 % of the protein was still extracted. At salt concentrations greater than 0.4 M NaCl, the extraction yield was greatly reduced.

A minimum surfactant concentration of 30 mM CTAB was found to exist for extraction yields of 80 % or higher. This minimum is very similar to that required for EGFP (30 mM CTAB required for 65 % extraction in EGFP). The optimum surfactant concentration was 100 mM CTAB with extraction yields around 90 %.

Overall the best conditions for the extraction of flavodoxin were with no salt in the aqueous phase at a pH of about 7, and with 100 mM CTAB in the organic phase.

Chapter 6: DsRed2 Experiments

6.1 Experimental Methods

DsRed2 was produced recombinantly in *E. coli* for use in the reverse micelle experiments. This protein was collected and purified through various methods including anion exchange chromatography, size exclusion chromatography, and ultrafiltration. Details on the production and purification processes can be found in Appendix B. The final concentration of the purified DsRed2 used in the experiments was ~ 2.4 g/L.

For the reverse micelle experiments using DsRed2 900 μL of the organic phase and 900 μL of the aqueous phase were contacted in a 2 mL glass vial (Fisher Scientific, Cat. No. 03-338AA). Three different experiments were conducted using DsRed2. The experiments performed varied pH, salt concentration, and surfactant concentration in the system. Each experiment was performed in triplicate to ensure the repeatability of the results.

The organic phase was the same as previously used in both the EGFP and flavodoxin experiments. The surfactant used in the organic phase was CTAB (Sigma-Aldrich, Product # H9151) in an alkane/alcohol solution. For the pH and salt variation experiments the organic phase consisted of 100 mM CTAB in 90 % isooctane (Sigma-Aldrich, Product # 258776) and 10 % hexanol (Sigma-Aldrich, Product # H13303), with a small amount of water. In the experiment varying surfactant concentration the organic phase consisted of CTAB in amounts varying from 5 to 100 mM, with 90 % isooctane, 10 % hexanol, and a small amount of water.

The aqueous phase consisted of a 50 mM carbonate buffer solution at a pH of ~ 9 with no salt and DsRed2 at ~ 2.4 g/L for the surfactant variation experiment. In the salt

variation experiment the aqueous phase was a 50 mM carbonate buffer solution at a pH of ~ 9, DsRed2 at ~ 2.4 g/L, and varying concentration of NaCl from 0 – 0.75 M. For the experiment varying pH, pH was varied from ~ 6 to ~ 10.5 in the aqueous phase using 50 mM acetate, phosphate, and carbonate buffers. Acetate buffer was used for the 3-5 pH range, phosphate buffer was used for the 6-8 pH range, and carbonate buffer was used for the 9-12 pH range. In the pH experiment no salt was used, and the DsRed2 concentration in the aqueous phase was again ~ 2.4 g/L.

In each experiment the aqueous phases and organic phases were contacted in glass vials through rotary inversion at 8 rpm for 1 hour to obtain equilibrium. The samples were then centrifuged to separate the phases for 5 minutes at 3000 rpm. Organic phases were then analyzed in an UV/vis Spectrophotometer at 280 and 561 nm.

6.2 Results

The results of the experiments using DsRed2 were interpreted through use of Beer's Law (Equation 4.1), as with EGFP and flavodoxin. The pathlength of the quartz cuvettes used was 0.5 cm, and the molar extinction coefficient for DsRed2 at 561 nm is 43800 $M^{-1}cm^{-1}$ (per tetramer DsRed2).[68] The molar concentration was converted to concentration in g/L using the molecular weight of DsRed2 of 103 kDa (per tetramer DsRed2).

Each experiment was performed in triplicate to ensure the accuracy of the results. To observe the general trend for each experiment the average result of the three replicates was found and is shown in each representative graph of results. The percent of DsRed2 that was extracted into the organic phase was determined through comparison with the original amount of DsRed2 in the aqueous phase of each sample (See Equation 3.1). In

each of the representative graphs of results, the standard deviation between the three replicates is shown as error bars. Detailed sample compositions and experimental data can be found in Appendix A.

6.2.1 pH Variation

As with both EGFP and flavodoxin, the pH in the system was the first thing to be investigated for the experiments with DsRed2. Here the pH was varied from ~6 to 10.5. Figure 6.1 below shows the results obtained, where there was 900 µL per phase and ~ 2.4 g/L of DsRed2 in the aqueous phase.

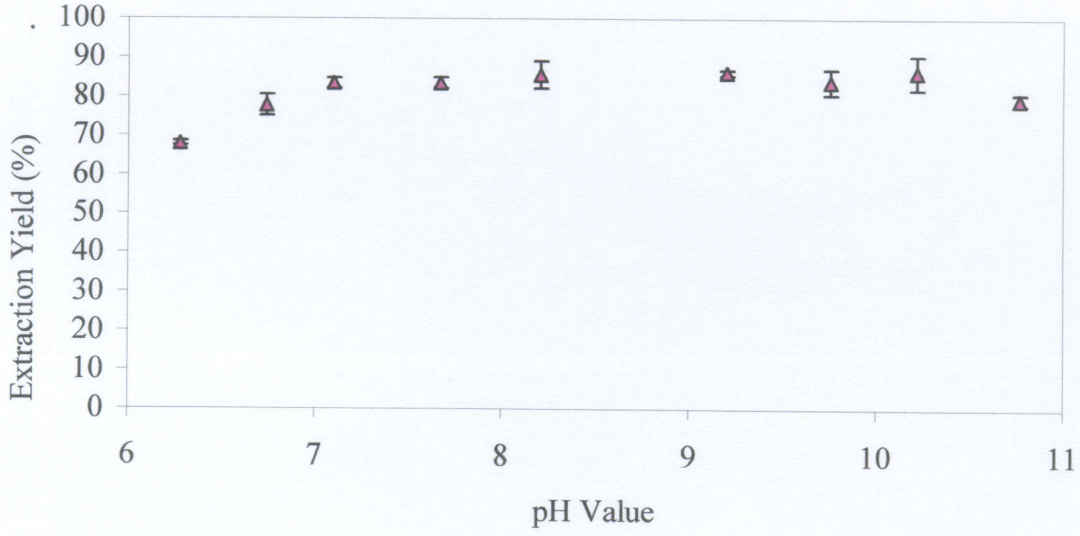

Figure 6.1: Effect of pH Variation on DsRed2 Extraction
Readings of Organic Phase at 561 nm
Organic Phase: 100 mM CTAB in 90 % isooctane, 10 % hexanol
Aqueous Phase: 50 mM Buffer with pH varied from ~ 6 - 10.5, ~ 2.4 g/L DsRed2, No Salt

DsRed2 was found to extract well in a wide range of pH values. At pH of ~ 7 – 10.5, greater than 80 % DsRed2 was extracted into the organic phase. The pH chosen for use in further experiments was 9, as the greatest amount extracted, with the least amount of error, was at this pH.

6.2.2 Salt Variation

Following experiments with pH, the salt concentration in the system was varied. Here the salt concentration was varied from 0 – 0.75 M NaCl. The results are shown in Figure 6.2 below. For the experiments there was 900 μL per phase, and ~ 2.4 g/L DsRed2 in the aqueous phase.

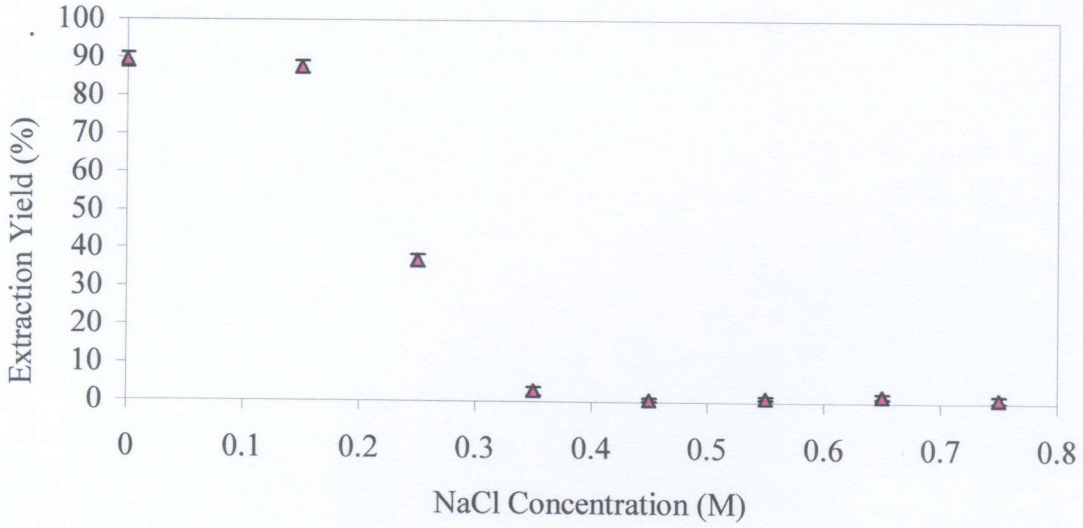

Figure 6.2: Effect of NaCl Variation on DsRed2 Extraction

Readings of Organic Phase at 561 nm

Organic Phase: 100 mM CTAB in 90 % isooctane, 10 % hexanol

Aqueous Phase: 50 mM Carbonate Buffer at pH ~ 9, ~ 2.4 g/L DsRed2, NaCl varied from 0 to 0.75 M

It was found that the best extraction yield occurred when there was very little or no salt in the system. With no salt in the aqueous phase ~ 89 % of the DsRed2 was extracted into the organic phase. When the salt concentration was increased to 0.15 M NaCl, there was very little change; ~ 88 % of the DsRed2 was extracted into the organic

phase. At a salt concentration of 0.25 M NaCl the extraction yield dropped to below 40 %, and at concentrations greater than 0.35 M NaCl less than 3 % was extracted.

6.2.3 Surfactant Variation

After the experiments with pH and salt concentration were completed, the surfactant concentration was then varied. The surfactant concentration was varied from 0 to 100 mM with no salt in the system, and at a pH ~ 9. The results are shown in Figure 6.3 below. For the experiments there was 900 µL per phase, and ~ 2.4 g/L DsRed2 in the aqueous phase.

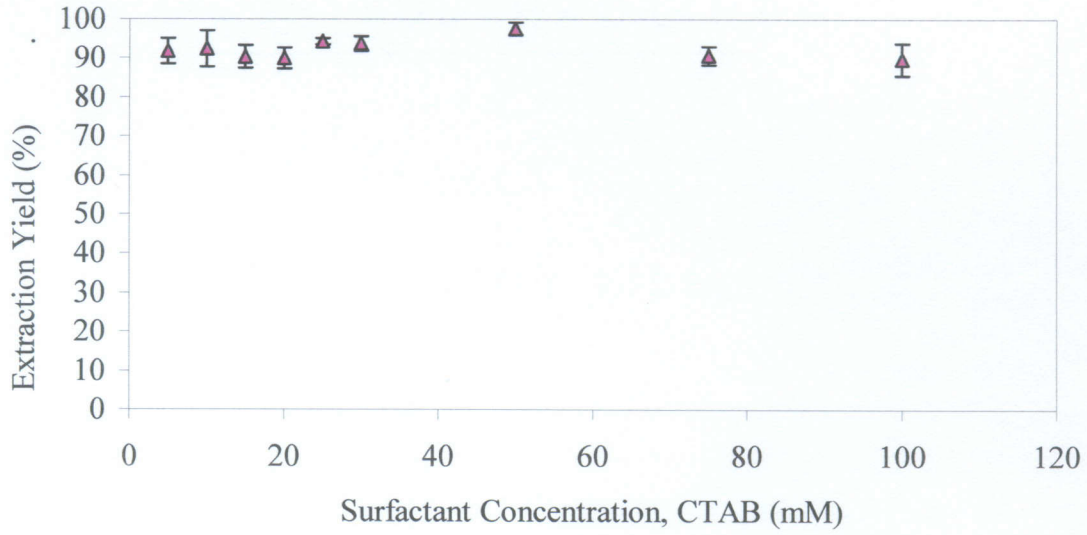

Figure 6.3: Effect of Surfactant Variation on DsRed2 Extraction
Readings of Organic Phase at 561 nm
Organic Phase: CTAB varied from 5 -100 mM in 90 % isooctane, 10 % hexanol
Aqueous Phase: 50 mM Carbonate Buffer with pH at ~ 9, ~ 2.4 g/L DsRed2, No Salt

No minimum surfactant concentration was found for DsRed2. For all of the surfactant concentrations tested greater than 90 % of the DsRed2 was extracted into the organic phase.

