Exploring the multiple applications of the membrane-disrupting Styrene Maleic Acid

Craig Harris

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School of Biosciences
University of Birmingham
Birmingham, B15 2TT

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Abstract

Poly(styrene-*co*-maleic) acid (SMA) has demonstrated great potential as an interrogation tool for cell membranes. SMA can insert into the cell membrane excising discs of lipid and any associated membrane proteins. The potential applications of SMA in membrane protein study and the specific release of the periplasmic fraction of *E. coli* were investigated in this project. There is a well-documented disparity in the number of structurally characterised soluble proteins versus membrane proteins due to a number of factors. Whilst solubilisation and subsequent characterisation of membrane proteins has been achieved, lack of a universal membrane solubilisation protocol is restricting the growth of membrane protein study. SMA was applied to three membrane proteins (FtsA, MurJ-mfGFP and FtsW-mfGFP) leading to their solubilisation and purification with conventional methods. FtsA and MurJ-mfGFP were also characterised with CD and svAUC.

Periplasmic targeting of recombinant proteins in *E. coli* offers many advantages over cytoplasmic production. Existing methods for selective periplasmic extraction are expensive, time consuming and inefficient. In this report we investigated whether SMA can disrupt the *E. coli* outer membrane to selectively release the periplasmic contents. We discovered that SMA performed favorably compared to an existing method at selectively releasing two periplasm-targeted proteins.

Keywords: SMALP; Membrane proteins; FtsA; MurJ; FtsW; Periplasmic release, superfolder-GFP; mCherry

Abbreviations

Apo A-1: Human apolipoprotein A-1

ATP: Adenosine triphosphate

bR: Bacteriorhodopsin

BCA: Bicinchoninic acid

BSA: Bovine serum albumin

CD: Circular dichroism

CMC: Critical micelle concentration

CTAB: Cyltrimethylammonium

DDM: n-dodecyl-β-D-maltoside

DSC: Differential scanning calorimetry

EDTA: Ethylenediaminetetraacetic acid

E. coli: *Escherichia coli*

Fts: Filamentous temperature sensitive

FtsA: Filamentous temperature sensitive protein A

FtsW: Filamentous temperature sensitive protein W

FT-ICR: Fourier transform ion cyclotron resonance

GFP: Green-fluorescent protein

GPCR: G-protein coupled receptors

IPTG: Isopropyl-β-D-thiogalactopyranoside

kDa: Kilodalton

LB: Luria-Bertani

M: Molar

mfGFP: Multifunctional-GFP

mg: Milligram

mL: Milliliter

mM: Millimolar

MOP: Multidrug/oligo-saccharidyl-lipid/polysaccharide

MSP: Membrane scaffold protein

MurJ: Muramyl ligase J

Peri-mCherry: Periplasm-targeted mCherry

Peri-sfGFP: Periplasm-targeted superfolder-GFP

PG: Peptidoglycan

pH: -log10 [H+]

SDS: sodium dodecyl sulfate

SMA: Poly(Styrene-*co*-maleic acid)

SMALP: Styrene maleic acid lipid particles

sfGFP: Superfolder-GFP

svAUC: Sedimentation velocity analytical ultracentrifugation

S. aureus: Staphylococcus *aureus*

tat: Twin-arginine translocation

T*. martima: Thermotoga martima*

TE: Tris-HCl EDTA

TM: Transmembrane

UV: Ultraviolet

µg: Microgram

µL: Microliter

µM: Micromolar

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CHAPTER 1:

Introduction

This report details two distinct studies that are joined by a common reagent - styrene maleic acid copolymer (SMA). SMA been used successfully for membrane protein solubilisation and here we investigate whether it can prove an effective mechanism for triggering periplasmic release. This introduction details existing literature surrounding membrane proteins and the solubilisation of them before providing a summary of the *Escherichia coli* (*E. coli*) periplasm and existing methods for releasing the periplasmic contents.

1.1 Membrane proteins

1.1.1 Why study membrane proteins

The development of biochemistry has been underpinned in recent years by a growth in structural studies. Such studies have been particularly successful when applied to soluble proteins. Thus far, studying membrane proteins has proved much more difficult. One of the key barriers to understanding membrane proteins is the lack of a simple and universal method for removing the membrane proteins from their native environment without negatively impacting structure and function (Jamshad *et al*., 2011). Membrane proteins are extremely important to cellular human biology playing pivotal roles in processes such as cellular signaling and transport (Musnier *et al*., 2010), respiration and cell division (Egan and Vollmer, 2013). The human genome sequence shows over 30 % of human genes encode membrane proteins (Stevens and Arkin, 2000). These proteins serve as targets for 60 % of approved pharmaceuticals illustrating how important membrane proteins are as therapeutic targets. (Hopkins and Groom, 2002).

The first high-resolution protein structure for an integral membrane protein was published in 1985 (Deisenhofer *et al.*, 1985). The protein acted as a photosynthetic reaction centre for the purple bacterium *Rhodopseudomonas viridis* and contained five transmembrane helices (Deisenhofer *et al.*, 1985). Water soluble proteins had been structurally resolved (at a minimum resolution of 2 Å) for the previous 25 years beginning with the X-ray crystallographic structure of myoglobin, an iron and oxygen binding soluble protein found in mammalian muscle tissue (Kendrew *et al.*, 1960). There is a well-documented disparity in the number of resolved soluble proteins versus resolved membrane proteins. A large number of factors combine to complicate the study of membrane proteins – their insolubility in water, complex native environment and lower native expression levels make their study a challenging task (Hunte *et al.*, 2003). In this chapter, difficulties in membrane protein study will be discussed and current methods of membrane protein study will be described.

1.1.2 The issues in membrane protein study and current techniques

Membrane protein research is made much more difficult than the study of soluble proteins due to a combination of factors. Figure 1-1 illustrates an example of a membrane protein experimental study, from primer design through to protein characterisation.

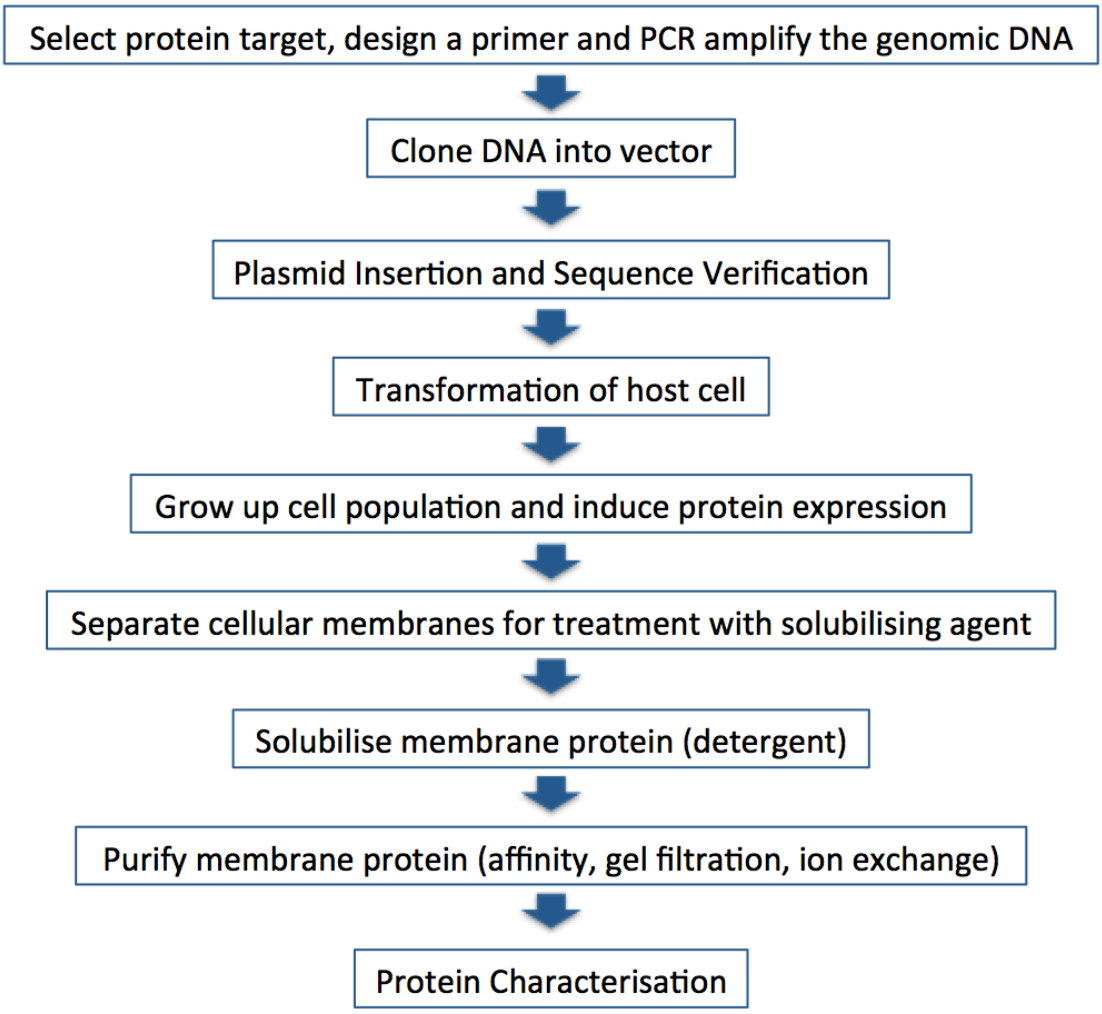


Figure 1-1. The process of membrane protein experimental study, from primer design through to protein characterisation.

1.1.2.1 Complications in membrane protein overexpression

Many factors combine to prevent efficient overexpression of membrane proteins in terms of protein yield and quality; these include poor expression levels, cellular toxicity and protein misfolding leading to aggregations (Junge *et al.*, 2008). To circumvent some of the issues in membrane protein production, a multitude of different expression systems have previously been exploited. Membrane proteins have been successfully expressed in various bacteria such as the gram-negative bacteria *E. coli* (Yin *et al.*, 2006) and in *Lactococcus lactis* a

gram-positive bacterium (Frelet-Barrand *et al.*, 2010). Yeasts (Fraser, 2006), insect cells (Wang *et al.*, 2010), mammalian cells (Reeves *et al.*, 2002) and cell free systems (Katzen *et al.*, 2009) have all been used for membrane protein expression with varying levels of success. When selecting an expression system to obtain high yields, post-translational modification and typical lipid composition of the strain must be taken into account as these factors all vary between the strains discussed and impact expression levels.

Escherichia coli (*E. coli*) is the most popular expression system in membrane protein experiments for a variety of reasons. Vast amounts of literature already exist detailing how *E. coli* reacts to overexpression of different protein types, use of different induction controllers, temperatures and pH levels. Furthermore the gram-negative bacterium itself is simple and with a fully sequenced genome provides a system that is flexible and easy to manipulate (Assenberg *et al.*, 2013). It is attractive for protein production as it uses low cost expression media and replicates quickly, resulting in large amounts of protein production over short time periods – again reducing experimental cost, an important factor from research to industry (Rosano and Ceccarelli, 2014). *E. coli* is also associated with higher product yields when compared to other expression systems, resulting in up to 10 times more protein being produced in identical growth conditions (Fraser, 2006, Singh *et al.*, 2008). Table 1-1 below describes the benefits and limitations of using *E. coli* as an expression system (Rosano and Ceccarelli, 2014).

Table 1-1. The benefits and limitations of *E. coli* as an expression system for recombinant proteins (Rosano and Ceccarelli, 2014).

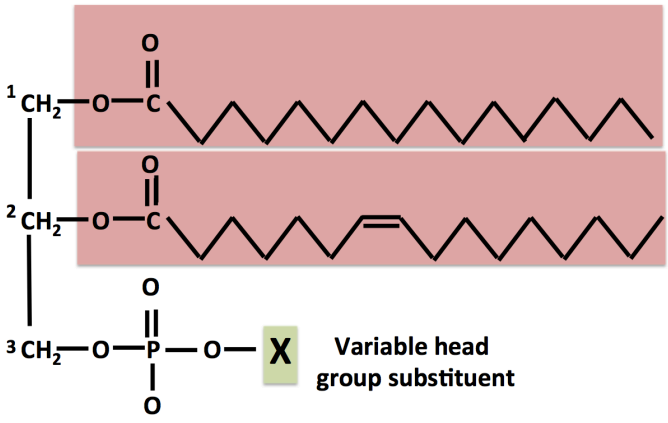
Expression system	Benefits	Limitations
E. Coli	Genome fully sequenced and large amounts of literature exist detailing its manipulability. Cheap to purchase the cell line and fast replication times result in economical experiments. Expression media is comparatively cheap and high cell density concentrations are easily achieved. Antibiotic tags and purification tags can be added and removed easily to aid downstream processing.	Difficult to implement post-translational modifications of proteins. Prokaryotes differ most from the human genome, can lead to folding issues due to size constraints. Prokaryotic membrane constitution is different to eukaryotes.

1.1.2.2 Membrane proteins and lipids

Membrane proteins fall into two categories depending on how tight their association is to the lipid membrane. Integral membrane proteins require disruption of the membrane with detergents or other chemical agents whilst peripheral membrane proteins can be isolated with mild environmental changes such as altering ionic strength or pH (Smith, 2011). The main constituents of the biological membrane are phospholipids, sphingolipids and sterols (Khan *et al*., 2013). These components combine to add support to hydrophobic sections of membrane proteins – impacting the structural stability of membrane proteins and their function (Carpenter *et al*., 2008). Figure 1-2 illustrates the three major constituents of the biological membrane (Khan *et al*., 2013).

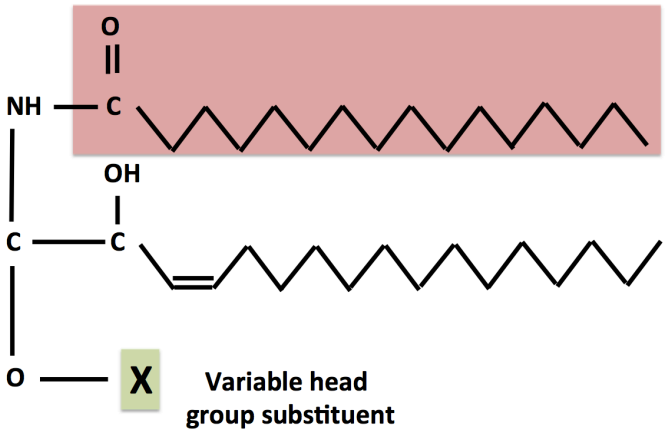
Phospholipids:

Built around an *sn*-glycerol-3-phosphate core that is esterified to fatty acids at positions C1 and C2 (highlighted in red). The variable head group substituent (X) can bind to a number of different components to make up different phospholipids.



Sphingolipids:

Built around a sphingosine backbone that is oxygen-linked, usually to a charged group (X). The backbone is also amide-linked to an acyl group, for example fatty acids (highlighted in red).



Sterols:

Sterols vary a lot more in structure than other membrane lipids. Their consistent feature is the hydrophobic moiety, which is composed of polycyclic structures. The prokaryotic sterol is lanosterol (shown opposite), whilst cholesterol is the most abundant sterol in eukaryotic membranes.

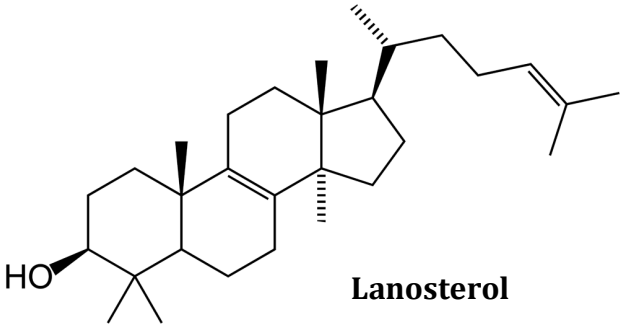


Figure 1-2. The three different types of lipid that populate biological membranes (Khan *et al.*, 2013).

Membrane lipids can be categorised depending on how closely they interact with membrane proteins. The majority of the lipid bilayer membrane fluidly interacts to create a lateral pressure important to the membrane proteins' ability to function and correctly fold (Lee, 2000, Seddon *et al.*, 2008). Lipids that are found close to membrane proteins are known as annular lipids; they usually have direct contact with the proteins and removal of these annular lipids from a membrane protein has been shown to impact protein function (Lee, 2004). The final type of membrane lipid are known as lipid cofactors and they need to be directly involved in membrane protein functionality to be classed as such. Examples of lipid cofactors are phosphatidylinositol-4-phosphate being key for Ca^{2+}–ATPase and cholesterol's association with the acetylcholine receptor (Starling, 1995, Fong and McNamee, 1986). This interactive lipid environment is important for maintaining protein fold and the functional activity of membrane proteins. If a membrane protein extraction method were to capture surrounding lipid and the target protein, it would clearly be beneficial to the protein's potential to be studied with any success downstream.

1.1.2.3 Current techniques for membrane protein solubilisation

In general, membrane proteins, much like soluble proteins, can be purified with a variety of different chromatographic techniques. These include affinity column chromatography, gel filtration and ion exchange. To use these methods the membrane proteins first have to be extracted from the membrane continuum; it is during this process that significant complications occur. A number of different techniques exist for isolating membrane proteins all with their own limitations and benefits – including the application of detergents, amphipols and protein-

scaffolded nanodiscs. However, completely removing surrounding lipids from a membrane protein often has detrimental effects to the native function of the protein (Jamshad *et al.*, 2011). Through the next three sections (1.1.2.3.1-3) the thesis contains a survey of the current methods for extracting membrane proteins from their native environment prior to their purification.