6.3 Discussion

The DsRed2 results show what the best conditions for extraction are in reverse micelle systems with conditions similar to those tested.

DsRed2 extracted well in a wide range of pH values. At pH of ~ 7 – 10.5, greater than 80 % DsRed2 was extracted into the organic phase. As seen with EGFP and flavodoxin, it was found that a pH about 2.5 - 3 points above the pI of the protein worked best. The pI of DsRed2 is 6.3.[59] A pH of 9 was found to have the best extraction with ~ 86 % of the protein being extracted into the organic phase.

It was interesting to find that the same amount of protein was extracted with no salt in the system, and with a low salt concentration of 0.15 M NaCl (~ 88 %). As with EGFP and flavodoxin however, salt concentrations higher than 0.25 M NaCl resulted in very poor extraction yields.

Unlike EGFP and flavodoxin, there was no minimum surfactant concentration needed for DsRed2 extraction. Of the conditions tested, all resulted in an extraction yield of 90 % or greater into the organic phase.

Overall the best conditions for the extraction of DsRed2 are with salt at 0 – 0.15 M NaCl in the aqueous phase at a pH of about 9, and with 5 - 100 mM CTAB in the organic phase.

Chapter 7: Crude and Mixed Protein Experiments

7.1 Crude Protein Experiments

7.1.1 Experimental Methods

An experiment was conducted using protein from crude lysate, which is protein collected after cell lysis. This experiment was conducted using DsRed2 crude lysate, to determine if the protein would be transferred into the reverse micelles. To begin, a Bradford assay was conducted using a Coomassie (Bradford) Protein Assay Kit (purchased from Pierce, Product # 23200) to determine the amount of DsRed2 present in the crude lysate as opposed to other proteins.

Following the Bradford assay a reverse micelle experiment was conducted using the protein from crude lysate as an aqueous phase along with 100 mM CTAB (Sigma-Aldrich, Product # H9151) in 90 % isooctane (Sigma-Aldrich, Product # 258776) and 10 % hexanol (Sigma-Aldrich, Product # H13303), with a small amount of water. The pH of the crude lysate (aqueous phase) was adjusted to ~ 9 with NaOH. The conductivity was also measured in the crude lysate (aqueous phase) following the pH adjustment. As in previous experiments using DsRed2 the organic and aqueous phase volumes were 900 μL and were contacted in a 2 mL glass vial (Fisher Scientific, Cat. No. 03-338AA). In each experiment the aqueous phases and organic phases were contacted in glass vials through rotary inversion at 8 rpm for 1 hour to obtain equilibrium. The samples were then centrifuged to separate the phases for 5 minutes at 3000 rpm. Organic phases were then analyzed in an UV/vis Spectrophotometer at 280 and 561 nm.

Back extraction of the DsRed2 from the organic phase to the aqueous phase was also performed. This was performed by contacting the organic phase containing DsRed2 with a fresh aqueous phase of pH ~ 9 using carbonate buffer, and 2 M NaCl, overnight. The organic phase following the extraction was analyzed using an UV/vis Spectrophotometer at 280 and 561 nm.

To further analyze the samples, gel electrophoresis (SDS-PAGE) was conducted using NuPage 10% Bis-Tris Gels (Invitrogen Cat. No. NP0303BOX).

7.1.2 Results

The Bradford assay was conducted on four separate 50 mL samples of DsRed2 crude lysate. From this analysis it was found that on average, about 79% of the total protein present in the sample was DsRed2, while about 21 % was other proteins. These results are shown in Table 7-1.

Table 7-1: Crude Lysate Protein composition based on Bradford Assay

% of Total Protein that is Other Protein	% of Total Protein that is DsRed2
11.95	88.05
20.66	79.34
25.39	74.61
26.30	73.70
Average % = 21	Average % = 79

Following the Bradford assay the pH of the crude lysate was adjusted to ~ 9 for use in the reverse micelle experiments. The conductivity of the sample was then measured and found to be ~ 11.1 at 22.7°C, which corresponds to ~ 0.13 M NaCl, based on a calibration curve.

The experiment was performed 9 times to ensure the repeatability of the results. Following the reverse micelle experiment it was found that on average ~ 72 % of the DsRed2 was extracted into the organic phase.

A back extraction experiment was also performed where the organic phase containing DsRed2 was contacted with a fresh aqueous phase overnight. The fresh aqueous phase had a pH of ~ 9 using carbonate buffer, and 2 M NaCl. This back extraction was performed with 3 samples and it was found that ~ 14 % of the DsRed2 was left behind in the organic phase.

A gel was also created using these samples, shown in Figure 7.1 below. The gel results also confirm that the majority of the DsRed2 was transferred into the organic phase through forward extraction, and back into an aqueous phase. The results also show that many unidentified proteins were left behind in the aqueous phase after forward extraction.

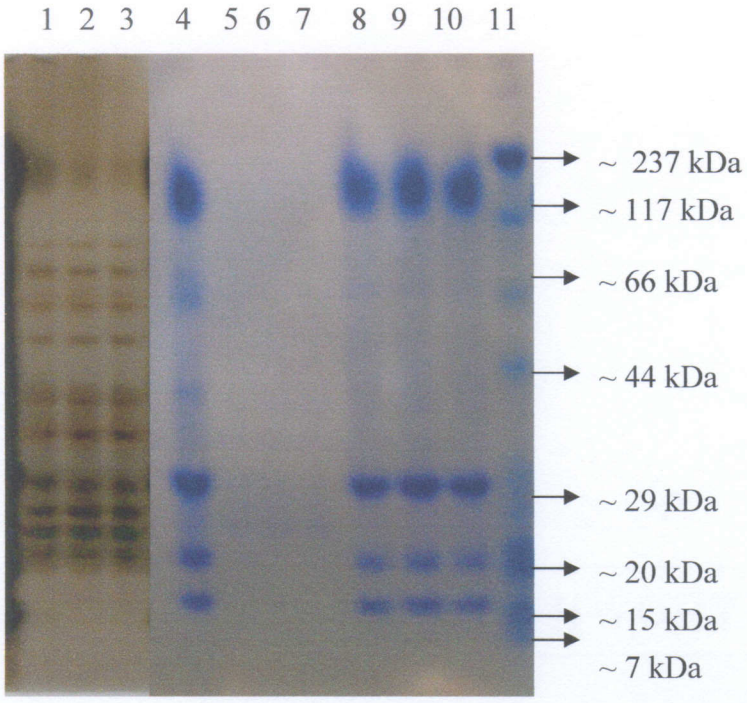

Figure 7.1: PAGE of DsRed2 Crude Lysate Experiment Results; Lanes 1-3 are silver stains of the aqueous phase leftover after forward extraction, the 4th lane is the original DsRed2 crude lysate sample by Coomassie blue dye stain, lanes 5-7 are again the aqueous phase leftover after forward extraction, lanes 8-10 are samples from the backward extracted aqueous phase containing DsRed2, and lane 11 is the molecular weight marker.

7.1.3 Discussion

The experiment with the DsRed2 crude lysate shows that the reverse micelle extraction works not only when the protein is pure, but when it is contaminated with other proteins as well. On average, 72% of the DsRed2 was extracted into the organic phase at a pH ~ 9 and conductivity equal to ~ 0.13 M NaCl. In the experiments using relatively pure DsRed2, ~ 88% of DsRed2 was extracted under similar conditions (pH ~ 9, 0.15 M NaCl). This shows that there is some interference due to the other proteins present in the system however, a decent quantity of the DsRed2 could still be extracted. This experiment also proved that the DsRed2 could be back-extracted into a fresh aqueous phase, with ~ 14 % being left behind in the organic phase. The results of the

electrophoresis gel shows that through forward extraction some of the contaminated proteins present can be separated from the DsRed2, and left behind in the aqueous phase.

7.2 Mixed Protein Experiments

7.2.1 Experimental Methods

Following the success of the reverse micelle experiments with individual proteins, experiments with mixed proteins were conducted. To begin the results were combined to easily identify the methods through which the proteins could be separated from one another. (See Figure 7.2-7.4)

The first experiment attempted was with both cytochrome *c* and DsRed2. The cytochrome *c* was to be removed first through the use of the surfactant AOT in isooctane as the organic phase (50 mM). The aqueous phase contained both cytochrome *c* and DsRed2 in 0.02 M Tris solution (pH ~ 8.5), 0.15 M NaCl. As in previous experiments, the organic and aqueous phase volumes were 900 μL each and were contacted in a 2 mL glass vial (Fisher Scientific, Cat. No. 03-338AA). In each experiment the aqueous phases and organic phases were contacted in glass vials through rotary inversion at 8 rpm for 1 hour to obtain equilibrium. The samples were then centrifuged to separate the phases for 5 minutes at 3000 rpm. Organic phases were then analyzed in an UV/vis Spectrophotometer at 280, 410, and 561 nm.

The organic phase containing cytochrome *c* was then back extracted through contact with a fresh aqueous phase with 1 M NaCl, carbonate buffer at pH ~11, overnight. The aqueous phase containing DsRed2 was contacted with a fresh organic phase of 100 mM CTAB (Sigma-Aldrich, Product # H9151) in 90 % isooctane (Sigma-

Aldrich, Product # 258776) and 10 % hexanol (Sigma-Aldrich, Product # H13303), with a small amount of water. Following this extraction, the organic phase was analyzed in an UV/vis Spectrophotometer at 280, 410, and 561 nm. The organic phase containing DsRed2 was then back extracted through contact with a fresh aqueous phase at 2 M NaCl, carbonate buffer at pH ~ 9, overnight.

Gel electrophoresis was also performed using samples from each aqueous phase throughout the process to further analyze the experimental results.

Following the success of this experiment, another experiment was performed using a protein mixture of cytochrome *c*, DsRed2, and flavodoxin. In this case the aqueous phase was again 0.02 M Tris solution (pH ~ 8.5), 0.15 M NaCl, along with the three proteins. Cytochrome *c* was first removed through contact with 50 mM AOT in isooctane. The cytochrome *c* was then back extracted through contact with a fresh aqueous phase with 1 M NaCl, pH ~11, overnight. The aqueous phase that remained was then contacted with 5 mM CTAB in 90 % isooctane and 10 % hexanol (and a small amount of water) in an attempt to remove the DsRed2 from the mixture. The protein that passed into the organic phase was back extracted using 1 M NaCl, carbonate buffer at pH ~9, overnight. The remaining aqueous phase was contacted with a fresh organic phase of 100 mM CTAB in 90% isooctane, 10% hexanol, with a small amount of water to remove the remaining protein. The protein transferred to the organic phase was back extracted through contact with a fresh aqueous phase at 2 M NaCl, carbonate buffer at pH ~ 9, overnight.

Throughout the experiment the organic phase samples were analyzed using an UV/vis Spectrophotometer at 280, 410, 464, and 561 nm. Gel electrophoresis was also

performed using samples from each aqueous phase throughout the process to further analyze the experimental results.

A second experiment was performed using the three protein mixture of cytochrome *c*, DsRed2, and flavodoxin as well. In this case the aqueous phase was again 0.02 M Tris solution (pH ~ 8.5), 0.15 M NaCl, along with the three proteins. Cytochrome *c* was first removed through contact with 50 mM AOT in isooctane. The cytochrome *c* was then back extracted through contact with a fresh aqueous phase with 1 M NaCl, pH ~11, overnight. The salt content of the aqueous phase that remained was then adjusted to ~ 0.35 M NaCl and contacted with 100 mM CTAB in 90 % isooctane and 10 % hexanol (and a small amount of water) in an attempt to remove the flavodoxin from the mixture. The flavodoxin that passed into the organic phase was then back extracted using 1 M NaCl, carbonate buffer at pH ~9, overnight. The remaining aqueous phase containing DsRed2 was then diluted with water to bring the salt content down to ~ 0.15 M NaCl. This volume was then divided into two sample vials of ~ 900 uL each and both of these aqueous phase samples were contacted with a fresh organic phase of 100 mM CTAB in 90% isooctane, 10% hexanol, with a small amount of water to remove the remaining protein. The protein transferred to the organic phase was back extracted through contact with a fresh aqueous phase at 2 M NaCl, carbonate buffer at pH ~ 9, overnight.

Throughout the experiment the organic phase samples were analyzed using an UV/vis Spectrophotometer at 280, 410, 464, and 561 nm. Gel electrophoresis was also performed using samples from each aqueous phase throughout the process to further analyze the experimental results.

7.2.2 Results

In order to determine a method by which the protein mixtures could be separated from one another, the results from the previous experiments were combined in Figures 7.2-7.4. The results from the experiments using cytochrome *c* were not included as they used a different surfactant.

The results of the pH experiments showed that flavodoxin could potentially be separated from EGFP and DsRed2 at a pH around 5, but some amount of DsRed2 and EGFP could also be present. (See Figure 7.2)

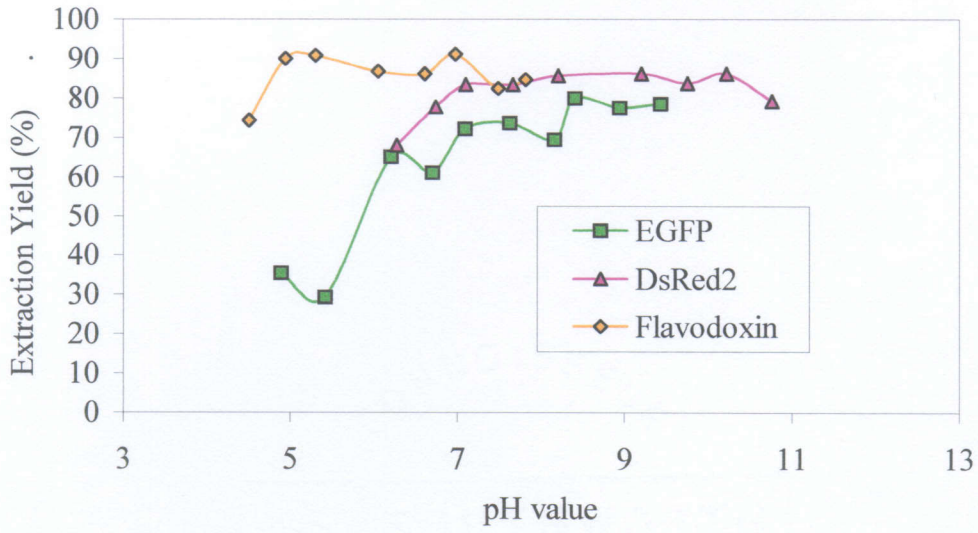

Figure 7.2: Combined pH variation results for EGFP, DsRed2, and Flavodoxin with 100 mM CTAB in 90% isooctane and 10% hexanol in the organic phase, and with no salt in the aqueous phase

The experiments with salt concentration variation showed another more promising possibility for the separation of flavodoxin from both DsRed2 and EGFP, at NaCl concentrations around 0.4 M. (See Figure 7.3)

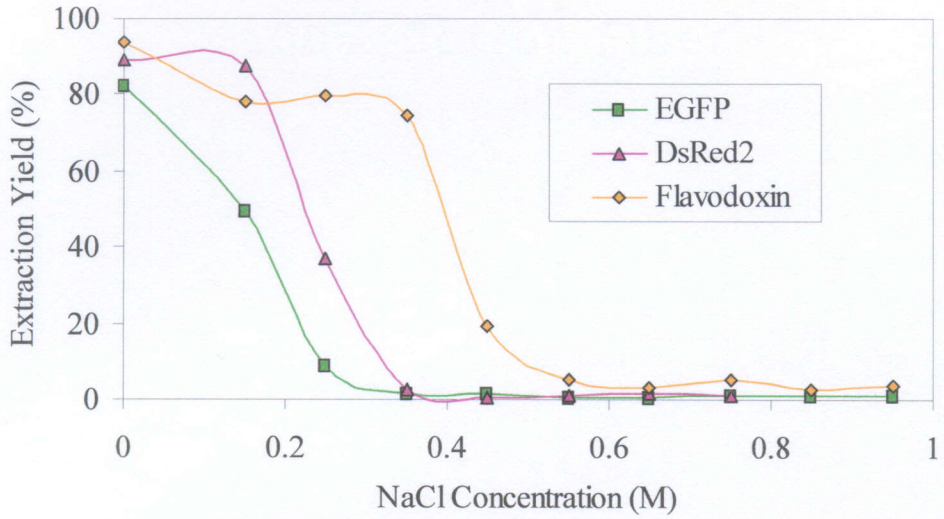

Figure 7.3: Combined NaCl variation results for EGFP, DsRed2, and Flavodoxin with 100 mM CTAB in 90% isooctane and 10% hexanol in the organic phase (EGFP experiments conducted at pH ~ 8, DsRed2 at pH ~ 9, and Flavodoxin at pH ~7)

The surfactant variation experiments showed a possibility for removing DsRed2 at low surfactant concentrations from EGFP and flavodoxin. (See Figure 7.4)

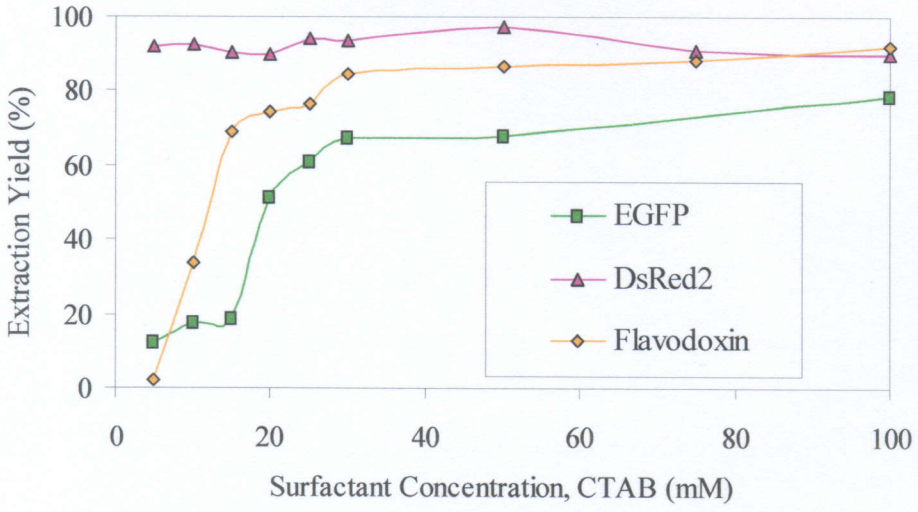

Figure 7.4: Combined surfactant variation results for EGFP, DsRed2, and Flavodoxin with no salt in the aqueous phase (EGFP experiments conducted at pH ~ 8, DsRed2 at pH ~ 9, and Flavodoxin at pH ~7)

It was decided that the separation of cytochrome *c* from DsRed2 would first be attempted. This was done to make sure that cytochrome *c* could be easily separated from the DsRed2 though the use of the surfactant AOT. Throughout the experiment the organic phase of the samples was read on the spectrophotometer at 410 nm and 561 nm, the results are as follows in Table 7-2. This experiment was performed twice to ensure the repeatability of the results.

Table 7-2: Two Protein Mixture Results, separation through use of different surfactants

Sample Description	Average % Cytochrome *c* Extracted	Average % DsRed2 Extracted
Forward Extraction #1	84	15
Forward Extraction #2	22	82
Backward Extraction #1	17	2
Backward Extraction #2	22	28

From the spectrophotometer readings it is observed that most of the cytochrome *c* was extracted in the first forward extraction, and most of the DsRed2 was extracted during the second forward extraction. Most of the cytochrome *c* and DsRed2 could be back extracted into a fresh aqueous phase again, leaving only a small amount behind in the organic phase. These readings cannot be considered entirely accurate since the total percent of protein adds up to over 100% with the cytochrome *c*, but are more of a rough estimate of the amount of protein extracted.

An electrophoresis gel was also run using the aqueous phase samples throughout the experiment. The results show that after the first forward extraction cytochrome *c* is removed from the aqueous phase, and the DsRed2 was left behind as expected. After the second forward extraction, all of the DsRed2 is transferred to the aqueous phase. Both

the cytochrome *c* and the DsRed2 were successfully back extracted into a fresh aqueous phase as well.

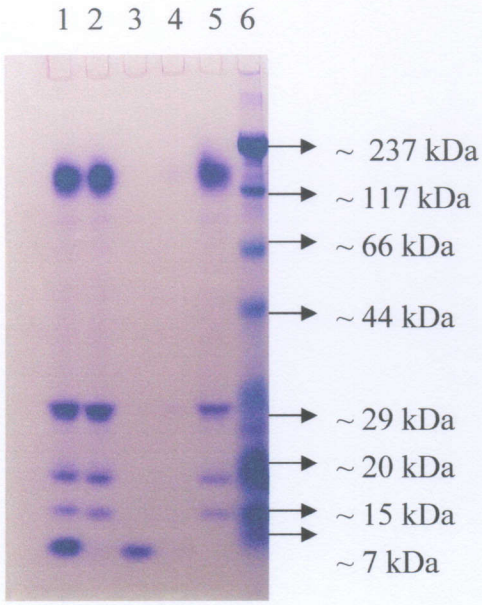

Figure 7.5: PAGE of cytochrome *c* and DsRed2 Experiment Results; Lane 1 is a sample of the aqueous phase with both cytochrome *c* and DsRed2, lane 2 is the aqueous phase after the first forward extraction, lane 3 is the protein back extracted from the organic phase after the first forward extraction, lane 4 is the aqueous phase after the second forward extraction, lane 5 is the protein back extracted from the organic phase after the second forward extraction, and lane 6 is the molecular weight marker.

Following the success with the separation of cytochrome *c* and DsRed2, an experiment was conducted to separated cytochrome *c*, DsRed2, and flavodoxin. First the cytochrome *c* was to be extracted using the surfactant AOT in isooctane. Next, the DsRed2 was to be removed through using very little surfactant CTAB, in isooctane/hexanol. Finally the flavodoxin was to be removed using 100 mM CTAB in isooctane/hexanol. The results of the experiment, however, were not as expected.

Throughout the experiment the organic phase of the samples was read on the spectrophotometer at 410 nm, 464 nm, and 561 nm, the results are as follows in Table 7-3. This experiment was performed twice to ensure the repeatability of the results.

Table 7-3: Three Protein Mixture Results, Part 1, separation through use of different surfactants and surfactant concentration variation

Sample Description	Average % Cytochrome *c* Extracted	Average % DsRed2 Extracted	Average % Flavodoxin Extracted
Forward Extraction #1	85	17	21
Forward Extraction #2	26	13	38
Forward Extraction #3	21	48	13
Backward Extraction #1	18	6	6
Backward Extraction #2	16	2	3
Backward Extraction #3	16	3	4

Most of the cytochrome *c* was removed with the first forward extraction as expected. On the second forward extraction it was expected that the DsRed2 would be removed into the organic phase, but mostly flavodoxin was removed instead. On the third forward extraction, mostly DsRed2 was found to be removed. In all of the back extractions most of the protein was removed into a fresh aqueous phase. Only small amounts were left behind in the organic phase. The results again cannot be considered entirely accurate, as the total % of cytochrome *c* accounted for adds up to well over 100%.

To further clarify the results an electrophoresis gel was ran using the aqueous phase samples. (See Figure 7.6) From the gel it appears that all of the cytochrome *c* is removed with the first forward extraction, but a trace of flavodoxin may come along with it. The second forward extraction removed not DsRed2 as expected, but mainly flavodoxin. The third forward extraction served to remove most of the DsRed2 which was left, but not all of the DsRed2 was able to be extracted in this step.

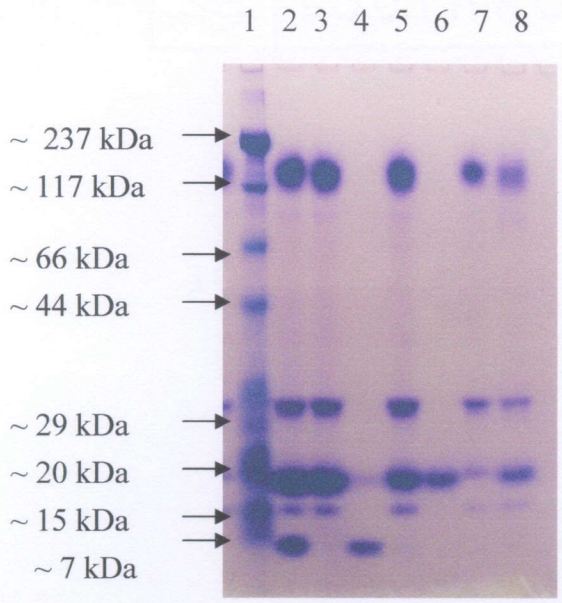

Figure 7.6: PAGE of cytochrome c, DsRed2, and flavodoxin Experiment Results (Part 1); Lane 1 is the molecular weight marker, lane 2 is a sample of the aqueous phase with all three proteins, lane 3 is the aqueous phase after the first forward extraction, lane 4 is the protein back extracted from the organic phase after the first forward extraction, lane 5 is the aqueous phase after the second forward extraction, lane 6 is the protein back extracted from the organic phase after the second forward extraction, lane 7 is the aqueous phase after the third forward extraction, and lane 8 is the protein back extracted from the organic phase after the third forward extraction.