Post isolation and purification, the membrane protein will often need reconstituting into a hydrophobic environment in order to become stable. This reconstitution into a model system can prove more difficult than the separation of the protein from impurities. When creating an ideal environment for reconstitution a multitude of factors can impact protein activity and longevity. Temperature and acidity levels remain important factors for reconstituted membrane proteins; they are most generally stable at 4^{o}C and between pH 6 and 8. Protease inhibitors and the presence of glycerol also have a positive impact on reconstituted membrane protein stability (Hunte *et al.*, 2003). Furthermore, addition of cholesterol has been shown to be key for mammalian membrane proteins (Opekarová and Tanner, 2003).

1.1.2.3.1 Detergent solubilisation of membrane proteins

Detergent solubilisation is the most popular method for isolating membrane proteins from the lipid bilayer (Luckey, 2014). Detergents are a combination of surface active agents (surfactants). It is the surfactants that work to integrate and disrupt the lipid bilayer membrane, however, scientists generally refer to them as detergents (Heerklotz, 2008).

Detergents fall into three categories: ionic detergents such as sodium dodecyl sulfate (SDS) and cyltrimethylammonium (CTAB), non-ionic detergents including Triton X-100 and n-dodecyl-β-D-maltoside (DDM), and zwitterionic detergents (Garavito and Ferguson-Miller, 2001). Ionic detergents operate by binding to hydrophobic membrane sections and interfere with ionic and hydrogen bonding with their innate charge. Non-ionic detergents interfere in a similar fashion; they tend to be long chain alcohols and have no net charge (Lichtenberg et al., 2013). Different detergents have unique limitations, for example SDS has been known to denature proteins, whilst Triton X-100 is aromatic and will interfere with biophysical studies such as circular dichroism (CD) (Luckey, 2014). Zwitterionic detergents operate similarly to non-ionic detergents as they too have no net charge but will always contain balanced numbers of charged groups, maintaining the protein's native charge once removed from the membrane (Garavito and Ferguson-Miller, 2001). There is no single, universal surfactant method making screening essential to identify suitable conditions for solubilising membrane proteins (Hunte *et al*., 2003).

Detergents operate by inserting into the lipid bilayer. At a sufficient concentration (known as the critical micelle concentration (CMC)) micelles will form. Below the CMC detergent molecules will insert into the membrane's lipid bilayer without causing any destabilisation. Membrane protein containing detergent micelles are often used to study the membrane, however, they lack a lipid bilayer and the environmental benefits that come with it (Seddon *et al*., 2004, le Maire *et al*., 2000). Figure 1-3 illustrates the formation of micelles around a membrane protein at a sufficient concentration of surfactant.

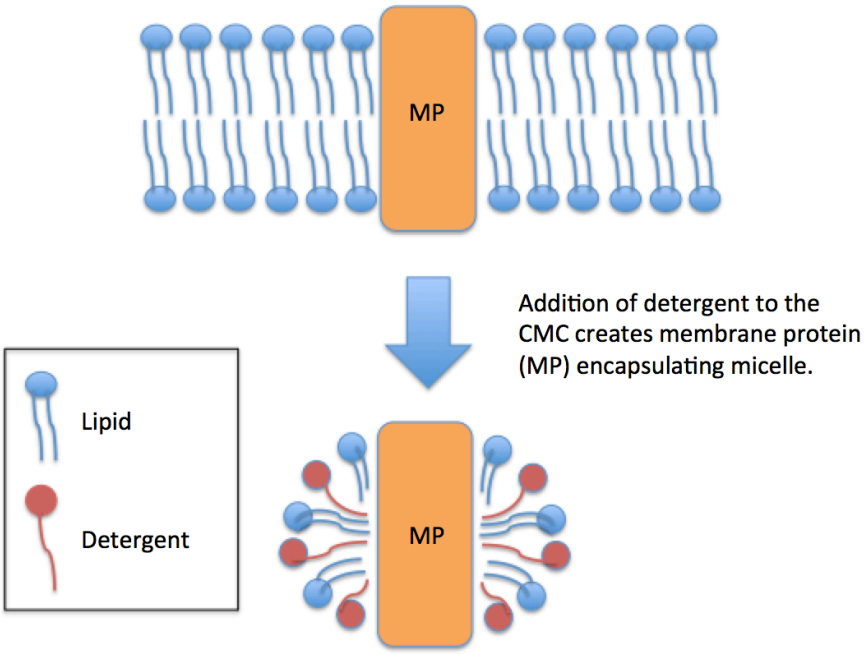
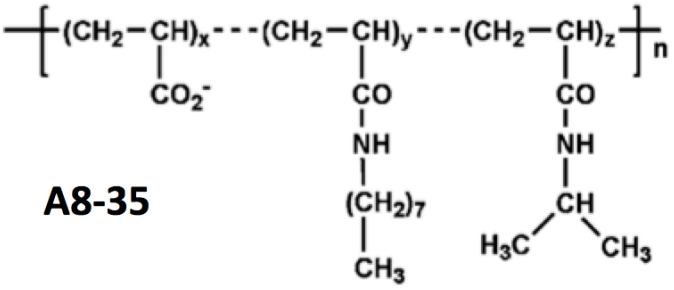


Figure 1-3. A diagram visualising how the addition of a detergent at the critical micelle concentration (CMC) forms membrane protein containing micelles.

1.1.2.3.2 Using amphipols to study membrane proteins

Amphipols are short chain amphipathic polymers that contain hydrophobic side chains branching from a hydrophilic backbone. They are highly soluble in water and have the ability to adsorb onto hydrophobic membrane segments (Tribet *et al.*, 1996, Popot, 2010). Amphipols act in a similar fashion to detergents in that they remove membrane proteins from their lipid bilayer. They allow the membrane protein to be encapsulated in a small hydrophilic complex along with its annular lipids (Picard *et al.*, 2006). The polymers can hypercoil around hydrophobic, transmembrane sections of membrane proteins, helping to retain protein folding without the lateral pressure from the membrane (Tribet *et al.*, 1996). A8-35 is the most studied amphipol and has been used to study multiple G-protein coupled receptors (GPCR) (Picard *et al.*, 2006). Figure 1-4 displays the structure of A8-35 (Picard *et al.*, 2006) .



Adapted from (Picard *et al*., 2006)

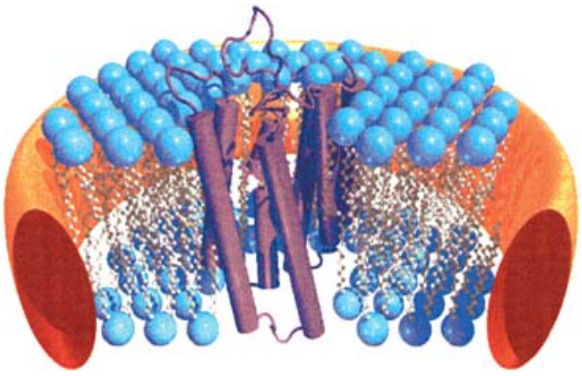
Figure 1-4. The structure of A8-35, the most popular amphipol for solubilising membrane proteins, adapted from (Picard *et al.*, 2006).

In some cases of amphipol driven membrane protein extraction protein activity is retained, however, A8-35 has also interfered with protein's intrinsic ATPase activity, as in the case of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (Picard *et al.*, 2006). The issue with using amphipols is that they still require the use of detergents in order to destabilise the membrane before the polymer can be applied, leading to all the drawbacks of detergent based membrane protein extraction (Rajesh *et al*., 2011). Furthermore, the polymer is expensive and it is difficult to ensure it remains monodispersed given that acidic conditions and divalent cations can interfere with amphipols causing polymer aggregation (Popot, 2010).

1.1.2.3.3 Protein-scaffolded nanodiscs

More recently, research into studying membrane proteins has led to the development of a novel nanodisc model system (Denisov *et al*., 2004, Bayburt and Sligar, 2003). In this method, the protein is first solubilised using detergents and then membrane scaffold proteins (MSP) are added, which combine with phospholipids to produce a membrane protein containing nanodisc. The

nanodisc is roughly 10 nm in diameter and 150 kDa in mass and has the ability to bind to one membrane protein (Leitz *et al*., 2006, Jamshad *et al*., 2011). The MSP is derived from the sequence of the human apolipoprotein A-1 (Apo A-1) and can be varied in length in order to alter the size of the nanodisc created (Denisov *et al*., 2004). The MSP has hydrophobic domains, which help stabilise membrane-spanning sections of the target membrane protein. Membrane proteins within nanodiscs have been proven to be functionally active, however, issues derived from the method limit the potential of MSP nanodiscs as a method to facilitate membrane protein study. These limitations include all the intrinsic flaws associated with solubilising membrane proteins with detergents. Furthermore, the addition of another protein (in the form of the MSP) acts as a contaminant which means extra processing steps are required downstream before most analytical tests can be completed (Leitz *et al*., 2006). Figure 1-5 demonstrates how the MSP scaffold encapsulates a target membrane protein with some surrounding lipid, extracted from (Bayburt and Sligar, 2003).



(Bayburt and Sligar, 2003)

Figure 1-5. A cutaway view of a target membrane protein (purple) captured with some lipids (blue) encircled by the Apo A-1 derived MSP (orange) in a 150 kDa nanodisc, extracted from (Bayburt and Sligar, 2003).

Table 1-2 summarises the current method for protein solubilisation, alongside their benefits and limitations.

Table 1-2. A summary of the current most popular methods of protein solubilisation, alongside their benefits and limitations.

Solubilising method	Benefits	Limitations
Detergents	Can extract proteins of any size. Ionic and non-ionic variants of detergent with vast literature library detailing previous work.	Removes all surrounding lipids and inherent benefits of the bilayer, nullifying lateral membrane pressures. Removal of annular lipids. Membrane protein aggregation can occur with lipid concentrations below the CMC.
Amphipols	Different polymers can extract proteins of different sizes Maintain lateral membrane pressure.	Polymer is known to aggregate. Can require destabilisation of membrane with detergents, sharing all their limitations.
Protein scaffolded nanodiscs	Proteins of different sizes can be extracted with different sized MSP. Lateral pressure is maintained by the MSP. Some annular lipid may be retained.	Not one universal disc that works on all proteins. MSP adds a contamination to be removed downstream, could interfere with structural and functional studies. Requires destabilisation of membrane with detergent, sharing all their limitations.

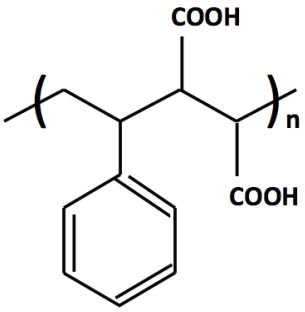
The lack of a single method for solubilising membrane proteins to facilitate their study leads to membrane proteins remaining an untapped resource for potential research. Consequently, research into this method remains a priority. This report will investigate the application of Styrene maleic acid lipid particles (SMALP) for that purpose.

1.1.3 Styrene maleic acid lipid particles (SMALP)

Research into a single, universal method for purifying membrane proteins remains a priority for facilitating membrane proteins study. This method ideally involves no detergent use and the ability to encapsulate the target membrane protein with its annular lipids in order to retain as much of the target protein's environment as possible. Poly(styrene-*co*-maleic acid) (SMA) has shown potential to be this all-important method due to its ability to create SMA lipid particles (SMALP) encapsulating membrane proteins and their annular lipids (Knowles *et al.*, 2009).

1.1.3.1 Poly(styrene-*co*-maleic acid) (SMA) the polymer

SMA is a polymer with a large range of applications in the plastics industry (Knowles *et al.*, 2009, Jamshad *et al.*, 2011). The polymer consists of alternating maleic acid residues (hydrophilic) and styrene moieties (hydrophobic) making the polymer amphipathic in nature when at neutral or alkali conditions (Tonge and Tighe, 2001). In an acidic environment, protonation of the maleic acid groups occur leading to the polymer becoming insoluble in water. Initial scientific research was completed with the SMA copolymer as a potential drug delivery system, utilising the polymer's ability to disrupt and move through cellular membranes – enhancing hydrophobic drug efficacy (Maeda *et al.*, 2009). The presence of divalent cations must be avoided when working with SMA as they can cause aggregation of SMA – much like amphipols (Knowles *et al.*, 2009). The chemical formula of the styrene-*co*-maleic acid (SMA) is illustrated in Figure 1-6 – adapted from (Banerjee *et al.*, 2012).



(Banerjee *et al.*, 2012)

Figure 1-6. Styrene-*co*-maleic acid (SMA) adapted from (Banerjee *et al.*, 2012).

1.1.3.2 Formation and applications of SMA lipid particles (SMALP)

Research has shown that the polymer can auto-assemble with lipid bilayers forming nanodiscs between 9 and 11 nm in diameter, creating SMA lipid particles (SMALP) (Knowles *et al.*, 2009). It is believed that in membranes these nanodiscs form as a spontaneous process, allowing the removal of membrane sections including any associated membrane proteins. The hypothesised formation of membrane protein containing SMALP is detailed in Figure 1-7.

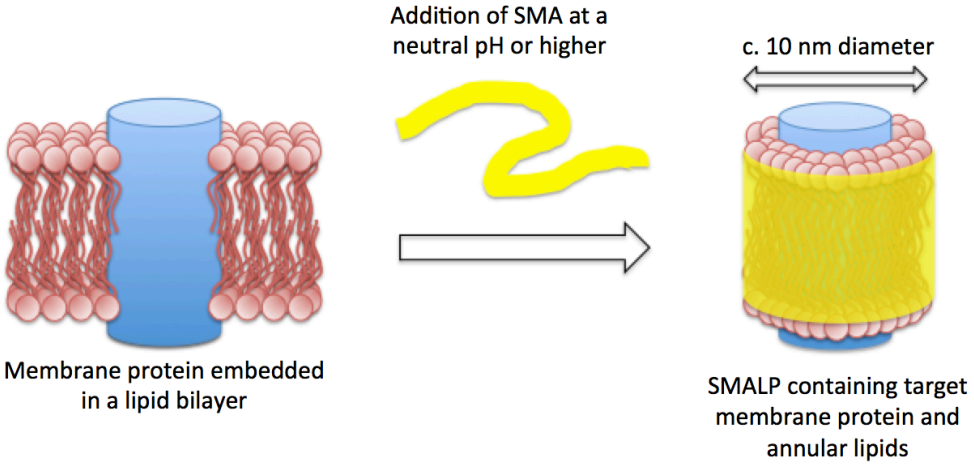


Figure 1-7. An illustration of how SMA copolymer is though to form SMALP containing membrane proteins and their annular lipids (Jamshad *et al.*, 2011).

The SMALPs containing membrane proteins are compatible with conventional purification methods such as affinity chromatography, gel filtration and size

exclusion (Jamshad *et al*., 2011 ,Lee *et al*., 2016b). Furthermore the SMALP are compatible with multiple biophysical techniques including analytical ultracentrifugation (AUC), circular dichroism (CD), differential scanning calorimetry (DSC) and fluorescence (Lee *et al*., 2016). Membrane proteins are also highly stable within the SMALP, with protein structure and function being retained at temperatures above 90^{o}C and post-desiccation through freeze drying to form powders (Jamshad *et al*., 2011).

1.1.3.3 Previously successful SMA applications

Applying SMA to solubilise membrane proteins has been successful with a variety of proteins across a range of expression systems. Bacteriorhodopsin (bR), a membrane protein containing 7 transmembrane helices - and PagP - a lipid A palmitoyltransferase containing an 8-stranded β-barrel - were two of the first proteins to be successfully integrated into SMALP and purified. PagP was expressed in *E. coli* in inclusion bodies whilst the bR was solublised from lyophilised purple membrane from *Halobacterium salinarum* (Knowles *et al*., 2009). SMALP were used to solubilise and purify a functional human adenosine A2A receptor expressed in yeast (Jamshad *et al*., 2015, Wheatley *et al*., 2016) and to extract an *E. coli* membrane protein AcrB, which subsequently studied with negative stain electron microscopy (Postis *et al*., 2015). SMA was also used for the isolation and characterisation of an *E. coli* tetrameric potassium channel before the membrane protein was reconstituted successfully into a planar lipid bilayer (Dörr *et al*., 2014). The versatility of the SMALP is further illustrated in the six different variants of eukaryotic ATP-binding cassette (ABC) transporters

that were expressed across six different expression systems: *High Five* insect cells, *H69AR* human lung-derived cells, *HEK* human kidney-derived cells, *Sf9* insect cells, *Saccharomyces cerevisiae* a strain of yeast and *MCF7-FLV* a human breast cancer-derived cell line. Finally work with *Staphylococcus aureus* and SMA has led to the isolation of an overexpressed penicillin-binding protein complex PBP2/PBP2a bound to the soluble protein FtsZ (Paulin *et al*., 2014). Solubilising associated proteins with a membrane protein complex is another illustration of the potential of SMA as a tool to interrogate membrane proteins.

1.1.4 Target protein selection: FtsA, MurJ and FtsW

1.1.4.1 Bacterial cell division and the divisome

Bacterial cell division is the process of a single cell dividing into two daughter cells, which occurs in all living organisms. In prokaryotes, cell division occurs with a process known as binary fission. The cell's DNA replicates semi-conservatively, before the two copies of the original genome are moved to the opposite poles of the cell. The physical cell division then begins and cell elongation occurs before the two daughter cells are formed around the two chromosomes (Fisher, 2001). In *E. coli*, several genes responsible for cell division were identified as temperature-sensitive through mutation studies; hence the name filamentous temperature sensitive mutation (Fts). FtsA, FtsB, FtsI, FtsK, FtsL FtsN, FtsQ, FtsW and FtsZ are examples of proteins coded for by Fts genes that are responsible for *E. coli* cell division (Lutkenhaus, 1993). FtsZ is the primary component in the formation of the cellular division site. The soluble protein forms an FtsZ-ring which acts as the septum and division point for

cellular division (Pichoff and Lutkenhaus, 2002, Rueda *et al.*, 2003). FtsZ is a tubulin homologue, which acts as a GTPase and is highly conserved amongst prokaryotes (Lutkenhaus *et al.*, 2012). FtsZ is highly abundant in *E. coli* with approximately 5000 copies per cell (Pla *et al.*, 1991, Cho, 2015). The formation of the FtsZ-ring occurs through the breakdown of GTP *via* the intrinsic GTPase activity of FtsZ (Kretschmer and Schwille, 2016). Figure 1-8 illustrates the polymerisation of FtsZ to construct the Z-ring, adapted from (Lutkenhaus, 1993).