A second experiment using the three proteins (cytochrome *c*, DsRed2, and flavodoxin) was conducted. First the cytochrome *c* was to be extracted using the surfactant AOT in isooctane. Next, the flavodoxin was to be removed through an increase in salt content. Finally the DsRed2 was to be removed through dilution of the sample and using 100 mM CTAB in isooctane/hexanol. When the DsRed2 was diluted, the resulting volume was divided into two sample vials.

Throughout the experiment the organic phase of the samples was read on the spectrophotometer at 410 nm, 464 nm, and 561 nm, the results are as follows in Table 7-4.

Table 7-4: Three Protein Mixture Results, Part 2, separation through use of different surfactants and salt concentration variation

Sample Description	Average % Cytochrome *c* Extracted	Average % DsRed2 Extracted	Average % Flavodoxin Extracted
Forward Extraction #1	88	14	19
Forward Extraction #2	22	4	36
Forward Extraction #3a	17	39	19
Forward Extraction #3b	17	34	18
Backward Extraction #1	22	7	10
Backward Extraction #2	18	3	7
Backward Extraction #3a	17	8	7
Backward Extraction #3b	16	6	5

Most of the cytochrome *c* was removed with the first forward extraction as expected. The second forward extraction resulted in mainly flavodoxin being extracted. And with the third forward extraction, mostly DsRed2 was removed. In all of the back extractions most of the protein was removed into a fresh aqueous phase. Only small amounts were left behind in the organic phase. The results again cannot be considered entirely accurate, as the total % of cytochrome *c* accounted for adds up to well over 100%.

To further clarify the results an electrophoresis gel was ran using the aqueous phase samples. (See Figure 7.7) From the gel it appears that all of the cytochrome *c* is removed with the first forward extraction, but a trace of either flavodoxin may come along with it. The second forward extraction removed mainly flavodoxin. The third forward extraction removed most of the DsRed2. There are two samples for the third forward and back extraction because the sample was diluted to decrease the salt content after the second step. The resulting volume was split into two sample vials.

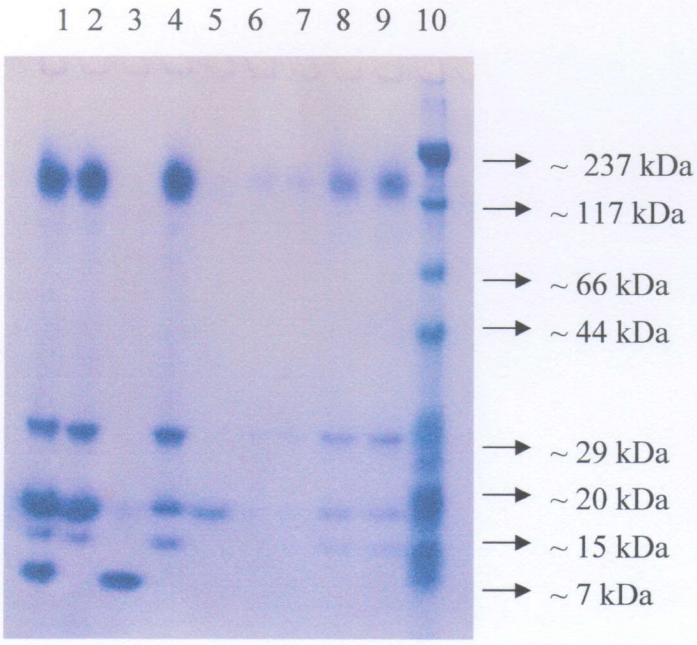

Figure 7.7: PAGE of cytochrome c, DsRed2, and flavodoxin Experiment Results (Part 2); Lane 1 is a sample of the aqueous phase with all three proteins, lane 2 is the aqueous phase after the first forward extraction, lane 3 is the protein back extracted from the organic phase after the first forward extraction, lane 4 is the aqueous phase after the second forward extraction, lane 5 is the protein back extracted from the organic phase after the second forward extraction, lanes 6 and 7 are the aqueous phases after the third forward extraction, and lanes 8 and 9 are the protein back extracted from the organic phases after the third forward extraction, lane 10 is the molecular weight marker.

7.2.3 Discussion

It was shown that DsRed2 and cytochrome *c* could be successfully separated from one another by varying the surfactant used in the organic phase. In the first forward extraction ~ 84 % of the cytochrome *c* was observed in the organic phase, with ~ 15 % DsRed2. In the second forward extraction ~ 82 % DsRed2 was extracted into the organic phase with ~ 22% cytochrome *c*. It was also shown that both the DsRed2 and cytochrome *c* could be back extracted into a fresh aqueous phase. In the first backward extraction only ~ 17 % cytochrome *c* was observed to be left behind in the organic phase,

along with ~ 2 % DsRed2. In the second backward extraction ~ 28% of the DsRed2 was observed to be left behind in the organic phase along with ~ 22% cytochrome *c*.

Problems arose when another protein was added to this mixture. As evidenced by the results of the first experiment with three proteins, it is obvious that DsRed2 cannot be selectively extracted using a low concentration of the surfactant CTAB. Instead, in the protein mixture flavodoxin was favored for extraction at low surfactant concentrations, a trend that was not previously observed in the individual experiments. This may be due to the fact that an experiment was not conducted varying surfactant concentration with flavodoxin at the exact pH at which the experiment was run. Tests were performed using low surfactant concentrations with flavodoxin at a pH ~ 7, and this experiment was performed at a pH of ~ 8.5. Therefore, it is thought that the results were not as expected either due to the difference in pH or because of protein-protein interactions.

In the first forward extraction ~ 85 % cytochrome *c* was removed based on the spectrophotometer readings, along with ~ 17 % DsRed2 and ~ 21 % flavodoxin. In the second forward extraction ~ 38 % flavodoxin was observed to be removed along with ~ 26 % cytochrome *c* and ~ 13 % DsRed2. In the third forward extraction ~ 48 % of the DsRed2 was observed to be removed along with ~ 21 % cytochrome *c* and ~ 13 % flavodoxin. All of these proteins were also successfully back extracted into a fresh aqueous phase leaving only a small amount of detectable protein behind in the organic phase.

A second experiment was performed with these three proteins in order to investigate an alternative pathway to separation that proved to be more productive. Cytochrome *c* was separated effectively using the surfactant AOT. Flavodoxin was then

removed through an increase in salt concentration, and DsRed2 was removed through lowering the salt concentration by dilution of the sample.

In the first forward extraction ~ 88 % cytochrome *c* was removed based on the spectrophotometer readings, along with ~ 14 % DsRed2 and ~ 19 % flavodoxin. In the second forward extraction ~ 36 % flavodoxin was observed to be removed along with ~ 22 % cytochrome *c* and ~ 4 % DsRed2. In the third forward extraction ~ 36.5 % of the DsRed2 was observed to be removed in each of the two samples, along with ~ 17 % cytochrome *c* and ~ 18.5 % flavodoxin. All of these proteins were also successfully back extracted into a fresh aqueous phase leaving only a small amount of detectable protein behind in the organic phase.

Chapter 8: Conclusion

The results of the experiments conducted show the ideal conditions for individual extraction of cytochrome *c*, EGFP, flavodoxin and DsRed2 from reverse micellar systems.

With cytochrome *c*, a minimum AOT concentration of ~ 20 mM was found necessary in order to have ≥ 70% extraction from a buffer solution at pH 9.7. Cytochrome *c* was also found to extract best with at low salt concentrations, and a pH between 8 to 10.5.

The optimum conditions for the extraction of EGFP were no salt, pH of about 8.3, and with 100 mM CTAB in the organic phase.

Flavodoxin extracted best when no salt was present in the system, but could also extraction under low salt concentrations up to about 0.35 M NaCl. The best conditions for the extraction of flavodoxin were with no salt in the aqueous phase at a pH of about 7, and with 100 mM CTAB in the organic phase.

For DsRed2 no minimum surfactant concentration was found. The optimum conditions for the extraction of DsRed2 were salt at 0 – 0.15 M NaCl in the aqueous phase at a pH of about 9, and with 5 - 100 mM CTAB in the organic phase.

With the individual protein experiments using the cationic surfactant CTAB it was found overall that the best pH for the process was around 2.5 to 3 units above the pI of the protein. Each of the proteins also did best with 100 mM CTAB and salt concentrations below 0.25 M NaCl.

Overall, the results of the pH experiments showed that flavodoxin could potentially be separated from a mixture of EGFP and DsRed2 by pH variation, but some amount of DsRed2 and EGFP could also be present. The experiments with salt concentration variation showed another more promising possibility for the separation of flavodoxin from both DsRed2 and EGFP, at NaCl concentrations around 0.4 M. The surfactant variation experiments showed a possibility for removing DsRed2 at low surfactant concentrations from EGFP and flavodoxin.

It was also observed that DsRed2 could be successfully extracted from crude lysate. The experiment with the DsRed2 crude lysate showed that the reverse micelle extraction works not only when the protein is pure, but also when it is contaminated with other proteins. On average, 72% of the DsRed2 was extracted into the organic phase at a pH ~ 9 and conductivity equal to ~ 0.13 M NaCl. In the experiments using relatively pure DsRed2, ~ 88% of DsRed2 was extracted under similar conditions (pH ~ 9, 0.15 M NaCl). This shows that there is some interference due to the other proteins present in the system however, a decent quantity of the DsRed2 could still be extracted. This experiment also proved that the DsRed2 could be back-extracted into a fresh aqueous phase, with only ~ 14% of the protein left behind in the organic phase. The results of the electrophoresis gel shows that through forward extraction some of the contaminated proteins present can be separated from the DsRed2, and left behind in the aqueous phase.

Cytochrome *c* and DsRed2 were extracted from one another relatively easily, but problems arose when flavodoxin was added to the mixture. DsRed2 and cytochrome *c* could be successfully separated from one another by varying the surfactant used in the organic phase. In the first forward extraction ~ 84 % of the cytochrome *c* was observed

in the organic phase, with ~ 15 % DsRed2. In the second forward extraction ~ 82 % DsRed2 was extracted into the organic phase with ~ 22% cytochrome *c*. It was also shown that both the DsRed2 and cytochrome *c* could be back extracted into a fresh aqueous phase with only small amounts of protein left behind in the organic phase.

When flavodoxin was added to the mixture, it was found that DsRed2 could not be selectively extracted using a low concentration of the surfactant CTAB. Instead, in the protein mixture flavodoxin was favored for extraction at low surfactant concentrations, a trend that was not previously observed in the individual experiments. In the first forward extraction ~ 85 % cytochrome *c* was removed based on the spectrophotometer readings, along with ~ 17 % DsRed2 and ~ 21 % flavodoxin. In the second forward extraction ~ 38 % flavodoxin was observed to be removed along with ~ 26 % cytochrome *c* and ~ 13 % DsRed2. In the third forward extraction ~ 48 % of the DsRed2 was observed to be removed along with ~ 21 % cytochrome *c* and ~ 13 % flavodoxin. All of these proteins were also successfully back extracted into a fresh aqueous phase leaving only a small amount of detectable protein behind in the organic phase.

Due to these results a second experiment was performed with these three proteins in order to investigate an alternative pathway to separation that proved to be more productive. In this case the cytochrome *c* was separated effectively using the surfactant AOT. Flavodoxin was then removed through an increase in salt concentration, and DsRed2 was removed through lowering the salt concentration by dilution of the sample.

In the first forward extraction ~ 88 % cytochrome *c* was removed based on the spectrophotometer readings, along with ~ 14 % DsRed2 and ~ 19 % flavodoxin. In the second forward extraction ~ 36 % flavodoxin was observed to be removed along with ~

22 % cytochrome *c* and ~ 4 % DsRed2. In the third forward extraction ~ 36.5 % of the DsRed2 was observed to be removed in each of the two samples, along with ~ 17 % cytochrome *c* and ~ 18.5 % flavodoxin. All of these proteins were also successfully back extracted into a fresh aqueous phase leaving only a small amount of detectable protein behind in the organic phase.

It is recommended that further experiments be conducted with each of the proteins to observe how each extracts from crude lysate as well. In the area of separation of protein mixtures more work is also needed in order to refine the extraction of the three proteins from one another, and then each of the four proteins from one another as well. Each mixture of proteins is very unique, and protein-protein interactions also need to be considered in these systems. While much more research is needed overall with reverse micelle systems, it does have definite potential for future use in the biotechnology industry.

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Appendices

Appendix A: Detailed Sample Compositions and Experimental Data

Cytochrome *c*

Surfactant Variation

Experiment #1

The aqueous phase was prepared by making a stock solution of 1 g/L cytochrome *c* in 80% 0.1 M Tris buffer and 20% 0.1 M NaCl. The organic phase was prepared by making a stock solution of 50 mM AOT in isooctane, and combining that stock solution with isooctane to obtain the desired AOT concentration as follows in Table A-1. For this experiment the total volume of each phase was 750 μL. Table A-2 shows the corrected absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-3 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-4.

Table A-1: Organic Phase of Samples for Surfactant Variation Experiment

Sample Number	AOT Conc. (mM)	Amt. Of 50 mM AOT stock added (μL)	Amt. Of Isooctane added (μL)
1	5	75	675
2	10	150	600
3	15	225	525
4	20	300	450
5	25	375	375
6	30	450	300
7	35	525	225
8	40	600	150
9	45	675	75
10	50	750	0

Table A-2: Corrected Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	AOT Conc. (mM)	A410 (nm)	A410 (nm)	A410 (nm)
1	5	0.0226	0.0057	0.3510
2	10	0.0619	0.0728	1.5656
3	15	2.6265	3.2485	7.0605
4	20	4.8185	6.0809	6.3124
5	25	7.0503	4.8044	7.1721
6	30	8.5501	4.9349	6.2187
7	35	8.2487	5.3562	6.8656
8	40	6.3696	5.3307	6.6621
9	45	6.9395	4.8530	7.1317
10	50	5.3887	4.6789	7.7236

Table A-3: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	AOT Conc. (mM)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Avg.	Std. Deviation
1	5	0.012	0.010	0.056	0.026	0.026
2	10	0.017	0.019	0.221	0.086	0.117
3	15	0.364	0.448	0.963	0.592	0.324
4	20	0.660	0.831	0.862	0.784	0.109
5	25	0.962	0.658	0.978	0.866	0.180
6	30	1.164	0.676	0.849	0.896	0.248
7	35	1.124	0.733	0.937	0.931	0.195
8	40	0.870	0.729	0.909	0.836	0.095
9	45	0.947	0.665	0.973	0.861	0.171
10	50	0.737	0.641	1.053	0.810	0.215

Table A-4: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	AOT Conc. (mM)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Avg.	Std. Deviation
1	5	1.114	0.904	5.209	2.41	2.43
2	10	1.605	1.741	20.353	7.90	10.79
3	15	33.580	41.335	88.861	54.59	29.93
4	20	60.909	76.647	79.534	72.36	10.02
5	25	88.734	60.733	90.252	79.91	16.62
6	30	107.432	62.359	78.365	82.72	22.85
7	35	103.675	67.612	86.431	85.91	18.04
8	40	80.247	67.295	83.894	77.15	8.72
9	45	87.353	61.339	89.749	79.48	15.76
10	50	68.018	59.168	97.128	74.77	19.86

Experiment #2

The aqueous phase was prepared by making a stock solution of ~ 0.22 g/L cytochrome *c* in 80% 0.1 M Tris buffer and 20% 0.1 M NaCl. The organic phase was prepared by making a stock solution of 50 mM AOT in isooctane, and combining that stock solution with isooctane to obtain the desired AOT concentration as follows in Table A-5. For this experiment the total volume of each phase was 900 μL. Table A-6 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-7 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-8.

Table A-5: Organic Phase of Samples for Surfactant Variation Experiment

Sample Number	AOT Conc. (mM)	Amt. Of 50 mM AOT stock added (μL)	Amt. Of Isooctane added (μL)
1	5	90	810
2	10	180	720
3	15	270	630
4	20	360	540
5	25	450	450
6	30	540	360
7	35	630	270
8	40	720	780
9	45	810	90
10	50	900	0

Table A-6: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	AOT Conc. (mM)	A410 (nm)	A410 (nm)	A410 (nm)
1	5	0.2209	0.3351	0.2426
2	10	0.4844	0.4567	0.3464
3	15	0.5166	0.5130	0.4492
4	20	0.6075	0.5467	0.4365
5	25	0.5920	0.3897	0.5790
6	30	0.5956	0.4559	0.6620
7	35	0.5911	0.5237	0.6084
8	40	0.6032	0.4591	0.7115
9	45	0.5921	0.5488	0.6390
10	50	0.5876	0.5919	0.6745

Table A-7: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	AOT Conc. (mM)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	5	0.111	0.137	0.116	0.12	0.01
2	10	0.170	0.164	0.139	0.16	0.02
3	15	0.177	0.177	0.162	0.17	0.01
4	20	0.198	0.184	0.159	0.18	0.02
5	25	0.194	0.149	0.191	0.18	0.03
6	30	0.195	0.164	0.210	0.19	0.02
7	35	0.194	0.179	0.198	0.19	0.01
8	40	0.197	0.165	0.221	0.19	0.03
9	45	0.194	0.185	0.205	0.19	0.01
10	50	0.193	0.194	0.213	0.20	0.01

Table A-8: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	AOT Conc. (mM)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	5	49.319	60.647	51.473	53.81	6.02
2	10	75.461	72.717	61.773	69.98	7.24
3	15	78.656	78.305	71.968	76.31	3.76
4	20	87.677	81.641	70.711	80.01	8.60
5	25	86.135	66.069	84.851	79.02	11.23
6	30	86.502	72.633	93.084	84.07	10.44
7	35	86.047	79.362	87.767	84.39	4.44
8	40	87.248	72.957	98.000	86.07	12.56
9	45	86.150	81.856	90.799	86.27	4.47
10	50	85.707	86.128	94.327	88.72	4.86

Experiment #3

The aqueous phase was prepared by making a stock solution of 1 g/L cytochrome *c* in 80% 0.1 M Tris buffer and 20% 0.1 M NaCl. The organic phase was prepared by making a stock solution of 50 mM AOT in isooctane, and combining that stock solution with isooctane to obtain the desired AOT concentration as in Table A-5. For this experiment the total volume of each phase was 900 μL. Table A-9 shows the corrected absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-10 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-11.

Table A-9: Corrected Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	AOT Conc. (mM)	A410 (nm)	A410 (nm)	A410 (nm)
1	5	0.036	0.080	0.044
2	10	0.157	0.074	0.115
3	15	2.846	2.497	2.275
4	20	3.360	2.822	2.984
5	25	3.203	2.535	3.357
6	30	3.761	2.030	3.001
7	35	2.775	3.192	2.809
8	40	3.965	2.825	2.888
9	45	3.211	3.247	2.530
10	50	3.400	2.729	2.905

Table A-10: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	AOT Conc. (mM)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	5	0.070	0.080	0.072	0.07	0.01
2	10	0.097	0.078	0.088	0.09	0.01
3	15	0.698	0.620	0.571	0.63	0.06
4	20	0.813	0.693	0.729	0.75	0.06
5	25	0.778	0.629	0.813	0.74	0.10
6	30	0.903	0.516	0.733	0.72	0.19
7	35	0.683	0.776	0.690	0.72	0.05
8	40	0.949	0.694	0.708	0.78	0.14
9	45	0.780	0.788	0.628	0.73	0.09
10	50	0.822	0.672	0.712	0.74	0.08

Table A-11: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	AOT Conc. (mM)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	5	7.201	8.199	7.384	7.59	0.53
2	10	9.982	8.073	9.018	9.02	0.95
3	15	71.954	63.923	58.808	64.89	6.63
4	20	83.802	71.399	75.152	76.78	6.36
5	25	80.202	64.785	83.737	76.24	10.08
6	30	93.049	53.166	75.533	73.92	19.99
7	35	70.324	79.931	71.108	73.79	5.33
8	40	97.749	71.470	72.922	80.71	14.77
9	45	80.365	81.208	64.680	75.42	9.31
10	50	84.733	69.277	73.314	75.77	8.02

Salt Variation

For the experiments varying salt concentration the organic phase was held at 50 mM AOT in isooctane. The aqueous phase was varied as follows in Table A-12. The cytochrome *c* stock used was ~ 0.6 g/L, and each sample had a final cytochrome *c* concentration of ~ 0.2 g/L. For this experiment the total volume of each phase was 900 µL. Table A-13 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-14 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-15.

Table A-12: Aqueous Phase of Samples for Salt Variation Experiment

Sample #	Final Salt Conc. (M)	2 M NaCl Added (µL)	DI H_2O Added (µL)	Cytochrome *c* Stock Added (µL)	1 M Tris Added (µL)
1	0	0	582	300	18
2	0.08	36	546	300	18
3	0.16	72	510	300	18
4	0.24	108	474	300	18
5	0.32	144	438	300	18
6	0.4	180	402	300	18
7	0.48	216	366	300	18
8	0.56	252	330	300	18
9	0.64	288	294	300	18
10	0.72	324	258	300	18
11	0.8	360	222	300	18

Table A-13: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	NaCl Conc. (M)	A410 (nm)	A410 (nm)	A410 (nm)
1	0.00	0.003	0.006	0.005
2	0.08	0.505	0.404	0.446
3	0.16	0.836	0.782	0.706
4	0.24	0.840	0.831	0.794
5	0.32	0.830	0.803	0.744
6	0.40	0.785	0.731	0.692
7	0.48	0.646	0.584	0.549
8	0.56	0.467	0.452	0.367
9	0.64	0.338	0.281	0.240
10	0.72	0.237	0.176	0.168
11	0.80	0.162	0.142	0.121

Table A-14: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	NaCl Conc. (M)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	0.00	0.062	0.063	0.063	0.06	0.0004
2	0.08	0.175	0.152	0.161	0.16	0.0114
3	0.16	0.249	0.237	0.220	0.24	0.0147
4	0.24	0.250	0.248	0.240	0.25	0.0054
5	0.32	0.247	0.241	0.228	0.24	0.0098
6	0.40	0.237	0.225	0.217	0.23	0.0104
7	0.48	0.206	0.192	0.185	0.19	0.0110
8	0.56	0.166	0.163	0.144	0.16	0.0121
9	0.64	0.137	0.125	0.116	0.13	0.0110
10	0.72	0.115	0.101	0.099	0.11	0.0084
11	0.80	0.098	0.093	0.089	0.09	0.0046

Table A-15: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	NaCl Conc. (M)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	0.00	22.753	23.598	23.464	23.27	0.45
2	0.08	63.682	56.802	60.269	60.25	3.44
3	0.16	90.654	88.370	81.989	87.00	4.49
4	0.24	91.013	92.438	89.407	90.95	1.52
5	0.32	90.166	90.088	85.214	88.49	2.84
6	0.40	86.467	84.120	80.863	83.82	2.81
7	0.48	75.188	71.814	68.913	71.97	3.14
8	0.56	60.591	60.822	53.710	58.37	4.04
9	0.64	50.046	46.509	43.135	46.56	3.46
10	0.72	41.810	37.791	37.087	38.90	2.55
11	0.80	35.702	34.880	33.169	34.58	1.29

pH Variation

For the experiments varying pH concentration the organic phase was held at 50 mM AOT in isooctane. For these tests the pH of the aqueous phase was varied from ~ 3 to 11 using phosphate, acetate, and carbonate buffers, while the salt concentration in the system was held at 0.08 M NaCl. Acetate buffer was used for the 3-5 pH range, phosphate buffer was used for the 6-8 pH range, and carbonate buffer was used for the 9-12 pH range. The cytochrome *c* stock was ~ 0.6 g/L, and each sample had a final cytochrome *c* concentration of ~ 0.2 g/L. For this experiment the total volume of each phase was 900 µL. Table A-16 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-17 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-18.