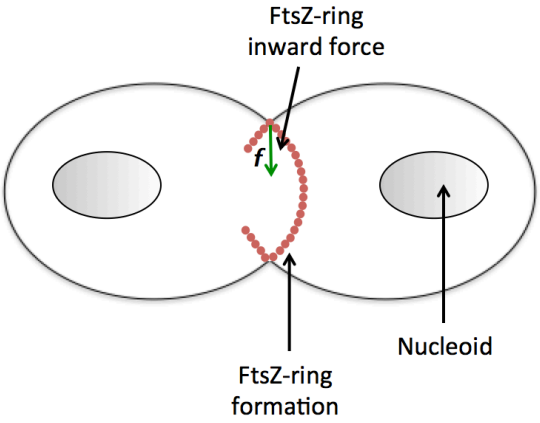


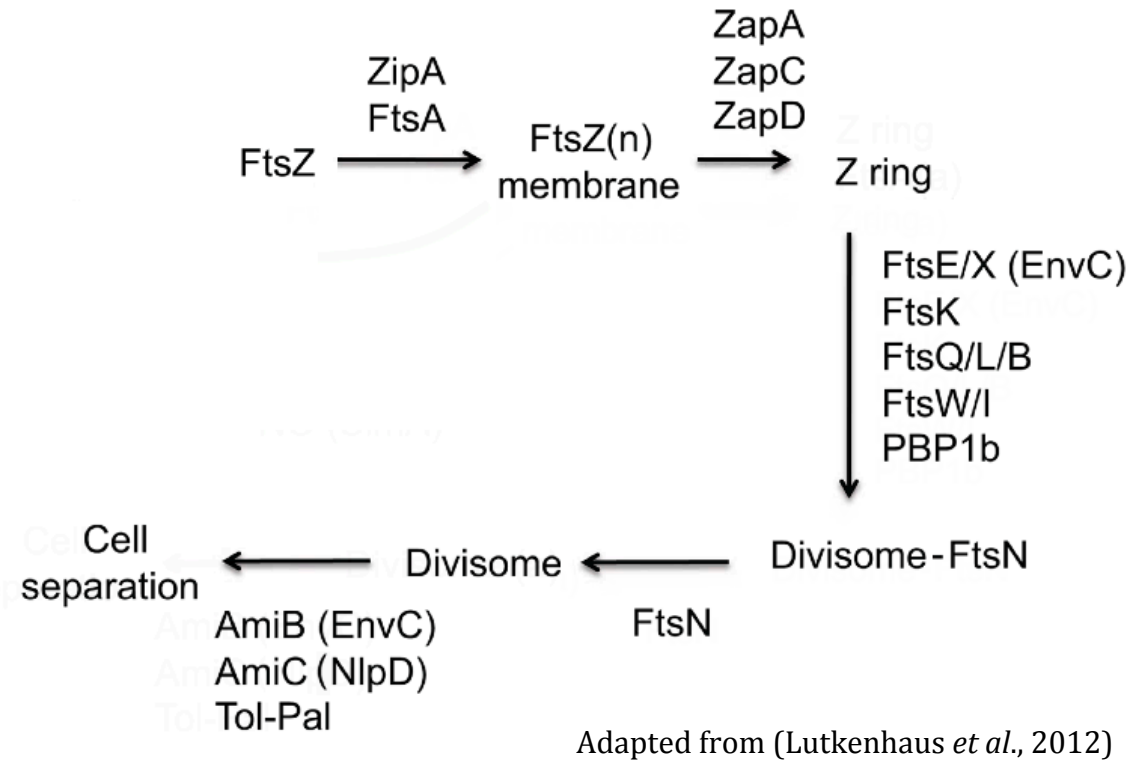
Figure 1-8. An illustration of the polymerisation of FtsZ to construct the Z-ring which drives cell septation, adapted from (Lutkenhaus, 1993).

ZipA acts as a membrane tether for the FtsZ ring whilst the proteins MinC, MinD and SlmA all have roles in the location of the FtsZ-ring within the replicating cell by driving the FtsZ-ring location towards the septation point of the dividing cell (Thanbichler and Shapiro, 2008). The next step in the creation of the divisome is the addition of more FtsZ interacting proteins ZapA, ZapC and ZapD. Although non-essential, these Zap proteins help to localise the FtsZ-ring and increase its integrity (Durand-Heredia *et al.*, 2012). The remaining Fts proteins and PBP1b are then recruited – the last of which is FtsN, which signals the maturation of the *E. coli* divisome. The roles of these proteins are described in Table 1-3.

Table 1-3. Summary of the proteins recruited to the divisome after ZipA, FtsA, ZapA, ZapC and ZapD have anchored and stabilised the FtsZ ring away from MinC, MinD and SlmA.

Protein	**Function**
FtsE/FtsX/EnvC	FtsE and FtsX encode subunits of an ABC transporter that removes lipoproteins from the cytoplasmic membrane. EnvC is recruited by the ABC transporter which activates a septal peptidoglycan amidase AmiB (Schmidt *et al*., 2004, Yang *et al*., 2011).
FtsK	FtsK is a DNA translocase that moves DNA away from the septum of the dividing cell (Bigot *et al*., 2005).
FtsQ	FtsQ acts as a physical linker between the FtsZ-ring and cellular peptidoglycan biosynthetic machinery (Goehring *et al*., 2006).
FtsL	FtsL acts as a physical linker between the FtsZ-ring and cellular peptidoglycan biosynthetic machinery (Goehring *et al*., 2006).
FtsB	FtsB acts as a physical linker between the FtsZ-ring and cellular peptidoglycan biosynthetic machinery (Goehring *et al.*, 2006).
PBP1b	PBP1b is a peptidoglycan synthase that operates around the septum, resulting in septation (Egan *et al*., 2014).
FtsI	FtsI operates like PBP1b and is activated by the recruitment of FtsN (Wissel and Weiss, 2004). It operates as a transpeptidase, moving elements across the cytoplasmic membrane for peptidoglycan synthesis (Wang *et al*., 1998). FtsI also recruits further proteins that aid septation (Gerding *et al.*, 2009).
FtsW	FtsW is an integral membrane which accommodates the transport of Lipid II through the bacterial cytoplasmic membrane (Mohammadi *et al*., 2014)
FtsN	FtsN is the last essential protein to arrive to the divisome. It activates multiple proteins of the divisome, recruits downstream proteins that are unessential and triggers septation (Bernard *et al*., 2007).

After the arrival of FtsN, the mature divisome activates septal peptidoglycan synthesis. The septal wall then begins to split upon the activation of AmiB and AmiC by EnvC and NlpD, respectively (Peters *et al*., 2011). The Tol-Pal complex facilitates efficient invagination of the outer membrane and the combination of these events leads to separation of the two daughter cells (Gerding *et al*., 2007). The order of divisome recruitment from FtsZ-ring formation through to cell separation is illustrated in Figure 1-9, adapted from (Lutkenhaus *et al*., 2012).

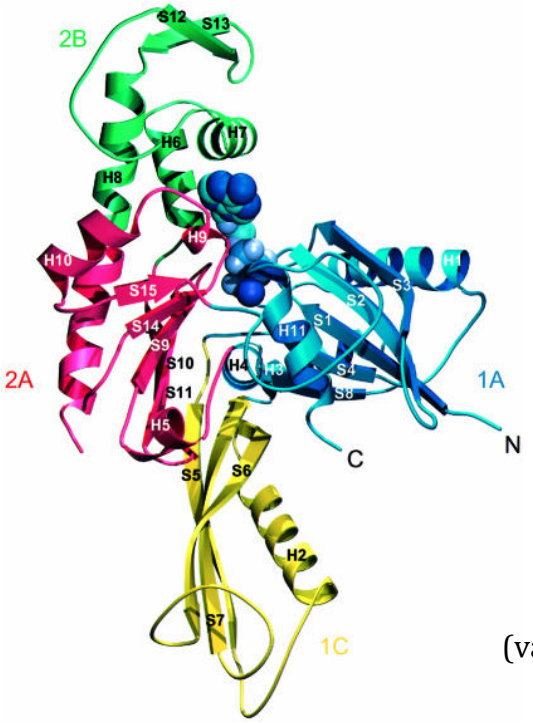


Adapted from (Lutkenhaus *et al*., 2012)

Figure 1-9. An illustration of FtsZ-ring formation followed by the maturation of the *E. coli* divisome leading to cell separation. FtsZ polymerises and anchors with the help of ZipA and FtsA into FtsZ(n) membrane. ZapA, ZapC and ZapD further stabilise the polymerised, anchored FtsZ to form the Z ring. FtsE/FtsX with EnvC, FtsK, FtsQ, FtsL, FtsB FtsI, FtsW and PBP1b combine to form an immature divisome (without FtsN). The recruitment of FtsN leads to peptidoglycan manipulation through AmiB, AmiC and Tol-Pal and cell separation (Lutkenhaus *et al*., 2012).

1.1.4.2 Filamentous temperature sensitive protein A (FtsA)

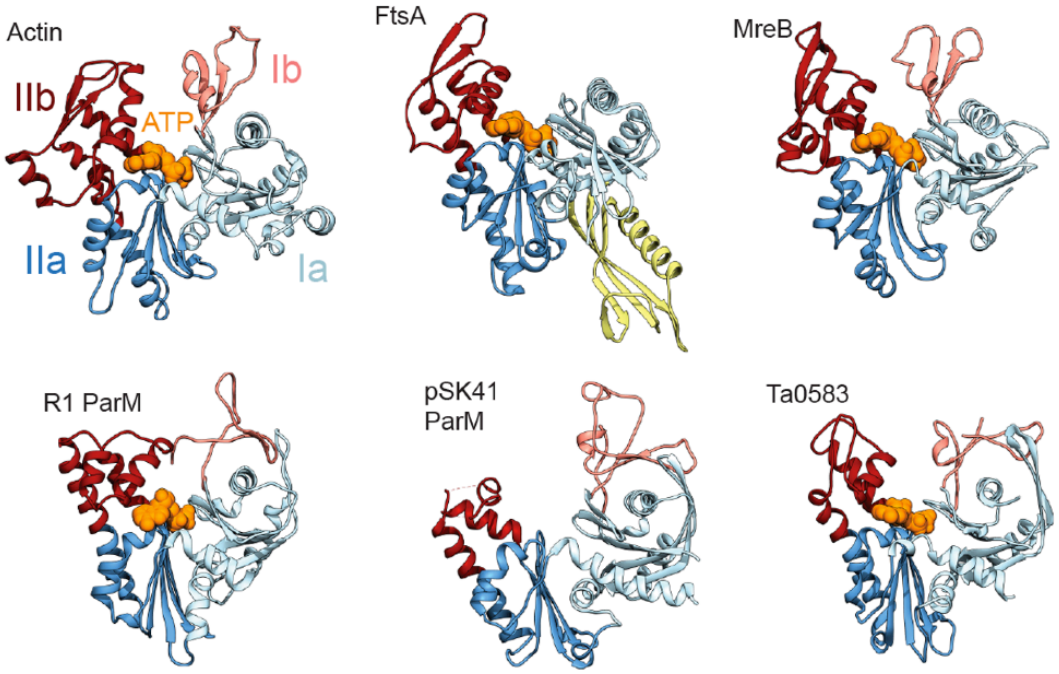
Filamentous temperature sensitive protein A (FtsA) is an essential, highly conserved cell division protein amongst prokaryotes with important roles in the membrane tethering and stabilising of the FtsZ-ring (Pichoff and Lutkenhaus, 2007). It is a peripheral membrane protein belonging to the actin/hsp70/sugar kinase family with the ability to bind ATP (Sánchez *et al*., 1994, Löwe and van den Ent, 2001). Upon the binding of ATP it undergoes a conformational change causing it to polymerise and be able to interact with the cell membrane (Krupka *et al*., 2014). The first crystal structure of FtsA was solved in 2000 by van den Ent and Löwe. The hyperthermophillic *Thermotoga martima* (*T. martima*) FtsA was solved in the apo and ATP-bound form. The crystal structure can be observed in Figure 1-10 (van den Ent, 2000).



(van den Ent, 2000)

Figure 1-10. Crystal structure of *T. martima* FtsA. The structure is separated into four domains like other Actin proteins; 1A (teal) 1C (yellow) that is unique to FtsA, subdomain 2A (red) and subdomain 2b (green). Secondary structure motifs are labeled depending on the proteins primary sequence. An ATP homologue is depicted in dark blue in its binding groove (van den Ent, 2000).

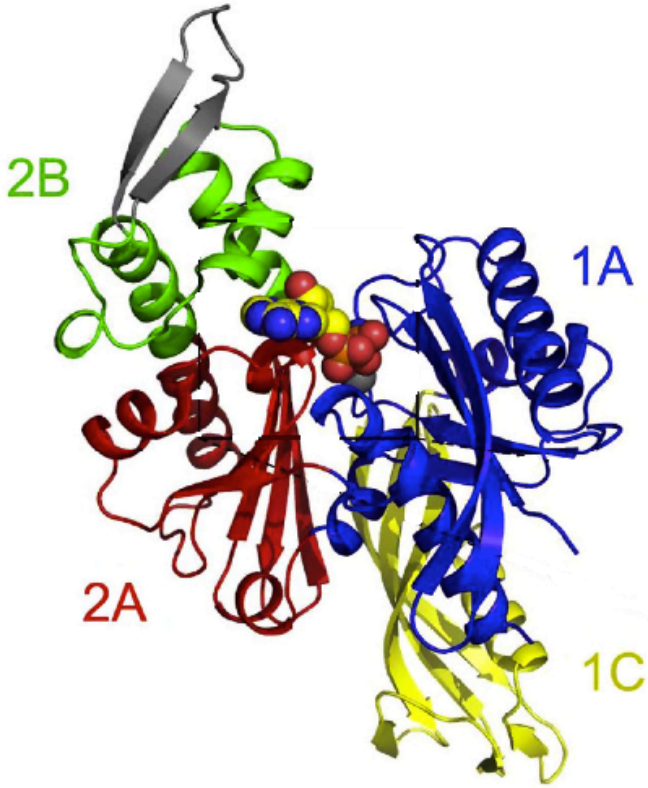
FtsA contains four protein domains that display an organisation resembling the actin fold. The FtsA specific domain, which is believed to be responsible for the interaction with the cell's membrane, allowing it to act as a membrane tether (van den Ent, 2000), is coloured yellow and labeled as 1C in Figure 1-11. To demonstrate the similarities in subdomain organisation across the actin protein family, the *T. martima* FtsA crystal structure is shown along with other bacterial actin-fold proteins in Figure 1-11 adapted from (Ozyamak *et al.*, 2013).



Adapted from (Ozyamak *et al.*, 2013)

Figure 1-11. A comparison of known crystal structures of bacterial actins, showing they all share a conserved structural core. The FtsA specific domain is coloured yellow to allow distinction. Adapted from (Ozyamak *et al.*, 2013).

Research into the ability of FtsA to interact with the divisome indicates that a highly conserved C-terminal motif that is 10-13 amino acids long is key to the formation of the divisome. Deletion of the residue lowers the protein's affinity to FtsZ; interfering with the cell division process (Gayda *et al*., 1992, Löwe and van den Ent, 2001). This motif is highly conserved and can be observed in the more recent FtsA crystal structure that was derived from the mesophillic *Staphylococcus aureus* (*S. aureus)* (Fujita *et al*., 2013, Fujita *et al*., 2014). This crystal structure is displayed in Figure 1-12, which was adapted from (Fujita *et al*., 2014).



(Fujita *et al*., 2014)

Figure 1-12. Crystal structure of *S. aureus* FtsA. The structure is separated into four domains like other Actin proteins; 1A (blue) 1C (yellow) that is unique to FtsA, subdomain 2A (red) and subdomain 2b (green). Secondary structure motifs are labeled depending on the proteins primary sequence. An ATP homologue is depicted as a globular molecule in its binding groove. The S12-S13 hairpin is depicted in grey; it is the major difference between *T. martima* and *S. aureus* FtsA (Fujita *et al*., 2014).