Table A-16: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	pH	A410 (nm)	A410 (nm)	A410 (nm)
1	3.371	0.104	0.087	0.055
2	3.909	0.124	0.057	0.055
3	4.914	0.121	0.109	0.097
4	5.970	0.246	0.279	0.266
5	6.910	0.440	0.417	0.377
6	7.830	0.561	0.533	0.493
7	9.008	0.472	0.468	0.383
8	10.005	0.543	0.541	0.472
9	10.635	0.517	0.531	0.459
10	10.952	0.137	0.143	0.112

Table A-17: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	pH	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	3.371	0.085	0.081	0.074	0.08	0.0055
2	3.909	0.089	0.074	0.074	0.08	0.0087
3	4.914	0.089	0.086	0.083	0.09	0.0027
4	5.970	0.117	0.124	0.121	0.12	0.0037
5	6.910	0.160	0.155	0.146	0.15	0.0071
6	7.830	0.187	0.181	0.172	0.18	0.0076
7	9.008	0.167	0.166	0.147	0.16	0.0112
8	10.005	0.183	0.183	0.167	0.18	0.0091
9	10.635	0.177	0.181	0.164	0.17	0.0086
10	10.952	0.092	0.094	0.087	0.09	0.0037

Table A-18: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	pH	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	3.371	37.992	36.354	33.123	35.82	2.48
2	3.909	40.005	33.314	33.166	35.50	3.91
3	4.914	39.691	38.507	37.318	38.51	1.19
4	5.970	52.217	55.493	54.269	53.99	1.66
5	6.910	71.652	69.299	65.387	68.78	3.16
6	7.830	83.742	80.909	76.947	80.53	3.41
7	9.008	74.845	74.416	65.954	71.74	5.01
8	10.005	81.996	81.802	74.821	79.54	4.09
9	10.635	79.360	80.734	73.527	77.87	3.83
10	10.952	41.289	41.981	38.864	40.71	1.64

EGFP

pH Variation

For the experiments varying pH concentration the organic phase was held at 100 mM CTAB in 90 % isooctane and 10 % hexanol with a small amount of H_2O. For the experiment varying pH, pH was varied from ~ 5 to ~ 9.5 in the aqueous phase using 50 mM acetate, phosphate, and carbonate buffers. Acetate buffer was used for the 3-5 pH range, phosphate buffer was used for the 6-8 pH range, and carbonate buffer was used for the 9-12 pH range. In the pH experiment no salt was used, and the EGFP concentration in the aqueous phase was ~ 0.36 g/L. For this experiment the total volume of each phase was 900 μL. Table A-19 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-20 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-21.

Table A-19: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	pH	A488 (nm)	A488 (nm)	A488 (nm)
1	4.889	0.141	0.133	0.123
2	5.418	0.099	0.118	0.112
3	6.204	0.214	0.270	0.245
4	6.705	0.224	0.220	0.240
5	7.096	0.319	0.245	0.244
6	7.631	0.265	0.309	0.250
7	8.165	0.254	0.262	0.261
8	8.412	0.292	0.306	0.296
9	8.944	0.338	0.287	0.242
10	9.432	0.345	0.292	0.243

Table A-20: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	pH	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	4.889	0.139	0.131	0.121	0.13	0.0088
2	5.418	0.097	0.116	0.110	0.11	0.0094
3	6.204	0.210	0.265	0.241	0.24	0.0277
4	6.705	0.220	0.216	0.236	0.22	0.0105
5	7.096	0.314	0.241	0.239	0.26	0.0424
6	7.631	0.260	0.303	0.245	0.27	0.0299
7	8.165	0.250	0.258	0.256	0.25	0.0044
8	8.412	0.286	0.300	0.291	0.29	0.0071
9	8.944	0.332	0.281	0.238	0.28	0.0472
10	9.432	0.339	0.287	0.238	0.29	0.0503

Table A-21: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	pH	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	4.889	37.922	35.662	33.094	35.56	2.42
2	5.418	26.618	31.649	29.950	29.41	2.56
3	6.204	57.245	72.310	65.684	65.08	7.55
4	6.705	60.006	58.950	64.352	61.10	2.86
5	7.096	85.606	65.753	65.354	72.24	11.58
6	7.631	71.117	82.743	67.003	73.62	8.16
7	8.165	68.134	70.351	70.016	69.50	1.19
8	8.412	78.218	82.016	79.467	79.90	1.94
9	8.944	90.728	76.813	64.973	77.50	12.89
10	9.432	92.481	78.264	65.011	78.59	13.74

Salt Variation

For the salt variation experiments the organic phase consisted of 100 mM CTAB in 90 % isooctane and 10 % hexanol, with a small amount of H_2O. The aqueous phase was a 50 mM Tris buffer solution at a pH of ~ 8.3, EGFP at ~ 0.36 g/L, and varying concentration of NaCl from 0 – 0.95 M, as shown in Table A-22. For this experiment the total volume of each phase was 900 µL. Table A-23 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-24 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-25.

Table A-22: Aqueous Phase of Samples for Salt Variation Experiment

Sample #	Final Salt Conc. (M)	2 M NaCl Added (µL)	DI H_2O Added (µL)	EGFP Stock Added (µL)	1 M Tris Added (µL)
1	0	0	755	100	45
2	0.15	67.5	687.5	100	45
3	0.25	112.5	642.5	100	45
4	0.35	157.5	597.5	100	45
5	0.45	202.5	552.5	100	45
6	0.55	247.5	507.5	100	45
7	0.65	292.5	462.5	100	45
8	0.75	337.5	417.5	100	45
9	0.85	382.5	372.5	100	45
10	0.95	427.5	327.5	100	45

Table A-23: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	NaCl Conc. (M)	A488 (nm)	A488 (nm)	A488 (nm)
1	0.00	0.335	0.272	0.315
2	0.15	0.182	0.176	0.195
3	0.25	0.034	0.030	0.036
4	0.35	0.008	0.003	0.009
5	0.45	0.004	0.004	0.009
6	0.55	0.004	0.001	0.003
7	0.65	0.004	0.001	0.001
8	0.75	0.007	0.002	0.001
9	0.85	0.004	0.004	0.002
10	0.95	0.007	0.007	0.000

Table A-24: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	NaCl Conc. (M)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	0.00	0.329	0.267	0.309	0.30	0.0316
2	0.15	0.179	0.173	0.192	0.18	0.0096
3	0.25	0.033	0.029	0.036	0.03	0.0032
4	0.35	0.008	0.003	0.009	0.01	0.0031
5	0.45	0.004	0.004	0.009	0.01	0.0028
6	0.55	0.003	0.001	0.003	0.00	0.0013
7	0.65	0.004	0.001	0.001	0.00	0.0015
8	0.75	0.007	0.002	0.001	0.00	0.0031
9	0.85	0.004	0.004	0.002	0.00	0.0011
10	0.95	0.006	0.007	0.000	0.00	0.0037

Table A-25: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	NaCl Conc. (M)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	0.00	89.787	72.924	84.426	82.38	8.62
2	0.15	48.839	47.269	52.364	49.49	2.61
3	0.25	9.116	8.030	9.735	8.96	0.86
4	0.35	2.072	0.752	2.359	1.73	0.86
5	0.45	1.205	1.067	2.470	1.58	0.77
6	0.55	0.954	0.326	0.885	0.72	0.34
7	0.65	1.009	0.315	0.303	0.54	0.40
8	0.75	1.776	0.411	0.261	0.82	0.83
9	0.85	1.193	1.097	0.635	0.98	0.30
10	0.95	1.755	1.992	0.114	1.29	1.02

Surfactant Variation

In the experiment varying surfactant concentration the organic phase consisted of CTAB in amounts varying from 5 to 100 mM, with 90 % isooctane, 10 % hexanol. and a small amount of water as shown in Table A-26. The aqueous phase consisted of a 50 mM phosphate buffer solution at a pH of ~ 8.3 with no salt and EGFP at ~ 0.36 g/L. For this experiment the total volume of each phase was 900 µL. Table A-27 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-28 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-29.

Table A-26: Organic Phase of Samples for Surfactant Variation Experiment

Sample Number	Final CTAB Conc. (mM)	Amt. Of 100 mM CTAB stock added (µL)	Amt. Of 90% Isooctane, 10% Hexanol solution added (µL)
1	5	45	855
2	10	90	810
3	15	135	765
4	20	180	720
5	25	225	675
6	30	270	630
7	50	450	450
8	100	900	0

Table A-27: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	CTAB Conc. (mM)	A488 (nm)	A488 (nm)	A488 (nm)
1	5	0.075	0.042	0.018
2	10	0.106	0.028	0.062
3	15	0.088	0.058	0.065
4	20	0.173	0.194	0.208
5	25	0.219	0.231	0.230
6	30	0.256	0.262	0.237
7	50	0.268	0.245	0.248
8	100	0.298	0.314	0.267

Table A-28: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	CTAB Conc. (mM)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	5	0.073	0.042	0.018	0.04	0.0278
2	10	0.105	0.028	0.061	0.06	0.0386
3	15	0.086	0.057	0.064	0.07	0.0150
4	20	0.170	0.190	0.204	0.19	0.0171
5	25	0.215	0.227	0.226	0.22	0.0065
6	30	0.251	0.258	0.233	0.25	0.0126
7	50	0.263	0.240	0.243	0.25	0.0122
8	100	0.292	0.308	0.262	0.29	0.0234

Table A-29: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	CTAB Conc. (mM)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	5	19.985	11.334	4.867	12.06	7.59
2	10	28.537	7.535	16.696	17.59	10.53
3	15	23.491	15.639	17.539	18.89	4.10
4	20	46.354	51.898	55.645	51.30	4.67
5	25	58.682	61.888	61.615	60.73	1.78
6	30	68.541	70.305	63.660	67.50	3.44
7	50	71.777	65.652	66.426	67.95	3.34
8	100	79.794	84.035	71.501	78.44	6.38

Flavodoxin

pH Variation

For the experiments varying pH concentration the organic phase was held at 100 mM CTAB in 90 % isooctane and 10 % hexanol with a small amount of H_2O. For the experiment varying pH, pH was varied from ~ 4.5 to ~ 8 in the aqueous phase using 50 mM acetate, phosphate, and carbonate buffers. Acetate buffer was used for the 3-5 pH range, phosphate buffer was used for the 6-8 pH range, and carbonate buffer was used for the 9-12 pH range. In the pH experiment no salt was used, and the flavodoxin concentration in the aqueous phase was ~ 2.15 g/L. For this experiment the total volume of each phase was 900 μL. Table A-30 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-31 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-32.

Table A-30: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	pH	A464 (nm)	A464 (nm)	A464 (nm)
1	4.510	0.171	0.243	0.253
2	4.945	0.265	0.263	0.281
3	5.300	0.282	0.280	0.253
4	6.050	0.278	0.254	0.247
5	6.612	0.269	0.268	0.236
6	6.980	0.266	0.278	0.275
7	7.490	0.241	0.242	0.257
8	7.820	0.244	0.239	0.277

Table A-31: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	pH	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	4.510	1.234	1.748	1.818	1.60	0.3189
2	4.945	1.908	1.892	2.021	1.94	0.0702
3	5.300	2.031	2.017	1.818	1.96	0.1191
4	6.050	2.004	1.829	1.776	1.87	0.1192
5	6.612	1.939	1.929	1.696	1.85	0.1376
6	6.980	1.913	2.001	1.978	1.96	0.0453
7	7.490	1.734	1.738	1.853	1.78	0.0674
8	7.820	1.755	1.721	1.992	1.82	0.1473

Table A-32: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	pH	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	4.510	57.269	81.113	84.373	74.25	14.80
2	4.945	88.557	87.813	93.791	90.05	3.26
3	5.300	94.275	93.601	84.379	90.75	5.53
4	6.050	92.983	84.874	82.415	86.76	5.53
5	6.612	89.977	89.536	78.705	86.07	6.38
6	6.980	88.791	92.843	91.791	91.14	2.10
7	7.490	80.468	80.672	85.986	82.38	3.13
8	7.820	81.437	79.880	92.425	84.58	6.84

Salt Variation

For the salt variation experiments the organic phase consisted of 100 mM CTAB in 90 % isooctane and 10 % hexanol, with a small amount of H_2O. The aqueous phase was a 50 mM phosphate buffer solution at a pH of ~ 7, flavodoxin at ~ 2.15 g/L, and varying concentration of NaCl from 0 – 0.95 M, as shown in Table A-33. For this experiment the total volume of each phase was 900 µL. Table A-34 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-35 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-36.

Table A-33: Aqueous Phase of Samples for Salt Variation Experiment

Sample #	Final Salt Conc. (M)	2 M NaCl Added (µL)	DI H_2O Added (µL)	Flavodoxin Stock Added (µL)	0.2 M Phosphate Added (µL)
1	0	0	575	100	225
2	0.15	67.5	507.5	100	225
3	0.25	112.5	462.5	100	225
4	0.35	157.5	417.5	100	225
5	0.45	202.5	372.5	100	225
6	0.55	247.5	327.5	100	225
7	0.65	292.5	282.5	100	225
8	0.75	337.5	237.5	100	225
9	0.85	382.5	192.5	100	225
10	0.95	427.5	147.5	100	225

Table A-34: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	NaCl Conc. (M)	A464 (nm)	A464 (nm)	A464 (nm)
1	0.00	0.250	0.299	0.292
2	0.15	0.241	0.223	0.240
3	0.25	0.231	0.241	0.245
4	0.35	0.219	0.219	0.230
5	0.45	0.047	0.062	0.064
6	0.55	0.014	0.017	0.017
7	0.65	0.010	0.007	0.013
8	0.75	0.011	0.015	0.020
9	0.85	0.009	0.006	0.007
10	0.95	0.006	0.008	0.018

Table A-35: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	NaCl Conc. (M)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	0.00	1.797	2.149	2.101	2.02	0.1911
2	0.15	1.733	1.606	1.725	1.69	0.0708
3	0.25	1.665	1.736	1.764	1.72	0.0510
4	0.35	1.580	1.579	1.654	1.60	0.0431
5	0.45	0.337	0.445	0.459	0.41	0.0667
6	0.55	0.102	0.120	0.123	0.12	0.0115
7	0.65	0.075	0.051	0.090	0.07	0.0200
8	0.75	0.081	0.109	0.143	0.11	0.0312
9	0.85	0.063	0.042	0.052	0.05	0.0106
10	0.95	0.042	0.058	0.132	0.08	0.0483

Table A-36: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	NaCl Conc. (M)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	0.00	83.387	99.746	97.498	93.54	8.87
2	0.15	80.428	74.547	80.031	78.34	3.29
3	0.25	77.259	80.545	81.854	79.89	2.37
4	0.35	73.304	73.271	76.754	74.44	2.00
5	0.45	15.661	20.668	21.319	19.22	3.10
6	0.55	4.727	5.560	5.726	5.34	0.54
7	0.65	3.495	2.351	4.187	3.34	0.93
8	0.75	3.742	5.037	6.631	5.14	1.45
9	0.85	2.946	1.966	2.430	2.45	0.49
10	0.95	1.937	2.677	6.133	3.58	2.24

Surfactant Variation

In the experiment varying surfactant concentration the organic phase consisted of CTAB in amounts varying from 5 to 100 mM, with 90 % isooctane, 10 % hexanol, and a small amount of water as shown in Table A-37. The aqueous phase consisted of a 50 mM phosphate buffer solution at a pH of ~ 7 with no salt and flavodoxin at ~ 2.15 g/L. For this experiment the total volume of each phase was 900 μL. Table A-38 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-39 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-40.

Table A-37: Organic Phase of Samples for Surfactant Variation Experiment

Sample Number	Final CTAB Conc. (mM)	Amt. Of 100 mM CTAB stock added (μL)	Amt. Of 90% Isooctane, 10% Hexanol solution added (μL)
1	5	45	855
2	10	90	810
3	15	135	765
4	20	180	720
5	25	225	675
6	30	270	630
7	50	450	450
8	75	675	225
9	100	900	0

Table A-38: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	CTAB Conc. (mM)	A464 (nm)	A464 (nm)	A464 (nm)
1	5	0.002	0.012	0.004
2	10	0.126	0.108	0.071
3	15	0.164	0.229	0.226
4	20	0.237	0.222	0.207
5	25	0.241	0.232	0.216
6	30	0.238	0.250	0.268
7	50	0.271	0.239	0.271
8	75	0.267	0.266	0.262
9	100	0.270	0.266	0.288

Table A-39: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	CTAB Conc. (mM)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	5	0.013	0.088	0.031	0.04	0.0387
2	10	0.905	0.776	0.508	0.73	0.2024
3	15	1.183	1.645	1.626	1.48	0.2611
4	20	1.704	1.594	1.493	1.60	0.1055
5	25	1.735	1.667	1.556	1.65	0.0903
6	30	1.714	1.801	1.930	1.81	0.1083
7	50	1.949	1.718	1.950	1.87	0.1337
8	75	1.921	1.912	1.886	1.91	0.0182
9	100	1.941	1.915	2.073	1.98	0.0848

Table A-40: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	CTAB Conc. (mM)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	5	0.626	4.065	1.452	2.05	1.80
2	10	42.016	35.991	23.593	33.87	9.39
3	15	54.921	76.340	75.445	68.90	12.12
4	20	79.062	73.989	69.276	74.11	4.89
5	25	80.512	77.376	72.212	76.70	4.19
6	30	79.553	83.578	89.546	84.23	5.03
7	50	90.455	79.730	90.505	86.90	6.21
8	75	89.165	88.731	87.535	88.48	0.84
9	100	90.064	88.891	96.219	91.72	3.94

DsRed2

pH Variation

For the experiments varying pH concentration the organic phase was held at 100 mM CTAB in 90 % isooctane and 10 % hexanol with a small amount of H_2O. For the experiment varying pH, pH was varied from ~ 6 to ~ 10.5 in the aqueous phase using 50 mM acetate, phosphate, and carbonate buffers. Acetate buffer was used for the 3-5 pH range, phosphate buffer was used for the 6-8 pH range, and carbonate buffer was used for the 9-12 pH range. In the pH experiment no salt was used, and the DsRed2 concentration in the aqueous phase was ~ 2.4 g/L. For this experiment the total volume of each phase was 900 μL. Table A-41 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-42 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-43.

Table A-41: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	pH	A561 (nm)	A561 (nm)	A561 (nm)
1	6.277	0.348	0.346	0.351
2	6.738	0.410	0.403	0.383
3	7.099	0.422	0.435	0.424
4	7.671	0.420	0.434	0.428
5	8.209	0.431	0.459	0.425
6	9.204	0.446	0.441	0.438
7	9.758	0.438	0.410	0.439
8	10.22	0.435	0.466	0.423
9	10.767	0.405	0.413	0.399

Table A-42: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	pH	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	6.277	1.638	1.627	1.652	1.64	0.0123
2	6.738	1.926	1.894	1.801	1.87	0.0651
3	7.099	1.986	2.044	1.996	2.01	0.0309
4	7.671	1.976	2.043	2.014	2.01	0.0337
5	8.209	2.027	2.159	2.001	2.06	0.0850
6	9.204	2.096	2.073	2.061	2.08	0.0177
7	9.758	2.061	1.928	2.065	2.02	0.0775
8	10.22	2.048	2.189	1.988	2.08	0.1033
9	10.767	1.906	1.943	1.877	1.91	0.0331

Table A-43: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	pH	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	6.277	67.925	67.500	68.518	67.98	0.51
2	6.738	79.904	78.552	74.699	77.72	2.70
3	7.099	82.385	84.791	82.812	83.33	1.28
4	7.671	81.977	84.765	83.540	83.43	1.40
5	8.209	84.071	89.574	83.002	85.55	3.53
6	9.204	86.925	86.002	85.477	86.13	0.73
7	9.758	85.475	79.989	85.639	83.70	3.22
8	10.22	84.956	90.815	82.471	86.08	4.28
9	10.767	79.041	80.598	77.865	79.17	1.37

Salt Variation

For the salt variation experiments the organic phase consisted of 100 mM CTAB in 90 % isooctane and 10 % hexanol, with a small amount of H_2O. The aqueous phase was a 50 mM carbonate buffer solution at a pH of ~ 9, DsRed2 at ~ 2.4 g/L, and varying concentration of NaCl from 0 – 0.75 M, as shown in Table A-44. For this experiment the total volume of each phase was 900 µL. Table A-45 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-46 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-47.

Table A-44: Aqueous Phase of Samples for Salt Variation Experiment

Sample #	Final Salt Conc. (M)	2 M NaCl Added (µL)	DI H_2O Added (µL)	DsRed2 Stock Added (µL)	0.1 M Carbonate Added (µL)
1	0	0	350	100	450
2	0.15	67.5	282.5	100	450
3	0.25	112.5	237.5	100	450
4	0.35	157.5	192.5	100	450
5	0.45	202.5	147.5	100	450
6	0.55	247.5	102.5	100	450
7	0.65	292.5	57.5	100	450
8	0.75	337.5	12.5	100	450

Table A-45: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	NaCl Conc. (M)	A561 (nm)	A561 (nm)	A561 (nm)
1	0	0.447	0.460	0.466
2	0.15	0.440	0.454	0.452
3	0.25	0.180	0.191	0.196
4	0.35	0.015	0.008	0.020
5	0.45	0.002	0.001	0.005
6	0.55	0.005	0.003	0.008
7	0.65	0.005	0.012	0.010
8	0.75	0.010	0.003	0.003

Table A-46: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	NaCl Conc. (M)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	0	2.101	2.165	2.192	2.15	0.0465
2	0.15	2.068	2.137	2.127	2.11	0.0376
3	0.25	0.846	0.896	0.921	0.89	0.0379
4	0.35	0.069	0.040	0.092	0.07	0.0262
5	0.45	0.010	0.005	0.024	0.01	0.0096
6	0.55	0.025	0.016	0.036	0.03	0.0100
7	0.65	0.023	0.055	0.045	0.04	0.0163
8	0.75	0.048	0.013	0.013	0.03	0.0202

Table A-47: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	NaCl Conc. (M)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	0	87.159	89.799	90.913	89.29	1.93
2	0.15	85.774	88.659	88.236	87.56	1.56
3	0.25	35.113	37.182	38.195	36.83	1.57
4	0.35	2.864	1.654	3.820	2.78	1.09
5	0.45	0.400	0.215	0.978	0.53	0.40
6	0.55	1.038	0.660	1.488	1.06	0.41
7	0.65	0.946	2.263	1.885	1.70	0.68
8	0.75	2.011	0.558	0.558	1.04	0.84

Surfactant Variation

In the experiment varying surfactant concentration the organic phase consisted of CTAB in amounts varying from 5 to 100 mM, with 90 % isooctane, 10 % hexanol, and a small amount of water as shown in Table A-48. The aqueous phase consisted of a 50 mM carbonate buffer solution at a pH of ~ 9 with no salt and DsRed2 at ~ 2.4 g/L. For this experiment the total volume of each phase was 900 μL. Table A-49 shows the absorbance values obtained from spectrophotometer readings on the organic phase of the samples following the experiment. In Table A-50 the calculated concentration values for the organic phase of each sample following the experiment are shown, along with the average value of those concentrations, and the standard deviation between those values. The extraction efficiency calculated for each sample along with the average value of those extraction efficiencies, and the standard deviation between those values are shown in Table A-51.

Table A-48: Organic Phase of Samples for Surfactant Variation Experiment

Sample Number	Final CTAB Conc. (mM)	Amt. Of 100 mM CTAB stock added (μL)	Amt. Of 90% Isooctane, 10% Hexanol solution added (μL)
1	5	45	855
2	10	90	810
3	15	135	765
4	20	180	720
5	25	225	675
6	30	270	630
7	50	450	450
8	75	675	225
9	100	900	0

Table A-49: Absorbance Values for Organic Phase

		Run #1	Run #2	Run #3
Sample #	CTAB Conc. (mM)	A561 (nm)	A561 (nm)	A561 (nm)
1	5	0.485	0.453	0.475
2	10	0.462	0.459	0.501
3	15	0.478	0.448	0.465
4	20	0.477	0.453	0.454
5	25	0.482	0.480	0.488
6	30	0.491	0.480	0.471
7	50	0.496	0.494	0.510
8	75	0.479	0.456	0.460
9	100	0.482	0.441	0.456

Table A-50: Calculated Concentration Values for Organic Phase

		Run #1	Run #2	Run #3		
Sample #	CTAB Conc. (mM)	Conc. (g/L)	Conc. (g/L)	Conc. (g/L)	Average	Std. Deviation
1	5	2.281	2.129	2.232	2.21	0.0777
2	10	2.172	2.157	2.358	2.23	0.1118
3	15	2.248	2.107	2.186	2.18	0.0708
4	20	2.243	2.131	2.135	2.17	0.0634
5	25	2.265	2.258	2.297	2.27	0.0207
6	30	2.307	2.258	2.216	2.26	0.0455
7	50	2.335	2.324	2.398	2.35	0.0401
8	75	2.250	2.145	2.163	2.19	0.0564
9	100	2.269	2.073	2.146	2.16	0.0990

Table A-51: Calculated Extraction Efficiencies

		Run #1	Run #2	Run #3		
Sample #	CTAB Conc. (mM)	Extraction Eff. (%)	Extraction Eff. (%)	Extraction Eff. (%)	Average	Std. Deviation
1	5	94.631	88.316	92.598	91.85	3.22
2	10	90.087	89.469	97.794	92.45	4.64
3	15	93.242	87.381	90.663	90.43	2.94
4	20	93.035	88.392	88.568	90.00	2.63
5	25	93.960	93.644	95.263	94.29	0.86
6	30	95.712	93.681	91.941	93.78	1.89
7	50	96.849	96.410	99.485	97.58	1.66
8	75	93.351	88.968	89.740	90.69	2.34
9	100	94.102	85.975	89.001	89.69	4.11

Appendix B: Protein Production and Purification

All proteins were produced recombinantly in *E. coli* for use in experiments, with the exception of horse heart cytochrome *c*, which was purchased from Sigma Aldrich (Product # C 7752, Lot # 102K7053). For fermentation processes several types of media were used for protein growth. Rich media, LB was used as well as the defined medias M9 and MR. (Media Recipes can be found at the end of this Appendix) These medias were supplemented with antibiotic (See Media Recipes for further information) After the fermentation process cells were harvested by centrifugation of the fermentation media. The cells were then lysed through a freeze/thawing process, and centrifuged again in order to separate the cell debris from the protein produced.