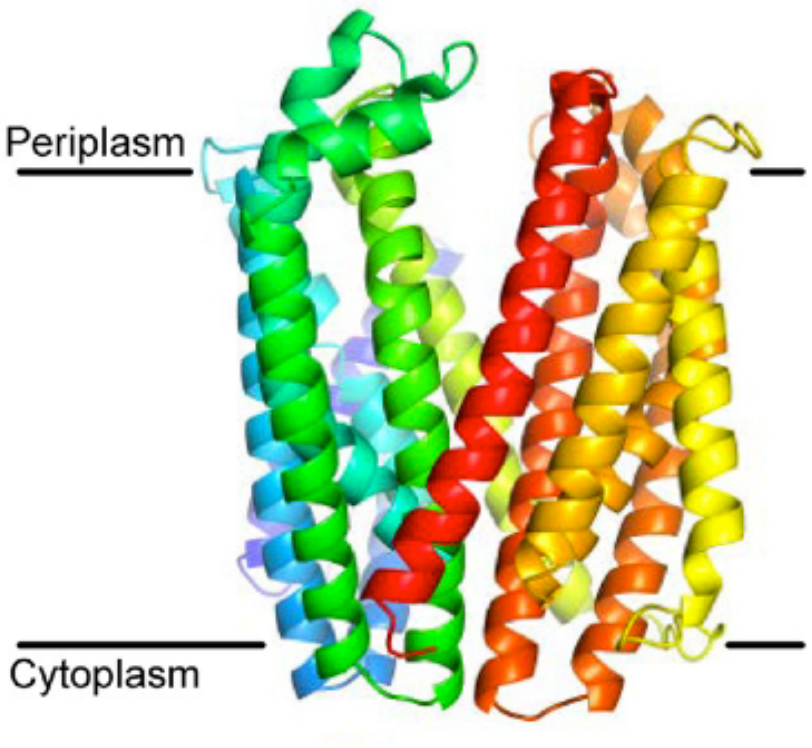
1.1.4.3 Muramyl ligase J (MurJ)

Muramyl ligase J (MurJ) (previously called MviN), is a polytopic integral membrane protein that belongs to the multidrug/oligo-saccharidyl-lipid/polysaccharide (MOP) exporter family, which are secondary active antiporters that utilise the electrochemical potential created by Na^+ or H^+ gradients (Hvorup *et al*., 2003, Kuroda and Tsuchiya, 2009, Islam and Lam, 2013, Sham *et al.*, 2014). The *E. coli* MurJ gene itself is located a long way away from other related genes on the *E. coli* genome, which led to it initially being named MviN (Fay and Dworkin, 2009). MurJ has proven to be essential to *E. coli*. MurJ deficient *E. coli* demonstrate a peptidoglycan precursor buildup and the synthesis of up to 70 % less mature peptidoglycan (Inoue *et al.*, 2008). Bleb formation and irregularities in cell morphology can also occur, these effects often lead to cell lysis (Ruiz, 2008).

Studying *E. coli* MurJ homologues across other bacteria has added some clarity to the function of the protein. *E. coli* MurJ is essential to cell division and hence bacterial survival. This is also true with all other conclusively tested gram-negative bacteria, including the MurJ (MviN) homologues of *Burkholderia cenocepacia* and *Sinorhizobium meliloti* (Rudnick *et al*., 2001, Fay and Dworkin, 2009, Mohamed and Valvano, 2014). In the gram-positive bacteria *Bacillus subtilis*, three homologues of MurJ exist and none of them are essential (Fay and Dworkin, 2009, Young, 2014). The high level of conservation of proteins responsible for peptidoglycan synthesis across Gram-positive and Gram-negative bacteria suggests a different protein group fulfills the unknown role of MurJ in

Lipid II manipulation throughout Gram-positive bacteria. The Gram-positive *Staphylococcus aureus* (*S. aureus*) protein SAV1754 is thought to have the same role as *E. coli* MurJ due to the similar responses to strains deficient in the respective proteins (Huber *et al*., 2009). *Streptococcus pyogenes* (*S. pyogenes)* is a Gram-positive bacteria that does not contain a conserved structural homologue of MurJ (Ruiz, 2009). Research into the *S. pyogenes* protein YtgP has suggested it fulfills the role of MurJ in gram-negative bacteria. This conclusion was reached by cloning the *S. pyogenes* YtgP into *E. coli* with and without active MurJ and observing the consequences (Ruiz, 2009).

Limited work with MurJ and its other bacterial homologues has been completed to this point due to complexities in extracting, solubilising and purifying the membrane protein. However, structural predictions suggest MurJ contains 14 transmembrane (TM) domains that coordinate to create a hydrophillic central cavity with a net positive charge, allowing transport through the cytoplasmic membrane (Ruiz, 2016). The arrangement of the TM domains in the membrane is illustrated in Figure 1-13, which was adapted from (Butler *et al.*, 2013).



Adapted from (Butler *et al*., 2013)

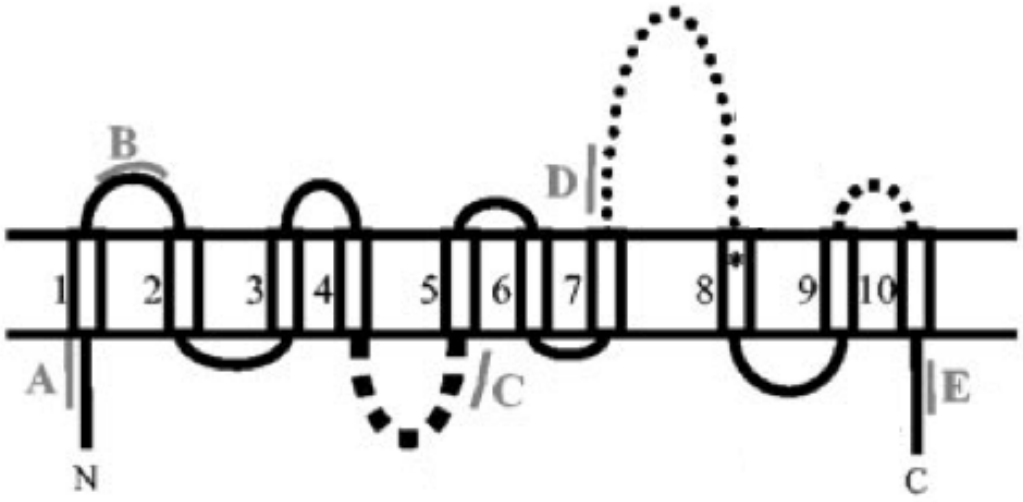
Figure 1-13. An I-TASSER model structure of the coordination of the MurJ TM domains positioned within the cytoplasmic membrane of *E. coli*. The image was adapted from (Butler *et al*., 2013).

The specific function of MurJ in *E. coli* is still heavily disputed in the literature. It appears to have a role in the transport of Lipid II, a peptidoglycan (PG) precursor as in the absence of MurJ, the buildup of other (PG) precursors occurs (Sham *et al*., 2014). Furthermore, the net positive charge inside the central cavity of MurJ would allow interactions with the negatively charged Lipid II (Young, 2014). It is hypothesised that MurJ facilitates the recycling of Lipid II components back into the cytoplasm (Personal communication, Dr D. Roper 2014).

1.1.4.4 Filamentous temperature sensitive protein W (FtsW)

FtsW has been known to be involved in the bacterial divisome through its localisation to the septum during cell division (Khattar *et al.*, 1994, Boyle *et al.*, 1997, Mercer and Weiss, 2002, Pastoret *et al.*, 2004, Mohammadi *et al.*, 2011). The specific function of FtsW was disputed until some clarity was provided by the work of Mohammadi *et al.*, 2011 (Mohammadi *et al.*, 2011). FtsW is an integral membrane that spans the cytoplasmic membrane, which accommodates the transport of Lipid II (a PG building block) through the bacterial cytoplasmic membrane (Mohammadi *et al.*, 2014). It is a member of the shape, elongation, division and sporulation family of proteins that also includes *E. coli* RodA and *Bacillus subtilis* SpoVE (Egan and Vollmer, 2013).

FtsW is highly conserved amongst all bacteria synthesising peptidoglycan-containing cell walls and has no counterpart in the human genome, making it a potentially ideal target for new antibiotics (Mercer and Weiss, 2002). FtsW spans the cytoplasmic membrane of prokaryotes and modeling predicts it to contain 10 TM helices (Lara and Ayala, 2002). Structural evidence is yet to be reported with respect to FtsW due to the inherent complications that result from studying membrane proteins, however, functional analysis of the protein in *E. coli* was published by Pastoret *et al.* in 2004 (Pastoret *et al.*, 2004). The proposed membrane topology of *E. coli* FtsW is illustrated in Figure 1-14 which is adapted from (Pastoret *et al.*, 2004).



Adapted from (Pastoret *et al*., 2004)

Figure 1-14. A diagram showing the membrane topology of *E. coli* FtsW. Amphiphilic sections are highlighted A through E and both the N and C terminus are shown to reside in the cells cytoplasm (Pastoret *et al*., 2004).

Mutagenesis studies have added extra insight to the proteins that interact directly with FtsW. TM9 and TM10 have been shown to be involved in the relationship between *E. coli* FtsW and PBP1B (Fraipont *et al.*, 2011). The periplasmic loop between TM7 and TM8 (specifically between residues Glu240 and Ala249) is essential for peptidoglycan assembly at the septation point during cell division and the first section of peptide from the N-terminus to TM1 is necessary for FtsW to associate with FtsQ (D'Ulisse *et al.*, 2007).

More recently, mutation studies were completed in combination with a fluorescent Lipid II analog to observe which amino acid residues and protein domains are key for *E. coli* FtsW to transport Lipid II into the periplasm (Mohammadi *et al*., 2014). It was discovered that TM4 is important for the transport activity of FtsW, in particular, the charged residues Arg145 and Lys 153 that sit within the domain. The positive charge of the arginine and lysine

residues is suggested to directly interact with the negatively charged Lipid II. Whilst the flipping and binding mechanisms of FtsW acting on Lipid II remain disputable, it is widely accepted that the 10 TM domains form a pore like structure, allowing the translocation of Lipid II from the cell's cytoplasm to its periplasm (Mohammadi *et al.*, 2014). Literature also suggests that FtsW associates with FtsI (previously known as PBP3) when both locate at the septum. They form a subcomplex that facilitates the transport of peptidoglycan building blocks across the bacterial cytoplasmic membrane (Wang *et al.*, 1998, Fraipont *et al.*, 2011).

As explained at the start of the introduction, the project which forms the basis of this thesis has two distinct subprojects linked by a common reagent (SMA). The first subproject involves a study of SMA as a membrane protein encapsulation method (see preceding section). The second subproject involves a study of SMA as a novel periplasmic release agent.

1.2 Periplasmic release

1.2.1 The potential of *E. coli* as an expression system

Over-expression of proteins in *E. coli* is extremely popular; the gram-negative bacterium has been one of the most commonly used expression systems since research on recombinant protein expression began (Choi and Lee, 2004, Schumann and Ferreira, 2004). Vast amounts of literature already exist detailing how *E. coli* reacts to overexpression of different protein types. As described in a previous section, *E. coli* provides a low cost protein production system from research to industry (Rosano and Ceccarelli, 2014). *E. coli* combines a fast replication rate with a higher product yield when compared to other expression systems which can result in up to 10 times more protein being produced in identical growth conditions (Fraser, 2006, Singh *et al*., 2008). It is also possible to produce proteins wherever desired within the bacterium – including the cytoplasm and periplasm or outside of the cell completely into the extracellular milieu (Assenberg *et al*., 2013).

1.2.1.1 Recombinant proteins in the cytoplasm

Cytoplasmic protein targeting is the most common method for recombinant protein expression in *E. coli*. However, over expression of recombinant proteins in the cytoplasm of cells can lead to aggregation and the growth of inclusion bodies, which can decrease the yield and adds extra downstream steps and costs to purify (Ventura and Villaverde, 2006). Another issue with cytoplasmic-targeted recombinant protein production is the reducing environment of the

cytoplasm (Mergulhão *et al*., 2005). In general, cytoplasmic proteins do not contain disulfide bonds, those that do are facilitated with specific cytoplasmic thioredoxins (Stewart *et al.*, 1998). This inability to spontaneously produce disulfide bonds could impact the tertiary structure of recombinant proteins targeted to the cytoplasm (Assenberg *et al*., 2013). Furthermore, the cytoplasm is home to a large percentage of the cell's proteins and proteases that could dilute the target recombinant protein or damage the product respectively *(French et al*., 1996). With the aforementioned taken into account, methods to target recombinant proteins to other areas of cells are growing in popularity. Gram-negative bacteria such as *E. coli* possess the ability to transport proteins into the cell's periplasmic space or out of the cell into the extracellular milieu (Kanonenberg *et al.*, 2013). This ability to move proteins across cellular compartments can be exploited to direct recombinant proteins to different sections of the cell.

1.2.1.2 Recombinant proteins secreted to the extracellular milieu

Proteins cross the cell membrane *via* secretion pathways mediated by protein-complexes that insert and span sections of the hydrophobic cell wall. In Gram-negative bacteria two types of secretion exist that enable protein transfer to the extracellular milieu; Type I secretion which is controlled by the HlyA/HiyD/TolC pathways in *E. coli* and Type II secretion which is regulated by secretion components known as the 'main terminal branch' (Kanonenberg *et al*., 2013, Korotkov *et al*., 2012). Manipulating proteins to moved across membranes is a complicated process, with the protein needing to be tagged correctly and not

over expressed to a level where the cell cannot secrete it fast enough (Mergulhão *et al*., 2005). However, it brings with it some inherent benefits in terms of protein quality and half-life. Once secreted, the recombinant proteins are separated from the reductive environment of the cytoplasm that also possesses the majority of the bacterial proteases. Post-secretion there is no need for cellular disruption to collect the target recombinant protein, resulting in a reduction in steps and nullifying the potential for the target protein to be damaged during the cell lysis stage (Shokri *et al*., 2003). Additionally, the transport process guarantees the cleavage of a signal sequence during the secretion; this ensures the N-terminal authenticity of the protein product (Mergulhão *et al*., 2005).

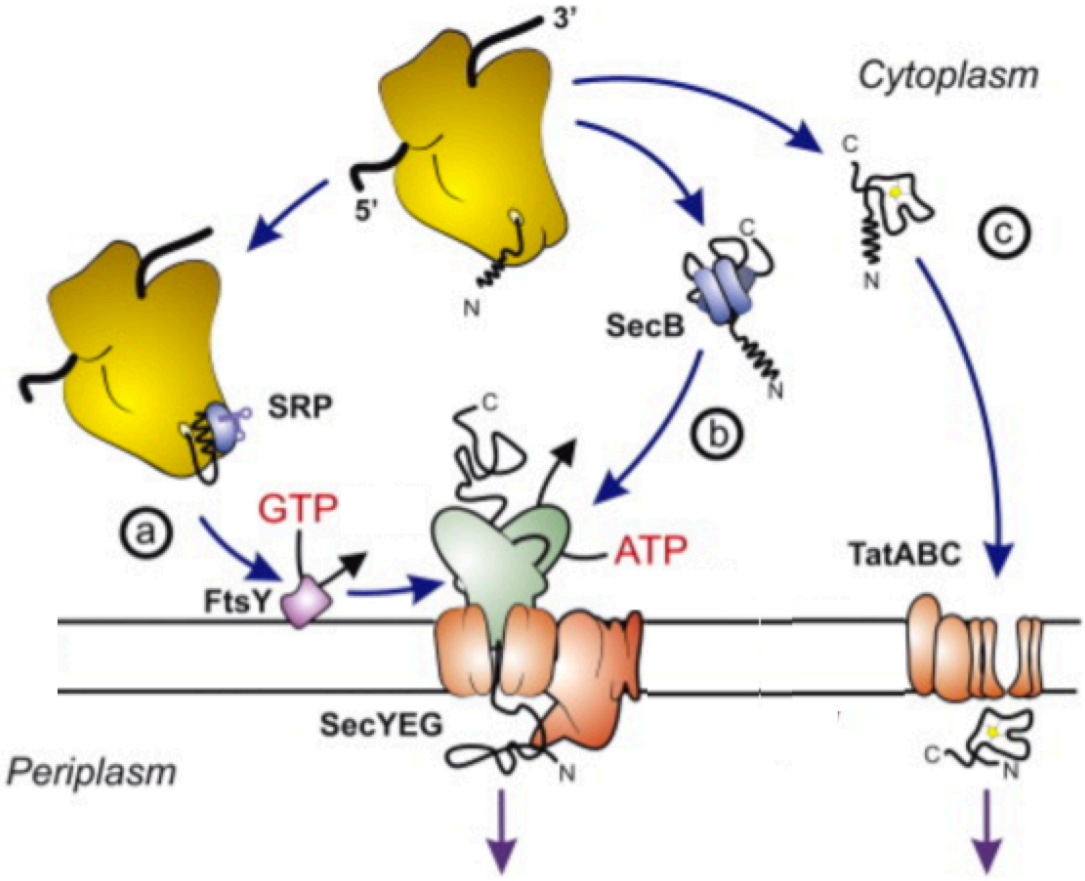
Whilst these secretion systems have been manipulated to secrete recombinant proteins into the extracellular milieu, they often malfunction and erroneously transfer proteins to the periplasm as opposed to the extracellular space (Kanonenberg *et al*., 2013). Additionally the transport of such a large volume of protein across the whole cell wall can put strain on cellular machinery, causing a protein back up in the cytoplasm which aggregates (Mergulhão *et al*., 2005). Furthermore, directing the protein to the media results in a large dilution of the product, necessitating expensive and time consuming concentration steps downstream (Mergulhão *et al.*, 2005). The inherent disadvantages associated with the targeting of recombinant proteins to both the cytoplasm and extracellular milieu have lead to other methods being exploited to maximise the efficiency of recombinant protein production. Perhaps the most promising of said other methods is the periplasmic targeting of recombinant proteins.

1.2.1.3 Recombinant proteins targeted to the periplasm

The periplasm is present in all gram-negative bacteria, it is a compartment sandwiched between the cytoplasmic membrane of the cell and the outer membrane (Beacham, 1979). In *E. coli*, approximately 450 proteins are naturally exported across the cytoplasmic membrane *via* the *sec* or *tat* pathways (Ehrmann, 2007). The main differences between the two pathways is that the *sec* pathway is utilised more frequently within prokaryotes and transports unfolded proteins to the periplasm, whereas the *tat* pathway transports fully folded proteins (Natale *et al.*, 2008).

The *sec* pathway transports protein across the cytoplasmic membrane with the Sec-translocase which is highly conserved throughout all prokaryotes and the endoplasmic reticulum of eukaryotes (Lee and Schneewind, 2001). The Sec-translocase comprises of three integral membrane proteins (SecY, SecE and SecG) which combine to form a protein conducting channel through the cytoplasmic membrane (Osborne *et al.*, 2005). Secretory proteins are directed to the Sec-translocase *via* two different methods: post-translational and co-translational targeting. In post-translational targeting the secretory protein is released from the ribosome before it is directed to the Sec-translocase with the aid of the protein chaperone SecB (Driessen, 2001). In co-translational targeting, the target protein remains in a complex with the ribosome. A signal recognition particle (SRP) binds the signal sequence of the target protein, which directs the complex with the help of FtsY to the Sec-translocase (Luirink *et al*., 2005).