Purification of the proteins obtained was varied according to the specific protein. Purification process used included ion exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatography, and ultrafiltration. The purity of the proteins obtained was then verified through uv/vis spectrophotometry and gel electrophoresis.

EGFP Production

EGFP (enhanced green florescent protein) was produced recombinantly in *E. coli* for use in experiments. The *E. coli* was grown in LB, M9, and MR medias for various fermentations. (See Media Recipes for further details)

Fermentation processes were carried out in both 1.5 L and 5 L bioreactor vessels (New Brunswick Scientific Co., Inc., BioFlo and BioFlo 3000 models). Overnight cultures were prepared prior to the fermentation and were added to the fermentation

vessels to begin the process. The fermentation was carried out at ~ 37 °C and an agitation rate of ~ 300 rpm. While the culture was grown in the fermentor, the optical density (OD) was monitored at 600 nm through use of a Hewlett Packard 8453 Spectrophotometer. When the OD reached about one, the culture was induced with 1 mL of 1 M IPTG (per 1 L media) (Isopropyl-beta-D-thiogalactopyranoside). The culture was then allowed to grow for another four and a half hours before the cells were harvested through centrifugation at 3000 rpm, 4°C, for 30 minutes. The collected cells were then resuspended in 50 mM Tris, pH 8.0, and the cells were freeze-thawed several times to lyse. The solution was then centrifuged again at 3000 rpm, 4°C, for 30 minutes, in order to remove the cell waste and the supernatant containing the EGFP was saved.

EGFP Purification

After the desired amount of protein was collected, purification began. The EGFP was first purified through anion exchange chromatography. For this the protein was loaded onto a DEAE Sepharose™, Fast Flow column (Amersham Biosciences, Cat. No. 17-0709-01) equilibrated with 10 mM sodium phosphate, 20 mM NaCl, pH 6.5. The column was then eluted with a gradient of 10 mM sodium phosphate, 20 mM NaCl, pH 6.5 to 10 mM sodium phosphate, 300 mM NaCl, pH 6.5. The fractions containing EGFP were collected.

Following the anion exchange chromatography procedure, EGFP was collected and concentrated using Millipore Centriplus Centrifugal Filter Units (10 kDa, Cat. No. 4411), and put into a high salt buffer (50 mM sodium phosphate, 2 M ammonium sulfate, pH 7.5). Then hydrophobic interaction chromatography was performed on a Butyl

Sepharose™, 4 Fast Flow (Amersham Biosciences, Cat. No. 17-0980-10) column equilibrated with 50 mM sodium phosphate, 2 M ammonium sulfate, pH 7.5. The column was then eluted with a gradient of 50 mM sodium phosphate, 2 M ammonium sulfate, pH 7.5, to 50 mM sodium phosphate, no salt, pH 7.5. Again the fractions containing EGFP were collected.

Finally size exclusion chromatography was performed on the protein using a Superdex™ 75 (Amersham Biosciences, Cat. No. 17-1044-01) column equilibrated with 10 mM sodium phosphate, 20 mM NaCl, pH 6.5, which was also the running buffer for the chromatography procedure. The fractions containing EGFP were collected and the protein was concentrated using Millipore Centriplus Centrifugal Filter Units (10 kDa, Cat. No. 4411).

Throughout the purification process the purity of the protein was monitored using uv/vis spectrophotometry and gel electrophoresis. With uv/vis spectrophotometry a comparison was made of the absorbance at 280 nm vs. the absorbance at 488 nm for EGFP purity. The absorbance at 488 nm divided by the reading at 280 nm gave a result of ~ 0.3 with EGFP crude lysate, ~ 0.5 after anion exchange chromatography, and ~ 1 after hydrophobic interaction and size exchange chromatography. After the previously mentioned purification processes, the proteins were determined to be pure enough for use in experiments.

DsRed2 Production

DsRed2 was produced recombinantly in *E. coli* for use in experiments. The *E. coli* was grown in LB media for various fermentations. (See Media Recipes for further details)

Fermentation processes were carried out in both 1.5 L and 5 L bioreactor vessels (New Brunswick Scientific Co., Inc., BioFlo and BioFlo 3000 models). Overnight cultures were prepared prior to the fermentation and were added to the fermentation vessels to begin the process. The fermentation was carried out at ~ 37 °C and an agitation rate of ~ 300 rpm. While the culture was grown in the fermentor, the optical density (OD) was monitored at 600 nm through use of a Hewlett Packard 8453 Spectrophotometer. When the OD reached about one, the culture was induced with 1 mL of 1 M IPTG (per 1 L media) (Isopropyl-beta-D-thiogalactopyranoside). The culture was then allowed to grow for another four and a half hours before the cells were harvested through centrifugation at 3000 rpm, 4°C, for 30 minutes. Harvested cells were resuspended in 50 mM Tris, pH 8.0 and freeze-thawed several times in order to lyse the cells. Afterwards the solution was centrifuged at 3000 rpm, 4°C, for 30 minutes to remove the cell waste, and the supernatant containing the DsRed2 was saved.

DsRed2 Purification

After the desired amount of protein was collected, purification began. The DsRed2 was first purified through anion exchange chromatography. For this the protein was loaded onto a DEAE Sepharose™, Fast Flow column (Amersham Biosciences, Cat. No. 17-0709-01) equilibrated with 25 mM Tris, 20 mM NaCl, pH 8.5. The column was

then eluted with a gradient of 25 mM Tris, 20 mM NaCl, pH 8.5 to 25 mM Tris, 300 mM NaCl, pH 8.5. The fractions containing DsRed2 were then collected.

Following the anion exchange chromatography, size exclusion chromatography was performed on the protein using a Superdex™ 75 (Amersham Biosciences, Cat. No. 17-1044-01) column equilibrated with 10 mM sodium phosphate, 20 mM NaCl, pH 8.5, which was also the running buffer for the chromatography procedure. The fractions containing DsRed2 were collected and the protein was concentrated using Millipore Centriplus Centrifugal Filter Units (100 kDa, Cat. No. 4414).

Throughout the purification process the purity of the protein was monitored using uv/vis spectrophotometry and gel electrophoresis. With uv/vis spectrophotometry a comparison was made of the absorbance at 280 nm vs. the absorbance at 561 nm for DsRed2 purity. The absorbance at 561 nm divided by the reading at 280 nm gave a result of ~ 0.1 with DsRed2 crude lysate, ~ 0.2 after anion exchange chromatography, and ~ 0.6 after size exchange chromatography. After the previously mentioned purification processes, the proteins were determined to be pure enough for use in experiments.

Flavodoxin Production

Flavodoxin was produced recombinantly in *E. coli* for use in experiments. The *E. coli* was grown in LB media for various fermentations. (See Media Recipes for further details)

Fermentation processes were carried out in both 1.5 L and 5 L bioreactor vessels (New Brunswick Scientific Co., Inc., BioFlo and BioFlo 3000 models). Overnight cultures were prepared prior to the fermentation and were added to the fermentation

vessels to begin the process. The fermentation was carried out at ~ 37 °C and an agitation rate of ~ 300 rpm. While the culture was grown in the fermentor, the optical density (OD) was monitored at 600 nm through use of a Hewlett Packard 8453 Spectrophotometer. When the OD reached about one, the culture was induced with 1 mL of 1 M IPTG (per 1 L media) (Isopropyl-beta-D-thiogalactopyranoside). The culture was then allowed to grow for another four and a half hours before the cells were harvested through centrifugation at 3000 rpm, 4°C, for 30 minutes. The harvested cells were resuspended in 10 mM Tris, 25 mM EDTA, pH 8.0, and freeze-thawed several times to lyse. Following this process the resulting solution was centrifuged to remove the cell waste. The collected supernatant was saved and flavin mononucleotide (Sigma-Aldrich, Cat. No. F1392) was added to a final concentration of 50 μM. This solution was then stirred in the dark for 2 hours. Afterwards the mixture was centrifuged at 3000 rpm, 4°C, for 30 minutes to remove excess flavin mononucleotide that did not bind to the flavodoxin. The flavin mononucleotide was added in order to give the flavodoxin its orange color, to be more easily monitored through uv/vis spectrophotometry.

Flavodoxin Purification

After the desired amount of protein was collected, purification began. The flavodoxin was purified through anion exchange chromatography. For this the protein was loaded onto a DEAE Sepharose™, Fast Flow column (Amersham Biosciences, Cat. No. 17-0709-01) equilibrated with 50 mM Tris, pH 8. The column was then eluted with a gradient of 0.1 M sodium acetate, pH 5 to 0.1 M sodium acetate, 0.5 M NaCl, pH 5.

The fractions containing flavodoxin were then collected, and the protein was concentrated using Millipore Centriplus Centrifugal Filter Units (10 kDa, Cat. No. 4411).

Throughout the purification process the purity of the protein was monitored using uv/vis spectrophotometry and gel electrophoresis. With uv/vis spectrophotometry a comparison was made of the absorbance at 280 nm vs. the absorbance at 464 nm for flavodoxin purity. The absorbance at 464 nm divided by the reading at 280 nm did not give a significant result with the flavodoxin crude lysate, due to the interference of unbound flavin. After anion exchange chromatography a factor of ~ 0.15 was achieved, and attempts at further purification did not raise this number. After the previously mentioned purification processes, the proteins were determined to be pure enough for use in experiments.

Media Recipes

LB Media

LB Media was mixed using Difco™ LB Broth, Lennox, purchased from Fisher Scientific (Cat. No. DF0402-17-0), and made up at 20 g/L. This media was supplemented with the antibiotic Carbenicillin at 75 mg per 1 L of media.

M9 Media

M9 Media was made using the following ingredients:

The ingredients listed below were combined and autoclaved for sterility

- 100 mL 10X M9 Stock (per 1 L media), which consists of (per 1 L 10X M9 Stock):
    - 128 g $Na_2HPO_4 * 7H_20$
    - 30 g KH_2PO_4
    - 5 g NaCl
- 880 mL DI Water
- 1 g NH_4Cl (per 1 L)
- NaOH was used to adjust the pH of the above combined ingredients to 7.4

The following ingredients were combined and the solution was brought to 20 mL with DI water and sterile filtered into the autoclaved media

- 4 g Glucose (per 1 L)
- 0.5 g $MgSO_4$ (per 1 L)

- 10 mg Thiamine (per 1 L)
- 10 mg Iron (per 1 L)
- 100 µL 1 M $CaCl_2$ (per 1 L)
- 100 mg Ampicillin (per 1 L)

MR media

MR Media was made using the following ingredients:

The ingredients listed below were combined and autoclaved for sterility

- 2 g $(NH_4)_2HPO_4$ (per 1 L media)
- 6.65 g KH_2PO_4 (per 1 L)
- 900 mL DI Water

The following ingredients were combined and the solution was brought to 100 mL with DI water and sterile filtered into the autoclaved media

- 10 g Glucose (per 1 L)
- 0.8 g Citric Acid (per 1 L)
- 0.7 g $MgSO_4 * 7H_2O$ (per 1 L)
- 3 mL trace metal solution (per 1 L)
    - Trace Metal Solution consisted of the following ingredients (per 1 L)
        - 10 g $FeSO_4 *7H_2O$
        - 0.5 g $MnSO_4*4H_2O$
        - 1.35 g $CaCl_2$
        - 2.25 g $ZnSO_4*7H_2O$
        - 1 g $CuSO_4*5H_2O$

- 0.106 g $(NH_4)_6Mo_7O_{24}*4H_2O$
- 0.23 g $Na_2B_4O_7*10\ H_2O$
- 10 mL of 35 % HCl

The pH was then adjusted to ~ 6.8 with NH_4OH

This media was supplemented with the antibiotic Ampicillin at 100 mg per 1 L of media.