The functional twin-arginine translocation (*tat*) pathway consists of three membrane integrated proteins (TatA, TatB and TatC) that together form a complex allowing the transport of fully folded proteins through the cytoplasmic membrane (de Keyzer *et al*., 2003). Figure 1-15 displays how both the *sec* and *tat* pathways secrete proteins into the periplasm.



Adapted from (Natale *et al*., 2008)

Figure 1-15. A diagram illustrating the *sec* and *tat* pathway for transporting proteins from the cytoplasm into the periplasm. (a) Co-translational transport to the Sec-translocase. (b) Post-translational transport to the Sec-translocase. (c) Protein secretion *via* the Tat-translocase. Adapted from (Natale *et al*., 2008)

The periplasmic space has a variety of features that make it a favorable final destination for recombinant proteins. The cell wall can be selectively removed

via a multitude of different methods, allowing release of the periplasmic contents whilst the cytoplasm remains intact. The periplasm makes up c. 4-8 % of the cell's total volume, therefore targeting proteins to this space has intrinsic purification benefits when compared to producing recombinant proteins in the comparably larger cytoplasm (French *et al*., 1996). Furthermore, in *E. coli* only 7 of the known 25 proteases inhabit the perisplasmic space. (Swamy and Goldberg, 1982). Therefore targeting recombinant proteins to this space goes some way to limit the potential for enzymatic damage. In direct contrast to the cell's cytoplasm, the periplasm is a naturally oxidising environment. This environment facilitates disulfide bond formation, aiding the correct initial folding of proteins and increasing their stability (Jalalirad, 2013). An example of this increased protein stability is the half-life of recombinant proinsulin increasing 10-fold when targeted to the periplasm compared to the cytoplasm (Talmadge and Gilbert, 1982). Periplasmic targeting also ensures N-terminal authenticity of the protein product *via* the guaranteed cleavage of a signal sequence, much like secreting target proteins to the extracellular milieu (Chatzi *et al*., 2013). As with secretion to the extracellular space, the targeting of overexpressed proteins to the periplasm puts extra strain on the *E. coli* cell machinery which can cause a backup of proteins in the cytoplasm that can aggregate (Mergulhão *et al*., 2005). The main limitation in the periplasmic targeting of recombinant proteins for higher yields comes in the lack of a single, universal method for compromising the cell wall whilst leaving the cell's cytoplasmic contents intact. Table 1-4 outlines the differences in recombinant protein expression in the *E. coli* cytoplasm, compared to secretion to its extracellular space or periplasm.

Table 1-4. The advantages and drawbacks of targeting recombinant proteins to the cytoplasm, extracellular milieu and periplasm.

Recombinant protein target location	Advantages	Drawbacks
Cytoplasm	Requires less protein engineering to target a protein to the cytoplasm. Most popular method of recombinant protein expression, lots of literature exists to review on them.	Reducing environment inhibits spontaneous disulfide bonds. Large number of cells proteases present in the cytoplasm. Home to large amount of cellular proteins, necessitating more purification steps. Proteins can be damaged during the breaking of cells to harvest target recombinant protein.
Extracellular milieu	No cell lysis necessary to harvest target protein. Cleavage of signal tag ensures N-terminal authenticity of the target recombinant protein.	Large scale product concentration necessary due to increased volume of the target space. Requires engineering of a sequence tag to move target proteins across membranes.
Periplasm	Naturally oxidising environment facilitates spontaneous disulfide bond formation. Periplasm c. 4-8 % of cell volume, inherent purification and concentration benefits. Less proteases compared to periplasm. Cleavage of signal tag ensures N-terminal authenticity of the target recombinant protein.	Requires engineering of a sequence tag to move target proteins across membranes. No single universal method available for releasing periplasm whilst leave the cytoplasm intact.

The next section of this report will assess the existing methods for specifically releasing cellular periplasmic contents from gram-negative bacteria and introduce SMA as a potential periplasmic release agent moving forward.

1.2.2 Periplasmic release methods

1.2.2.1 Existing methods for periplasmic release

Once targeted to the periplasm the next processing step involves releasing the targeted protein from the periplasm whilst maintaining the integrity of the inner membrane, keeping the cytoplasmic fraction contained. Methods exist for periplasmic release based around osmotic shock of the bacterium, usually with Tris-HCl based buffers with EDTA and lysozyme (Middelberg, 1995). EDTA removes divalent cations which interferes with the cell wall allowing the lysozyme to disrupt the peptidoglycan which compromises the periplasmic compartment (Jalalirad, 2013, Rathore *et al.*, 2003). The ratios of these osmotic shock ingredients change from method to method with varying levels of success from one target protein to the next (Quan *et al.*, 2013). ideally one universal method can be discovered that is scalable and robust. Furthermore these methods are expensive and time-consuming, and whilst effective in the laboratory setting, scale-up remains challenging. To illustrate the financial scale-up challenges, lysozyme costs £2,000 per 200 g (Sigma) and is usually required at 0.5 mg/mL (Pierce *et al.*, 1997). In a 1000 L fermenter, 0.5 kg of lysozyme would be required at a cost £5,000 for lysozyme alone. Whilst discounts for bulk purchase have been ignored in these calculations, as have the need for further time and labour intensive separation steps with various sucrose buffers (Pierce

et al., 1997). The financial factors and procedural scale up issues lead to it being common practice in industry to target proteins to the periplasm for the protein folding and quality benefits, before harvesting *via* complete cell rupture. This essentially nullifies the inherent purification and compartmentalisation benefits of the process (Personal communication, Andrew Collins – GSK).

Periplasmic expression systems are failing to reach their potential due to the lack of a scalable, robust method for selective periplasmic release. This report will now focus on the potential of SMA as a periplasmic release agent.

1.2.2.2 SMA as a periplasmic release agent

The SMA copolymer has demonstrated the ability to adsorb on to cell membranes and disrupt them by excising discs of lipids and any associated proteins within a SMALP (Knowles *et al*., 2009). In this thesis we investigate whether SMA at the right concentration could compromise the outer membrane of *E. coli* whilst retaining the integrity of the cytoplasmic contents. Figure 1-16 illustrates this hypothesis; that SMA could interfere with the outer cell wall releasing only the periplasmic contents of a gram-negative bacteria.

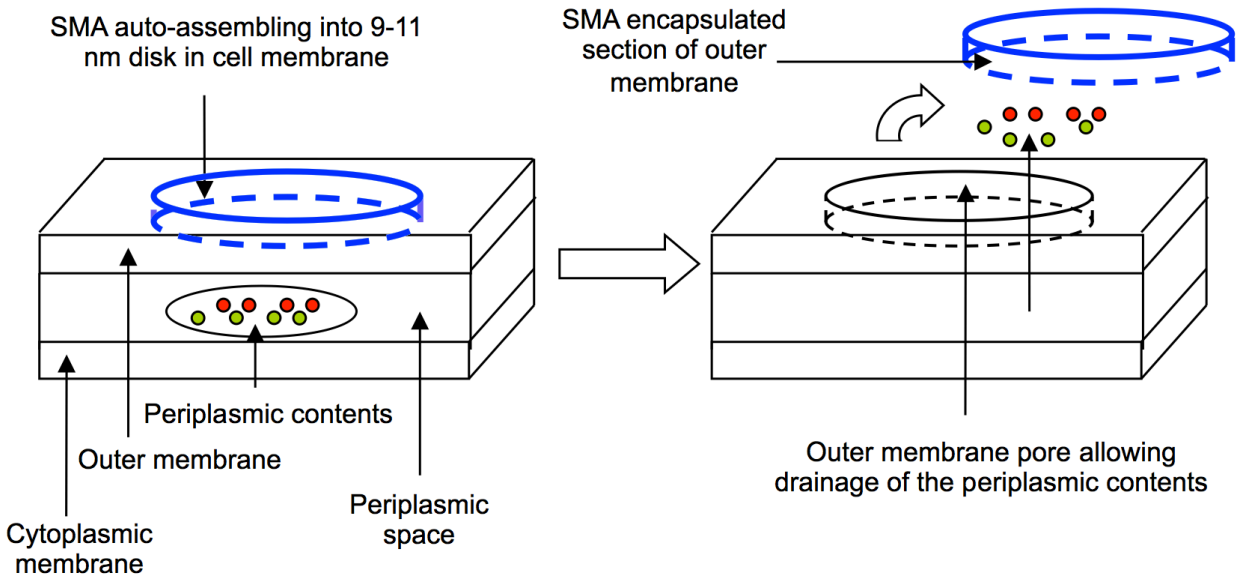


Figure 1-16. A diagram of the rationale behind how the SMA copolymer forming SMA lipid particles could operate as a periplasmic release agent.

In order to investigate the potential of SMA as a periplasmic release agent, we expressed two proteins that were targeted to the periplasm *via* the *sec* pathway. Both secreted proteins are fluorescent which facilitates tracking of the protein within the cell and post release from the periplasm. These proteins are a green-fluorescent protein (GFP) derivative targeted to the periplasm - periplasmic superfolder-GFP (Peri-sfGFP) and a periplasm-targeted mCherry - periplasmic mCherry (Peri-mCherry).

1.2.3 Target protein selection:

1.2.3.1 Periplasmic superfolder-GFP (Peri-sfGFP)

Green fluorescent protein (GFP) has long been exploited as a tool for observing the internal biology of bacteria (Feilmeier *et al*., 2000). GFP is a popular option for subcellular localisation studies within bacteria, however, folding complications occur when trying to express it outside of the cell's cytoplasm (Feilmeier *et al*., 2000). When unfolded, GFP is transported to the periplasm *via* the *sec* pathway, it fails to fold correctly and therefore does not fluoresce (Feilmeier *et al*., 2000). To circumvent these issues, alternatives such as red fluorescent proteins and GFP have been secreted with mixed success through the *tat* system (Lewenza *et al*., 2006, Santini *et al*., 2001). Another attempt to avoid the folding issues of GFP was to engineer a superfolding version of the protein (sfGFP) which could be directed to the periplasm of cells and still fluoresce (Pédelacq *et al*., 2006).

Superfolder-GFP has been engineered into a plasmid containing a signal sequence that directs the protein into the periplasmic space *via* the *sec* pathway (Aronson *et al*., 2011). The signal sequence used in this clone was originally engineered by Lee and Bernstein. The signal sequence MBP*1 was optimised for efficient transport of proteins to the periplasm (Lee and Bernstein, 2001). This clone facilitates the tracking of cellular periplasmic contents *via* simple colorimetric methods.

1.2.3.2 Periplasmic mCherry (Peri-mCherry)

Another popular fluorescent protein due to its folding efficiency, photostability and genetic manipulability is mCherry (Shaner *et al*., 2004, Winterflood and Ewers, 2014). Existing work proves mCherry folds correctly in the periplasm and that its fluorescence is unaffected by the environment change *(*Aronson *et al*., 2011). To target mCherry to the periplasm, the protein was engineered into a plasmid containing a signal sequence, triggering its secretion *via* the *sec* pathway (Aronson *et al*., 2011). The signal sequence used in this clone was originally engineered by Lee and Bernstein. The signal sequence MBP*1 was optimised for efficient transport of proteins to the periplasm (Lee and Bernstein, 2001). This clone facilitates the tracking of cellular periplasmic contents *via* simple colorimetric methods.

1.3 Objectives

1.3.1 Membrane protein work

Membrane proteins still remain valuable targets for therapeutic research. However, the inherent difficulties associated with studying membrane proteins have resulted in a lack of data on their structure and function. In this report we further explore the novel reagent SMA to extract membrane proteins to circumvent some of the issues associated with membrane protein study. The retaining of annular lipid and the ability to subject the membrane protein containing SMALP to traditional purification methods were exploited in the over-expression, purification and characterisation of three, essential *E. coli* proteins. The SMALP purification system was used to solubilise the peripheral membrane protein FtsA. The polymer was also used to purify two other *E. coli* membrane proteins fused to multifunctional-GFP (mfGFP) - MurJ-mfGFP and FtsW-mfGFP.

1.3.2 Periplasmic release work

Periplasmic targeting of proteins for the associated advantages is still an appealing concept, despite the lack of a single universal method to trigger the release of cellular periplasmic contents whilst leaving the cytoplasm intact. In this report, we explore the potential of SMA as an outer membrane disruptor to selectively release the periplasmic contents of *E. coli*. Specifically designed clones of sfGFP and mCherry target the fluorescent proteins to the periplasm, allowing the tracking of the periplasmic contents throughout the process.

CHAPTER 2:

Materials and methods

2.1 Materials

Chemicals used in this project were purchased from Sigma-Aldrich (Dorset, UK) and Fischer Scientific (Leicestershire, UK) unless stated otherwise.

2.1.1 Reagents for membrane protein expression

One Shot® TOP10 Chemically Competent *E. coli* and One Shot® BL21 (DE3) (F^- *omp*T *hsdS*$_B$($r_B^-m_B^-$)*gal dcm*(DE3)pLysS (Cam^R)) Chemically Competent *E. coli* cells were purchased from Invitrogen (Paisley, UK). OverExpress™ C43 (DE3) (F^- *omp*T $hsdS_B(r_B^- m_B^-)$ *gal dcm*) cells were sourced from Lucigen (Cambridge, UK). The *E. coli* FtsA expression plasmid used was generated by Dr Mohammed Jamshad. Both the *E. coli* MurJ-mfGFP and FtsW-mfGFP expression plasmids were generous gifts from the lab of Dr D. Roper of Warwick University. Luria-Bertani (LB) medium and the relevant antibiotics used (Ampicillin and Kanamycin) were sourced from Sigma-Aldrich (Dorset, UK). Isopropyl-β-D-thiogalactopyranoside (IPTG) for induction was purchased from Bioline (London, UK). Glycerol and methanol were sourced from Fisher Scientific (Leicestershire, UK).

2.1.2 Reagents for membrane protein purification

Styrene maleic anhydride (SMA 2000P) was purchased from Cray Valley (Philadelphia, USA). Ni^{2+}-NTA was sourced from Qiagen (Crawley, UK). Precast Tris-Glycine 12 % gels were purchased from Nu-Sep (Wasserburg, Germany). HyperPAGE prestained protein marker was purchased from Bioline (London, UK). NuPAGE LDS sample buffer (4x) was sourced from Novex® of Life

Technologies (Paisley, UK). InstantBlue protein stain was purchased from Expedeon (Cambridge, UK). BCA protein assay reagents were sourced from Thermo Fisher scientific (Northumberland, UK). Nitrocellulose membrane was purchased from Sigma-Aldrich (Dorset, UK). For Western blotting, 6xHis Monoclonal antibody produced in Mouse was sourced from Clontech (Takara) (Saint-Germain-en-Laye, France). Anti-GFP N-terminal antibody produced in rabbit was purchased from Sigma-Aldrich (Dorset, UK). Anti-Mouse IgG HRP-conjugated antibody and Anti-Rabbit IgG HRP-conjugated antibody were both purchased from Sigma-Aldrich (Dorset, UK). EZ-ECL detection solution was purchased from Geneflow (Lichfield, UK). Pierce Protein Concentrators (20K MWCO) were purchased from Thermo-scientific (Illinois, USA). Dialysis membranes (14K MWCO) were purchased from Thermo Fisher Scientific (Illinois, USA).

Reagents for Periplasm targeted protein expression and release

E. coli BL21 Codon Plus (DE3) (F^- *omp*T *hsd*S($r_B^-m_B^-$) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte [*argU proL* Cam^r] [*argU ileY leuW* Strep/$Spec^r$]) were sourced from Stratagene (California, USA). Both the peri-sfGFP and per-mCherry expression plasmids were generous gifts from Erik L. Snapp of the Einstein College of Medicine, New york.

2.2 Methods

2.2.1 Membrane protein expression, purification and characterisation

Protein expression and purification conditions were very similar across the three membrane proteins studied in this report. Methods will be listed generically for all three of the proteins purified.

2.2.1.1 FtsA vector

An FtsA expression vector was synthesised by Dr Mohammed Jamshad within our laboratory. The vector pET-32 Xa/LIC Vector (Invitrogen) was manipulated to express *E. coli* FtsA. Expression of the vector is controlled by the T7 RNA polymerase; hence the target protein can be induced with IPTG. A 6xHis tag was added at either end of the coded protein to facilitate protein purification. The FtsA vector is resistant to ampicillin allowing screening and selection of successfully transformed colonies. The sequence of the *E. coli* FtsA (45 kDa) is shown in Figure 2-1.

MIKATDRKLVVGLEIGTAKVAALVGEVLPDGMVNIIGVGS
CPSRGMDKGGVNDLESVVKCVQRAIDQAELMADCQISS
VYLALSGKHISCQNEIGMVPISEEEVTQEDVENVVHTAKS
VRVRDEHRVLHVIPQEYAIDYQEGIKNPVGLSGVRMQAK
VHLITCHNDMAKNIVKAVERCGLKVDQLIFAGLASSYSVL
TEDERELGVCVVDIGGGTMDIAVYTGGALRHTKVIPYAG
NVVTSDIAYAFGTPPSDAEAIKVRHGCALGSIVGKDESVE
VPSVGGRPPRSLQRQTLAEVIEPRYTELLNLVNEEILQLQE
KLRQQGVKHHLAAGIVLTGGAAQIEGLAACAQRVFHTQ
VRIGAPLNITGLTDYAQEPYYSTAVGLLHYGKESHLNGEAE
VEKRVTASVGSWIKRLNSWLRKEF

Figure 2-1. The amino acid sequence of *E. coli* FtsA a 45 kDa membrane protein.

2.2.1.2 MurJ vector

The MurJ expression vector was a generous gift from the laboratory of Dr D. Roper of Warwick University. An adapted pET-28(a+) derived GFPx8His fusion Vector (Novagen) was manipulated to express *E. coli* MurJ (Drew *et al.*, 2006). The GFP in the vector was removed and replaced with a multifunctional-GFP (mfGFP) which contained a 8xHis tag (Kobayashi *et al.*, 2008). The amino acid sequence of the mfGFP (34 kDa) including its multifunctional insertion tag is illustrated in Figure 2-2 which was adapted from (Kobayashi *et al.*, 2008).

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWP
TLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD
TLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGS
GHHHHHHHHGSMDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPSHGSEQKLIS
EEDLGSGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVT
AAGITLGMDELYKG

Figure 2-2. The amino acid sequence of mfGFP. Peptide tags inserted between Asp173 and Gly174 (boxed) are shown in color: 8×His in light blue, streptavidin-binding peptide (SBP) in yellow, c-Myc tag in green, and linkers in grey, this was adapted from (Kobayashi *et al*., 2008).

Expression of the protein is controlled by the T7 RNA polymerase and its corresponding promoter sequence; hence the target protein can be induced with IPTG. The 8xHis tag was exploited for protein purification and its resistance to kanamycin allowing screening and selection of successfully transformed colonies. The sequence of the *E. coli* MurJ a 55 kDa membrane proteins is shown in Figure 2-3.

MNLLKSLAAVSSMTMFSRVLGFARDAIVARIFGAGMAT
DAFFVAFKLPNLLRRIFAEGAFSQAFVPILAEYKSKQGEDA
TRVFVSYVSGLLTLALAVVTVAGMLAAPWVIMVTAPGFA
DTADKFALTSQLLKITFPYILLISLASLVGAILNTWNRFSIPA
FAPTLLNISMIGFALFAAPYFNPPVLALAWAVTVGGVLQL
VYQLPHLKKIGMLVLPRINFHDAGAMRVVKQMGPAILG
VSVSQISLIINTIFASFLASGSVSWMYYADRLMEFPSGVLG
VALGTILLPSLSKSFASGNHDEYNRLMDWGLRLCFLLALPS
AVALGILSGPLTVSLFQYGKFTAFDALMTQRALIAYSVGLI
GLIVVKVLAPGFYSRQDIKTPVKIAIVTLILTQLMNLAFIGPL
KHAGLSLSIGLAACLNASLLYWQLRKQKIFTPQPGWMAF
LLRLVVAVLVMSGVLLGMLHIMPEWSLGTMPWRLLRLM
AVVLAGIAAYFAALAVLGFKVKEFARRTV

Figure 2-3. The amino acid sequence of *E. coli* MurJ, a 55 kDa membrane protein.

2.2.1.3 FtsW vector

The FtsW expression vector that was a generous gift from the laboratory of Dr D. Roper or Warwick University. An adapted pET-28(a+) derived GFP-8xHis fusion Vector (Novagen) was manipulated to express *E. coli* FtsW (Drew *et al.*, 2006). The GFP in the vector was removed and replaced with mfGFP which contains a 8xHis tag (Kobayashi *et al.*, 2008). Expression of the vector is controlled by the T7 RNA polymerase; hence the target protein can be induced with IPTG. The 8xHis tag was exploited for protein purification and its resistance to kanamycin allowing screening and selection of successfully transformed colonies. The sequence of the *E. coli* FtsW a 46 kDa membrane protein is shown in Figure 2-4.

MRLSLPRLKMPRLPGFSILVWISTALKGWVMGSREKDTD
SLIMYDRTLLWLTFGLAAIGFIMVTSASMPIGQRLTNDPF
FFAKRDGVYLILAFILAIITLRLPMEFWQRYSATMLLGSIILL
MIVLVVGSSVKGASRWIDLGLLRIQPAELTKLSLFCYIANYL
VRKGDEVRNNLRGFLKPMGVILVLAVLLLAQPDLGTVVVL
FVTTLAMLFLAGAKLWQFIAIIGMGISAVVLLILAEPYRIRR
VTAFWNPWEDPFGSGYQLTQSLMAFGRGELWGQGLG
NSVQKLEYLPEAHTDFIFAIIGEELGYVGVVLALLMVFFVA
FRAMSIGRKALEIDHRFSGFLACSIGIWFSFQALVNVGAA
AGMLPTKGLTLPLISYGGSSLLIMSTAIMMLLRIDYETRLEK
AQAFVRGSR

Figure 2-4. The amino acid sequence of *E. coli* FtsW, a 46 kDa membrane protein.

2.2.1.4 Transformation of BL21 (DE3) One Shot *E. coli* cells

BL21 (DE3) *E. coli* cells (Invitrogen) were used to increase expression levels with the bacteriophage T7 promoter, which controls the expression of the protein. Recombinant DNA of the desired membrane protein (2 µL) at an approximate concentration of 100 ng/mL was added to a 50 µL aliquot of BL21 (DE3) One Shot competent *E. coli* cells. The inoculated *E. coli* cells were then incubated on ice for 0.5 h. The cells were then subjected to heat-shock at 42°C for 45 s in a water bath, before being placed immediately on ice for at 120 s. 1 mL of Luria-Bertani (LB) medium (no antibiotic) was added before a 1 h incubation at 37°C, 200 RPM to recover the plasmid. 100 µL of mixture is plated onto an LB agar plate with the relevant antibiotic (e.g. 100 µg/mL ampicillin, 50 µg/mL kanamycin); and incubated at 37°C overnight.

2.2.1.5 Transformation of BL21 OverExpress C43 *E. coli* cells

BL21 OverExpress C43 (Lucigen) were used as they have previously shown increased success overexpressing membrane proteins. Recombinant DNA of the desired membrane protein (2 µL) at an approximate concentration of 100 ng/mL was added to a 50 µL aliquot of BL21 (DE3) One Shot competent *E. coli* cells. The inoculated *E. coli* cells were then incubated on ice for 0.5 h. The cells were then subjected to heat-shock at 42°C for 45 s in a water bath, before being placed immediately on ice for at least 120 s. 1 mL of sterile LB medium (no antibiotic) was added before a 1 h incubation at 37°C, 200 RPM to recover the plasmid. 100 µL of mixture was plated onto an LB agar plate with the relevant antibiotic (100 µg/mL ampicillin, 50 µg/mL kanamycin); then incubated at 37°C overnight.

2.2.1.6 Small-scale colony screening

Ten transformant colonies were selected from the LB-antibiotic plate and each was used to inoculate 10 mL of LB, containing the corresponding concentration of the relevant antibiotic (100 μg/mL ampicillin, 50 μg/mL kanamycin). The small-scale colony screenings of the protein were then incubated at 37°C, 200 RPM overnight. 0.5 mL was extracted from the 10 flasks and added to a further 10 mL of LB-Amp for 3 h at 37°C, 200 RPM. IPTG was added (final concentration 0.25 mM) and the flasks incubated for a further 3 h at 25°C, 200 RPM. 1 mL from each flask was removed and separate by centrifugation at 12,000 x g for 300 s. The pellets can then be loaded onto an SDS to observe colony expressions levels. Glycerol stocks are made by 700 μL of sample being added to 300 μL sterile glycerol and flash freezing in liquid nitrogen before storage at -70°C.

2.2.1.7 Large-scale flask culture

After selecting a colony from the small-scale screening, 5 mL of the original uninduced culture from the colony was added to 200 mL of LB (supplemented with the relevant antibiotic at the following concentrations; 100 μg/mL ampicillin or 50 μg/mL kanamycin). This mixture was incubated overnight at 37°C, 200 RPM. 25 mL of the overnight culture was added to six aliquots of LB (800 mL in volume, supplemented with the relevant antibiotic at the following concentrations; 100 μg/mL ampicillin or 50 μg/mL kanamycin). The 6 flasks are monitored at OD_{600} until the cultures reach an OD_{600} of 0.6, at which point 0.25 mM IPTG is added to induce FtsA expression. The cultures are induced overnight

at 25°C, 200 RPM. The cultures were all separated by centrifugation at 15,000 x g for 0.5 h before the cells pellets were separated and stored at -70°C.

2.2.1.8 *E. coli* membrane preparation

All work from this point on was carried out at 4°C to prevent any protein degradation. A frozen cell pellet, typically 15 g from c. 5 L of culture, was thawed gently on ice in 100 mL of 50 mM Tris-HCl, 150 mM NaCl, pH 8.0. Protease inhibitor (Invitrogen) was added before the solution is thoroughly mixed and poured into an Avestin-C3 cell disruptor (Avestin). The cells in solution were broken by 8 passes through the refrigerated breaking cell at approximately 25,000 psi. Cell debris and unbroken cells were sedimented by a low-speed centrifugation (10,000 x g in a Beckman JA-25.5 rotor for 0.75 h). The supernatant from this separation was then subjected to a high-speed separation step (100,000 x g in a Beckman Type 70.1 Ti rotor for 1 h). The resultant membrane pellet was suspended in 50 mM Tris-HCl, pH 8 at a concentration of 40 mg/mL; ready for application of SMA copolymer.

2.2.1.9 Preparation of SMA polymer

Styrene maleic anhydride (SMA 2000P – Cray Valley) requires hydrolysis to become the styrene maleic acid (SMA) we add to disrupt cell membranes. A 10 % SMA-2000P polymer was prepared by dissolving in 1 M NaOH with stirring at room temperature overnight. The mixture was then refluxed for 2 h before being refrigerated at 4°C for 48 h. The polymer was then stored at −70°C until required. When necessary, the stock SMA polymer was dialysed overnight into

50 mM Tris-HCl, pH 8 with dialysis membranes (14,000 MWCO, Thermo Fisher Scientific) to remove the NaOH pre application to cellular membranes.

2.2.1.10 Solubilisation with SMA

Solubilisation with SMA copolymer is achieved by adding a 2.5 % w/v concentration of powdered SMA copolymer to the *E. coli* cell membrane pellet that was previously suspended in 50 mM Tris-HCl, pH 8. The mixture was incubated overnight at room temperature with stirring, leading to clarification of the solution over the time course. After the incubation, the mixture was subjected to another high-speed separation step (100,000 x g in a Beckman Type 70.1 Ti rotor for 1 h). The membrane pellet produced in this step was notably smaller and the supernatant will contain the SMALP.

2.2.1.11 Purification of membrane proteins

When membrane proteins are encapsulated within the SMALP, they can be subjected to conventional purification methods. The target membrane proteins were purified initially with affinity chromatography, before gel filtration was applied to achieve higher levels of protein purity.

2.2.1.12 Affinity chromatography of membrane proteins

The target protein (2.5 % SMA, 50 mM Tris-HCl, pH 8) was supplemented with 500 mM NaCl before being added to 2 mL bed volume of Ni-NTA resin (Qiagen). The 2 mL bed volume of resin was prepared by washing 4 mL of Ni-NTA slurry

(1:1 is 20 % ethanol) twice in 50 mL distilled H_2O, before finally being primed by a wash in 50 mL of 50 mM Tris-HCl, 500 mM NaCl, pH 8. The bed resin and target protein within the SMALP were incubated with regular inversions overnight at 4°C. The histidine tag on the construct forms a chelate with Nickel molecules attached to the NTA resin. An increasing imidazole gradient was applied to elute the target protein from the Ni-NTA resin; imidazole at a high enough concentration will have a higher affinity for the Ni-NTA than the histidine tags of the target protein construct, resulting in the protein being eluted from the column. The base purification buffer 50 mM Tris-HCl, 500 mM NaCl, 10 % glycerol, pH 8 was supplemented with the following range of imidazole concentrations: 5 mM, 10mM, 25 mM, 50mM, 100 mM, 200 mM, 300 mM, 400 mM and 500 mM. The stepwise increase in imidazole concentration results in the target protein being eluted in certain fractions, which were identified with either coomassie stained gel or Western blot.

2.2.1.13 Gel filtration of target proteins

In order to gel filter the protein samples, the NaCl, glycerol and imidazole were removed *via* dialysis or by concentration and addition of new buffers. All samples loaded for the procedure were in the following buffer: 50 mM Tris-HCl, 150 mM NaCl, pH 8. The protein samples were loaded into a Superdex 200 10/300 GL column, which was operated by a ÄKTA™ purifier FPLC purification system (GE Healthcare). The fractions were eluted with 50 mM Tris-HCl, 150 mM NaCl, pH 8 and collected every 0.5 mL.

2.2.1.14 SDS-PAGE and Western blotting

Unless noted otherwise, work in this section was performed at room temperature. Whole cell pellets (usually 1 mL culture sedimented) were prepared for SDS-PAGE by suspending in 100 µL distilled H_2O, 35 µL NuPAGE LDS sample buffer (4x) and 7 µL of NuPAGE LDS sample buffer β-mercaptoethanol mix. The solution was mixed by pipetting before being heated at 95°C for 600 s followed by a 30 s centrifugation at 5,000 x g. Samples containing no whole cells (pure protein samples) were prepared as follows: 15 µL of the pure proteins were combined with 5 µL NuPAGE LDS sample buffer (4x) and 1 µL of NuPAGE LDS sample buffer β-mercaptoethanol mix. The solution was mixed by pipetting before being left to equilibrate at room temperature for 600 s. Bioline HyperPAGE prestained protein marker (Range 10 – 178 kDa) was used as a molecular weight marker for the loaded proteins.

SDS-PAGE gels were made as per Table 2-1 (Heidcamp, 1995). Some Precast Tris-Glycine 12 % gels were purchased from Nu-Sep (Wasserburg, Germany). The PAGE was run for approximately 0.75 h in 1x Laemmli electrophoresis running buffer that was made as per the recipe detailed in Table 2-2 (Heidcamp, 1995). The protein was visualised with 20 mL of InstantBlue protein stain (Expedeon). For Western blotting, the proteins and markers on the gel were transferred onto a nitrocellulose membrane (Sigma-Aldrich) in transfer buffer. The transfer was done with an ice block over 1 h at 100 V. The transfer buffer is detailed in Table 2-3.

Table 2-1. Components of 12 % resolving gel and 4 % stacking gel used to create SDS-PAGE gels (Heidcamp, 1995).

12 % resolving gel (volumes)	**Components**	**4 % stacking gel (Volumes)**
5.1 mL	Distilled H_2O	6.4 mL
6 mL	30 % (w/v) Acrylamide 0.8 % Bisacrylamide	1 mL
3.75 mL	Lower Tris: (1.5 M Tris-base, 0.4 % SDS, pH 8.8)	-
-	Upper Tris: (0.5 M Tris-HCl, 0.4 % SDS, pH 6.8)	2.5 mL
150 µL	10 % (w/v) Ammonium persulfate	100 µL
6 µL	TEMED	5 µL

Table 2-2. Recipe for 1X Laemmli running buffer (Heidcamp, 1995).

Components	**Amounts**
Tris-base	3 g
Glycine	14.4 g
SDS	1 g
Dissolve in 1 L distilled H_2O and set pH of the buffer to 8.3	

Table 2-3. Recipe for Transfer buffer (Western blotting) (Heidcamp, 1995).

Components	**Amounts**
Tris-base	3.3 g
Glycine	14.4 g
Methanol	150 mL
Make up to 1 L with distilled H_2O	

The nitrocellulose membrane was transferred into a blocking solution (1xPBS, 0.5 % Tween-20 and 5% non-fat dried milk (Marvel)) for 1 h to reduce non-specific binding. The membrane was then placed for 1 h at 4°C in blocking solution with a 1:5,000 dilution of 6xHis Monoclonal antibody (Clontech). Two 600 s washes with wash buffer (1xPBS, 0.5 % Tween-20) were then completed to remove any excess antibody. The membrane was then placed in a secondary incubation, for 1 h at 4°C in blocking solution, this time with a 1:10,000 dilution of Anti-Mouse IgG HRP-conjugated antibody (Sigma-Aldrich). Two more 600 s washes with wash buffer were completed before EZ-ECL detection solution (Geneflow) was applied as per manufacturer instructions. The ECL solution was visualised with a Gel documentation system (UVItec).

2.2.1.15 Identification of target protein

The band corresponding to the expected mass of the target protein (with additional purification tag) was excised with a scalpel from an SDS-PAGE gel and then identified by the Functional Genomics and Proteomics Laboratory (central services) within the School of Biosciences at the University of Birmingham. The sample was digested with trypsin before identification was obtained using Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry. Mascot search was used to identify amino acid fragments and the relevant probabilities and data were further analysed using MassLynx software.

2.2.1.16 Protein quantification

In order to quantify protein concentration ultraviolet (UV) spectroscopy absorption at 280 nm was used to monitor the concentration of protein. UV spectroscopy is quick and easy to employ and uses only a very small amount of protein; 2 μL is sufficient to get a reading in the nanodrop machine used. However, scattering from the SMALP itself has caused inaccuracies on previous UV spectroscopy work completed by our laboratory. A BCA assay has been shown to provide a much better estimation of protein concentration within SMALP. During this assay, the protein present reduces the copper which interacts with the BCA causing a colour change that can be monitored at 362 nm (Smith *et al.*, 1985). The BCA assay was completed as per the manufacturer's instructions (Thermo Fisher scientific). However, the results can be influenced by presence of certain lipids (Okuno *et al*., 2008). These two methods were combined in unison to best quantify protein concentration.

2.2.1.17 Circular dichroism

Circular dichroism (CD) spectroscopy has been widely applied to investigate the secondary structure makeup of proteins. Depending on which secondary structures are present, varying levels of absorbance occur at set wavelengths for right and left circularly polarised light – allowing conclusions to be drawn on the presence of secondary folds such as α-helices and β-sheets (Serdyuk *et al*., 2007). The far-UV region (190 nm – 260 nm) section of the CD spectra allows the identification of these secondary structures (Nordén *et al*., 2010).

The expected CD spectrum of an α-helix shows a double minimum at 208 nm and 222 nm and positive CD signals between 190 nm and 200 nm (Harding and Chowdhry, 2001). β-sheets are not so easily defined; it is generally accepted that they produce a minimum between 216 nm and 220 nm with a positive around 195 nm (Serdyuk *et al*., 2007). CD spectra of β-turns can again prove difficult to define due to all the different kind of turns in a protein. They are usually associated with a positive peak between 200 nm and 205 nm and a negative between 220 nm and 225 nm. Furthermore a negative maximum signal between 180 and 190 nm is seen with a β-turn fold (Harding and Chowdhry, 2001). Finally a polyproline fold can be identified by observing a protein's CD spectrum – this structure is responsible for a positive peak between 225 nm and 230 nm (Serdyuk *et al.*, 2007).

To prepare the protein sample, the solution containing the protein was dialysed into 50 mM sodium phosphate, 150 mM NaCl, pH 8 using dialysis membrane (14,000 MWCO, Thermo Fisher scientific). Higher NaCl concentrations and any Tris based buffer can result in high absorbance over the CD spectra range. The far-UV (190 nm to 260 nm) CD spectrum was recorded in a JASCO J-715 spectrophotometer using a 1 mm pathlength quartz cuvette.

2.2.1.18 Sedimentation velocity analytical ultracentrifugation

Sedimentation velocity analytical ultracentrifugation (svAUC) provides information on protein characterisation within its native state in the solution. Sedimentation velocity experiments exploit molecules sedimenting at different

rates depending on their mass and shape. The sedimentation rate when processed correctly gives information about solution homogeneity, any potential protein oligomerisation states, protein mass and any potential protein aggregation (Serdyuk *et al*., 2007).

To prepare the protein sample for the application of svAUC, the solution was dialysed into 50 mM sodium phosphate, 150 mM NaCl, pH 8 using dialysis membrane (14,000 MWCO, Thermo Fisher scientific). Higher NaCl concentrations than 150 mM and any Tris based buffer can result in inaccuracies at lower protein concentrations. Velocity experiments were executed in a Beckman Coulter XL-1 analytical ultracentrifuge, in a Ti50 rotor at 40,000 rpm. Protein concentration in the cell was determined by absorption at 280 nm. The gathered data were analysed using distribution plots (c(S) and c(M)) implemented within SEDFIT (Schuck, 2000). Variable parameters for FtsA vbar and buffer density and viscosity were formulated using SEDNTERP. The vbar for the SMALP particle itself (excluding the FtsA protein) is incalculable.

2.2.1.19 Concentrating pure proteins in solution

Different methods and processes require proteins to be at certain concentrations to produce viable data – for instance CD and svAUC. To concentrate dilute protein samples they were added to Pierce protein concentrators (20 kDa MWCO) and subjected to centrifugation as per the manufacturer's protocol until the sample volume has been reduced sufficiently to reach the desired protein concentration.

2.2.2 Periplasmic release

Protein expression and extraction conditions were identical across the two strains used to study periplasmic release with SMA. This allowed comparisons between the two proteins. Methods will be listed generically for the two proteins released from the *E. coli* periplasm.

2.2.2.1 Peri-sfGFP and Peri-mCherry expression vectors

A Peri-mCherry expression vector was provided by Erik L. Snapp of the Einstein College of Medicine, New York (Aronson *et al*., 2011). The plasmid was synthesised by inserting a MBP*1 signal sequence (Tian and Bernstein, 2009) into a Clontech pEcoli-Cterm 6xHN vector with *Nco*I/*Eco*RI restriction sites to create a peri-PEcoli-Cterm 6xHN vector (Aronson *et al*., 2011). The sfGFP and mCherry fragments were inserted into this vector *via Eco*RI/*Pst*I restriction sites to produce the Peri-sfGFP and Peri-mCherry expression vectors, respectively. This expression vector is resistant to ampicillin, allowing screening and selection of successfully transformant colonies. The sequence of the Peri-sfGFP and Peri-mCherry expression vectors with added purification tags are shown in Figure 2-5 (Tian and Bernstein, 2009).

(a)

HNHNHNHNHNHNMSKGEELFTGVVPILVELDGDVNGHKFSV
RGEGEGDATNGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSR
YPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEG
DTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGI
KANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQ
SVLSKDPNEKRDHM VLLEFVTAAGITHGMDELYK

(b)

HNHNHNHNHNHNMVSKGEEDNMAIIKEFMRFKVHMEGSVN
GHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFM
YGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQ
DSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMY
PEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYN
VNIKLDITSHNEDYTIVEQ YERAEGRHSTGGMDELYK

(c)

MKIKTGARILALFALMLMMFSASALA

Figure 2-5. The sequence of the Peri-sfGFP (green) and Peri-mCherry (red) expression vectors with added purification tags (blue) and the MBP*1 signal sequence (Tian and Bernstein, 2009).

2.2.2.2 Transformation of *E. coli* BL21 Codon Plus

BL21 Codon Plus *E. coli* cells (Stratagene) were used to increase expression levels with the bacteriophage T7 promoter, which controls the expressions of the pET plasmid encoding the desired protein. Recombinant DNA of the desired periplasm-targeted protein (2 µL) at an approximate concentration of 100 ng/mL was added to a 50 µL aliquot of BL21 (DE3) codon plus competent *E. coli*

cells. The inoculated *E. coli* cells were then incubated on ice for 0.5 h. The cells were then subjected to heat-shock at 42°C for 45 s in a water bath, before being placed immediately on ice for 120 s. 1 mL of sterile LB medium (no antibiotic) is added before a 1 h incubation at 37°C, 200 RPM is completed to recover the plasmid. 100 µL of the mixture is plated onto an LB agar plate with 100 µg/mL ampicillin. The plate is then incubated at 37°C overnight.

2.2.2.3 Small scale expression of periplasm-targeted proteins

Ten transformant colonies were selected from the LB-ampicillin agar plate and used to inoculate 10 x 5 mL aliquots of LB supplemented with 100 µg/mL ampicillin and 50 µg/mL chloramphenicol (as per Stratagene protocol). These 10 cultures were then incubated overnight at 37°C, 200 RPM. 100 µL of these overnight cultures were used to inoculate 10 more 5 mL LB aliquots (without antibiotic) that were incubated for 2 h at 37°C, 200 RPM. The cultures were then induced with an addition of IPTG to a final concentration of 1 mM. These cell cultures expressing periplasmic-targeted fluorescent proteins can then be assessed for protein expression.

2.2.2.4 Scaled-up expression of periplasm-targeted proteins

Three transformant colonies were selected from the LB-ampicillin plate and each were used to inoculate 100 mL of LB with 100 µg/mL ampicillin and 50 µg/mL chloramphenicol at 37°C, 200 rpm overnight. 25 mL of the overnight culture was added to 500 mL of LB containing no selection antibiotics. The inoculated LB was

grown for 2 h at 37°C and 200 rpm. After 2 h, gene expression was induced by the addition of IPTG to a final concentration of 1 mM. The induced culture was incubated for 3 h at 37°C and 200 rpm before being separated into 50 mL aliquots. The 50 mL aliquots were separated by centrifugation at 8,000 x g, 4°C for 600 s; the supernatants were discarded and the pellet (approximately 0.3 g dry cell mass) was subjected to periplasmic extraction.

2.2.2.5 Periplasmic protein extraction

The existing Tris-HCL EDTA (TE) buffer used as a release agent was 100 mM Tris-HCl, 10 mM EDTA at pH 7.5. The SMA based periplasmic release protocol we investigated used 2.5 % SMA in 50 mM Tris-HCl at pH 8. Two 50 mL *E. coli* culture pellets were gently resuspended in 500 µL of both the TE and SMA extraction buffers. Immediately after resuspension, one culture pellet for each of the extraction buffers was subjected to separation by centrifugation at 8000 x g at 4°C for 300 s. The supernatants from these pellets were collected and tested for any initial periplasmic leakage caused by the resuspension process (0 h samples). The other two culture pellets were incubated for 2 h at 37°C and 200 RPM to release the periplasmic contents. Post-incubation, the supernatant is isolated for future study *via* separation by centrifugation at 8000 x g at 4°C for 300 s (2 h samples). This experimental protocol is illustrated in Figure 2-6.

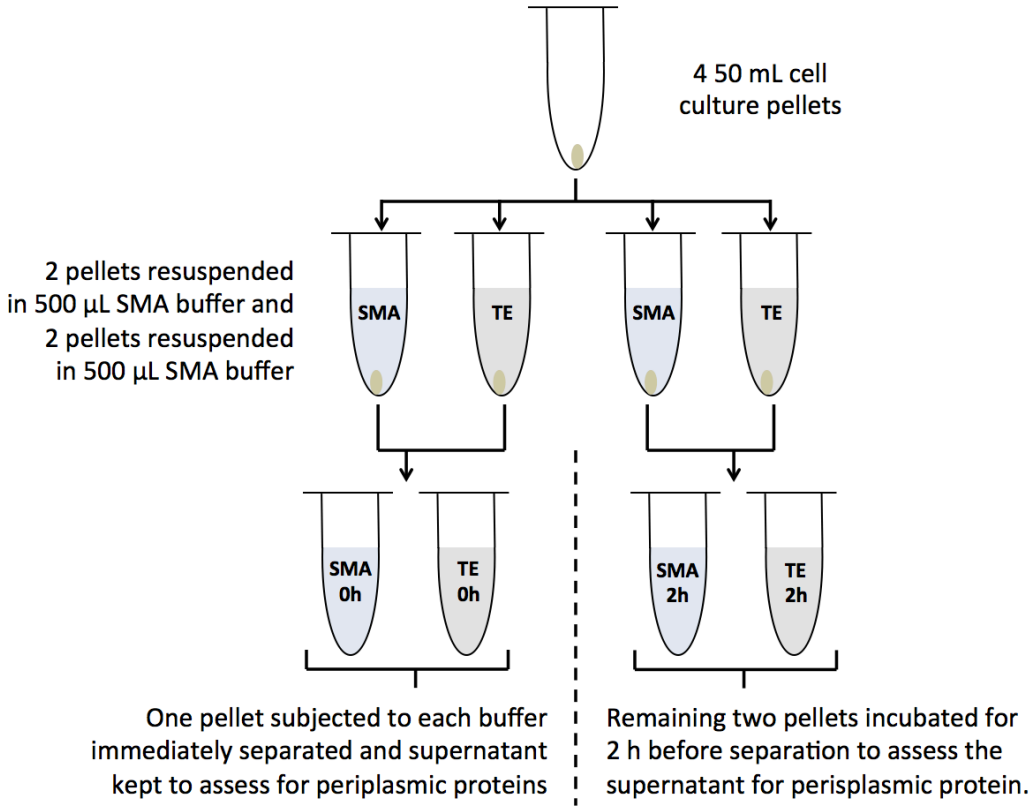


Figure 2-6. A diagram depicting the experimental protocol used to study periplasmic release across the Peri-sfGFP and Peri-mCherry strains with an SMA and TE based extraction buffer.

2.2.2.6 Fluorescence spectroscopy

Fluorescence studies were completed as an indication of the amount of periplasmic proteins released by treatment with the two periplasmic release buffers. These experiments were completed in the PerkinElmer Luminescence Spectrometer LS50B. 50 µL of sample were pipetted into a Starna 0.045 mL, 3 mM quartz fluorimeter Sub-Micro Cell. Each fluorescence spectrum was measured in triplicate then averaged to produce a smoother curve. All experiments were run at room temperature. The same cuvette was used for all experiments after being cleaned thoroughly with 100 % ethanol and pressurised air. The instrument settings used for fluorescence measurements of both sfGFP and mCherry are displayed in Table 2-4.

Table 2-4. The fluorimeter instrument settings used for fluorescence measurements of both sfGFP and mCherry.

	sfGFP	mCherry
Start	500 nm	600 nm
End	600 nm	700 nm
Excitation	490 nm	590 nm
Excitation slit	5 nm	4 nm
Emission slit	5 nm	4 nm
Scan speed	200 nm/min	200 nm/min

2.2.2.7 Bicinchoninic acid assay (BCA assay)

A BCA assay (Thermo Fisher scientific) can be used to indicate protein concentration based on comparisons to known concentrations of bovine serum albumin (BSA) (Smith *et al.*, 1985). We carried out the BCA assay as per the manufacturer's instructions (Thermo Fisher scientific). A BSA standard curve, the samples and the reagent blanks were prepared in duplicate at multiple final concentrations in a 96-well plate (Sterlin flatbottom). 200 µL of BCA reagent were added and the plate was incubated for 30 min at 37 °C before absorption was measured at 620 nm (Anthos, zenith 340rt).

CHAPTER 3:

Expression, solubilisation, purification and characterisation of FtsA

3.1 Introduction

There is a well-documented disparity in the number of structurally characterised soluble proteins versus membrane proteins. A large number of factors combine to complicate study of membrane proteins – their insolubility in water, complex native environment and lower native expression levels make their study a challenging task (Hunte *et al.*, 2003). Despite these obstructions, membrane protein study remains an important field due to their key roles within cells. Membrane proteins are key components in cellular human biology, playing pivotal roles in processes such as cellular signaling and transport (Musnier *et al.*, 2010), respiration and cell division (Egan and Vollmer, 2013).

The major problem impacting membrane study is their insolubility in water; this issue has been circumvented with the use of detergents, amphipols and protein scaffold containing nanodiscs (Jamshad *et al.*, 2011). These methods have only been applied with moderate success due to all their inherent limitations, resulting in the need for a single, universal method to solubilise membrane proteins to facilitate downstream study. Styrene maleic acid (SMA) has demonstrated potential to be this breakthrough method. SMA has the ability to spontaneously insert into the cell membrane excising 9-11 nm discs of lipid and any associated membrane proteins with SMA lipid particles (SMALP) (Knowles *et al.*, 2009).

In this chapter, SMA is applied to a bacterial cell division protein, *E. coli* FtsA which binds to FtsZ acting as a membrane tether for it; FtsA is highly conserved

throughout prokaryotes (Pichoff and Lutkenhaus, 2007). FtsA is a peripheral membrane protein with an actin fold that demonstrates the ability to bind ATP (Fujita *et al*., 2013). It has four protein domains that display an organisation resembling the actin fold (Sánchez *et al*., 1994). FtsA homologues from *T. martima* and *S. aureus* have been purified and crystal structures are available, (van den Ent, 2000, Fujita *et al*., 2013). No structural data of *E. coli* FtsA is available; we study this FtsA homologue in this chapter.

3.2 Results

3.2.1 Optimisation of FtsA expression

An *E. coli* FtsA expression vector was transformed into BL21 (DE3) One Shot *E. coli* cells. A large number of transformant colonies were observed after incubation at 37^0C overnight on an LB agar plate supplemented with 100 µg/mL ampicillin. An expression screen was completed to identify colonies producing the largest amount of protein. Target protein amount was estimated with a Western blot, using antibodies to the 6xHis tags at the N and C termini of the FtsA vector. The Western blots showed a band at 58 kDa. This was slightly larger than expected as untagged, wild type FtsA has a molecular weight of 45 kDa. However, membrane proteins have been observed running heavier than they are on Coomassie gels previously (Knowles *et al*., 2009). An examination of FtsA expression by Western blotting using anti-His-tagged monoclonal antibody is shown in Figure 3-1.

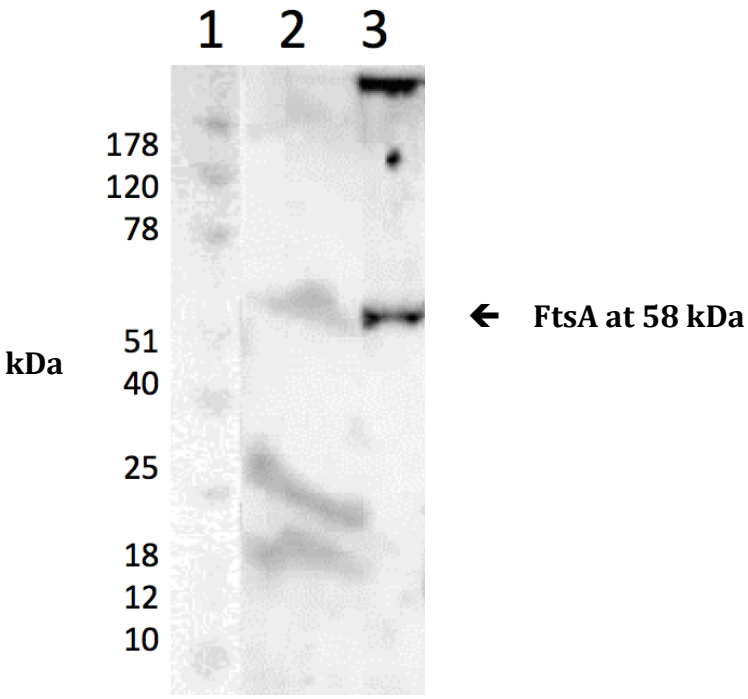


Figure 3-1. Western blotting using an anti-His-tagged antibody to probe for FtsA protein expression:
Lane 1 is 5 µL of HyperPAGE prestained protein marker.
Lane 2 contains an uninduced 1 mL cell culture pellet at and OD_{600} of 0.6.
Lane 3 contains a 1 mL cell pellet after induction at an OD_{600} of 0.6 for 3 h.

After successfully expressing FtsA at a small scale, we scaled up experiments before attempting purification *via* the N and C terminal 6xHis tags used to identify the target protein on the Western blot.

3.2.2 Large-scale expression and purification of FtsA

Typically six flasks containing 4.8 L of *E. coli* culture between them were inoculated and induced to produce 15-20 g of dry cell pellet. The cells were broken and the membrane was separated by a series of centrifugations. These cell membranes were resuspended at a concentration of 40 mg/mL in SMA solution (2.5 % SMA, 50 mM Tris-HCl at pH 8) and incubated at room temperature with stirring for 2 h.

Post incubation, the membrane that was not solublised by the SMALP was removed by another centrifugation step, leaving a supernatant containing the solublised FtsA. Using a Ni^{2+}-NTA resin, we then purified the solublised FtsA. Five milliliter elution volumes with increasing concentrations of imidazole were used to elute the His tagged protein from the resin. A SDS-PAGE gel was completed running samples from the purification and it illustrated the gradual release of a 58 kDa sized band as imidazole concentration increased. The retention of the protein by the resin at the previously observed size means conclusions can be drawn that FtsA has been successfully expressed and purified. An SDS-PAGE gel illustrating the FtsA elution profile from Ni^{2+}-NTA resin is shown in Figure 3-2.

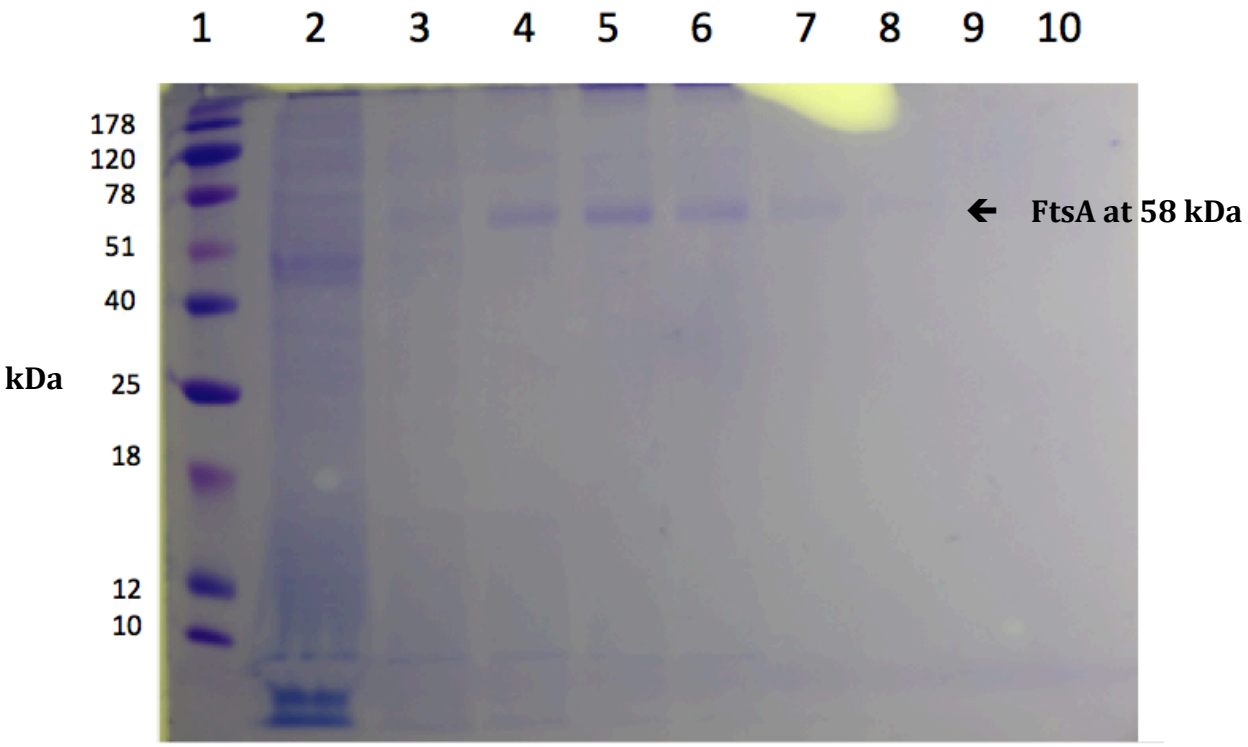


Figure 3-2. An SDS PAGE gel displaying the elution profile of FtsA with increasing imidazole concentration from Ni^{2+}-NTA:
Lane 1 is 5 µL of HyperPAGE prestained protein marker.
Lane 2 is the column run off containing proteins that didn't bind the resin.
Lane 3 contains a 25 mM elution fraction from the Ni^{2+}-NTA.
Lane 4 contains a 50 mM elution fraction from the Ni^{2+}-NTA.
Lane 5 contains a 100 mM elution fraction from the Ni^{2+}-NTA.
Lane 6 contains a 200 mM elution fraction from the Ni^{2+}-NTA.
Lane 7 contains a 300 mM elution fraction from the Ni^{2+}-NTA.
Lane 8 contains a 400 mM elution fraction from the Ni^{2+}-NTA.
Lane 9 contains a 500 mM elution fraction from the Ni^{2+}-NTA.
Lane 10 contains the Ni^{2+}-NTA resin (suspended 1:1 in 20 % ethanol).

Elution fractions containing FtsA (50 mM – 400 mM elution fractions) were collected and concentrated before being loaded on to a Superdex 200 gel filtration column to remove the residual contaminant proteins. The gel filtration profile of FtsA is illustrated in Figure 3-3.

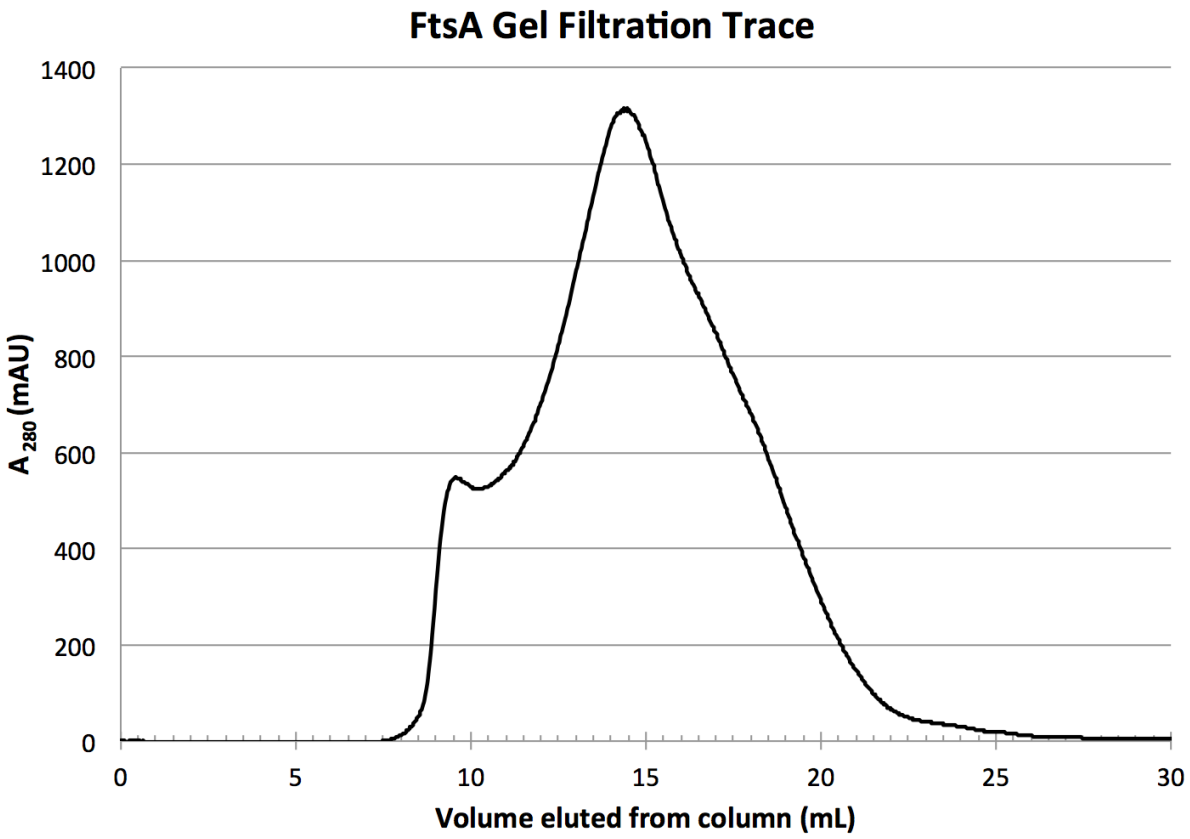


Figure 3-3. A gel filtration profile of FtsA. The Ni^{2+}-NTA elution fractions containing FtsA were combined and concentrated before loading onto the gel filtration column. The progress was monitored by observing absorbance at 280 nm. The experiments were completed on a Superdex 200 10/300 GL column, which was operated by a ÄKTA™ purifier FPLC purification system (GE Healthcare). The fractions were eluted with 50 mM Tris-HCl, 150 mM NaCl, pH 8 and collected every 0.5 mL.

The gel filtration fractions across the peak were examined on a SDS-PAGE gel to identify which of them contained pure FtsA. Those that did were collected, concentrated and examined on the SDS-PAGE gel in Figure 3-4.

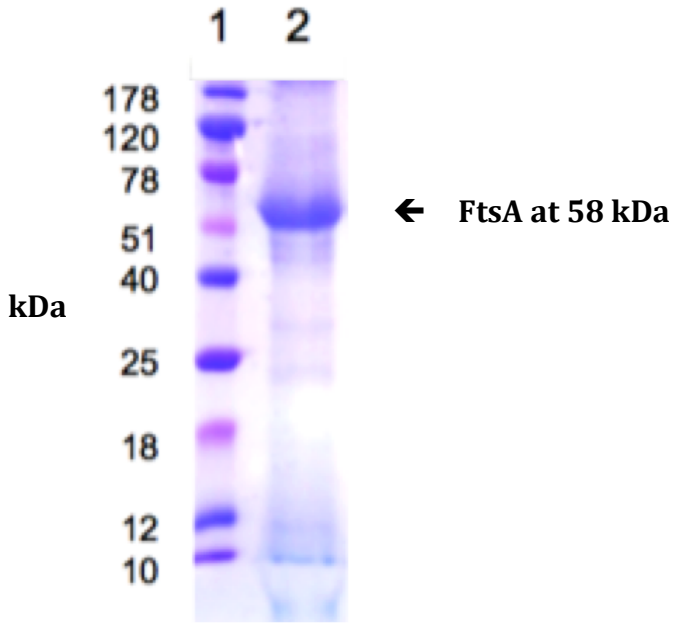


Figure 3-4. An SDS PAGE gel displaying FtsA purified by Ni^{2+}-NTA affinity chromatography and size exclusion chromatography through gel filtration.
Lane 1 contains 5 µL of HyperPAGE prestained protein marker.
Lane 2 is the pure FtsA protein.

All evidence to this point suggests that FtsA has been successfully solubilised in complex with SMA before being purified with affinity and size exclusion chromatography. To ensure the band at 58 kDa is FtsA, we completed FT-ICR mass spectrometry on a gel plug extracted from the gel pictured in Figure 3-4.

3.2.3 Protein identification *via* mass spectrometry and Western blot

The band observed at 58 kDa on the SDS-PAGE gel display in Figure 3-4 appears at a different size to the actual molecular weight of FtsA (45 kDa). In order to be certain the SDS-PAGE gel band was the purified FtsA within the SMALP, the band was excised with a scalpel and subjected to tryptic digestion for a FT-ICR mass spectrometry experiment. Six peptides from the tryptic digest were cross-referenced with the *E. coli* proteome allowing the protein to be identified as FtsA. Furthermore these peptide sequences show a relatively even spread throughout the 420 amino acid membrane protein. The peptides identified through FT-ICR mass spectrometry are listed in Table 3-1.

Table 3-1. FtsA-derived peptides present in the 58 kDa gel plug identified by FT-ICR mass spectrometry.

Peptide Fragments	Peptide Sequence	Peptide Location (amino acid)
1	VAALVGEVLPDGMVNIIGVGSCPSR	19-43
2	HISCQNEIGMVPISEEEVTQEDVENVVHTAK	87-117
3	NPVGLSGVR	145-154
4	ELGVCVVDIGGGTMDIAVYTGGALR	203-228
5	YTELLNLVNEEILQLQEK	301-318
6	HHLAAGIVLTGGAAQIEGLAACAQR	325-350

To further ensure the protein we purified is FtsA within the SMALP, the sample loaded onto Figure 3-4 was also observed on a Western blot using anti-His-tagged monoclonal antibody. The Western blot is displayed in Figure 3-5.

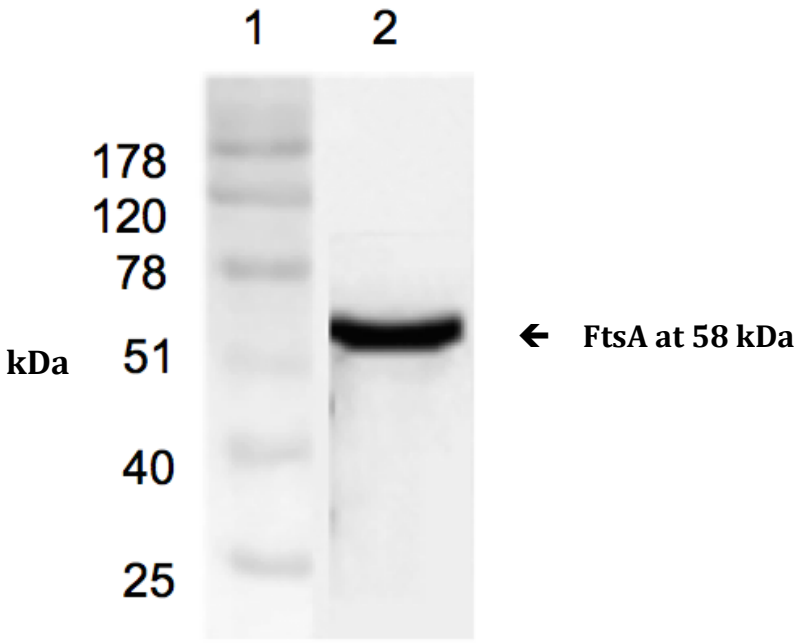


Figure 3-5. Western blotting using anti-His-tagged antibody to probe for the presence of FtsA in the fractions collected after the size exclusion chromatography detailed in Figure 3-3. This Western blot was completed with the same samples visualised *via* the SDS-PAGE gel detailed in Figure 3-4.
Lane 1 contains 5 µL of HyperPAGE prestained protein marker.
Lane 2 is the pure FtsA protein.

The results presented in this chapter prove we have expressed and solubilised the peripheral *E. coli* membrane protein FtsA with SMA. We then purified the SMALP-FtsA with affinity and size exclusion chromatography. Next this thesis will focus on characterising FtsA within the SMALP.

3.2.4 Characterisation of FtsA

3.2.4.1 Circular dichroism of FtsA

Circular dichroism (CD) spectroscopy has been widely applied to investigate the secondary structure makeup of proteins. Depending on which secondary structures are present, varying levels of absorbance occur at set wavelengths for right and left circularly polarised light – allowing conclusions to be drawn on the presence of secondary folds such as α-helices and β-sheets (Serdyuk *et al.*, 2007). In order to measure the CD spectrum of our SMALP-FtsA, it was dialysed into a more CD friendly buffer (50 mM sodium phosphate, 150 mM NaCl, pH 8). The CD data was recorded 16 times and averaged to produce the spectra in Figure 3-6.

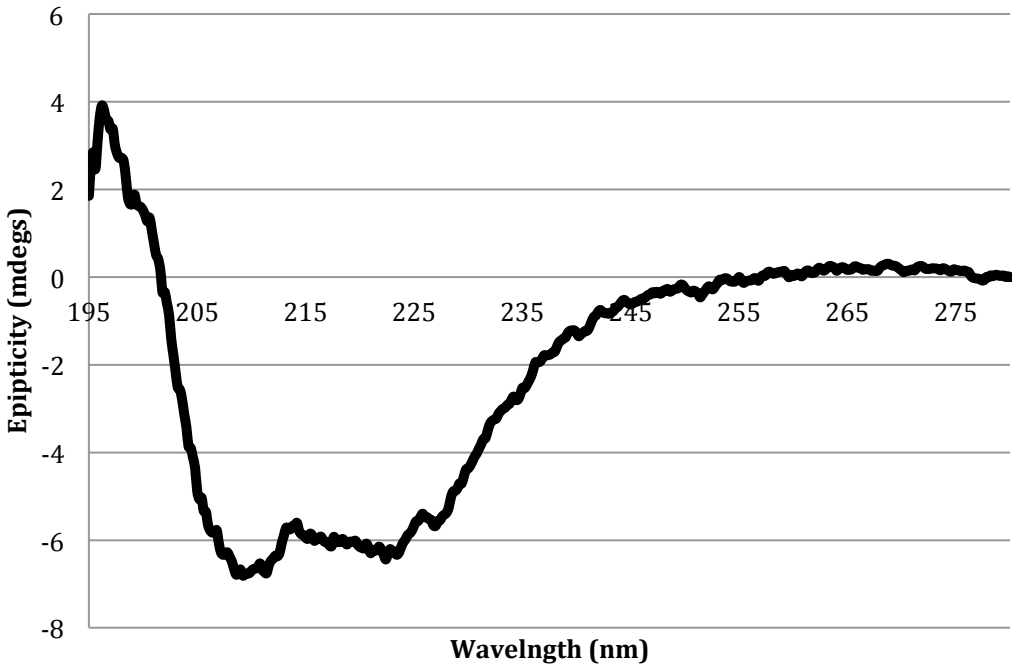


Figure 3-6 CD spectrum of purified SMALP-FtsA. The CD data was collected using the JASCO J-715 spectrophotometer using a 1 mm pathlength quartz cuvette. The concentration of the SMALP-FtsA was 0.05 mg/mL.

The CD spectrum of SMALP-FtsA presents a native folded protein. Strong evidence of α-helix structures exists in the presence of a double minimum at 208 nm and 222 nm and positive CD signals between 190 nm and 200 nm (Harding and Chowdhry, 2001). It is also worth noting that fully folded proteins should present a positive signal between 195 and 200 nm, suggesting that the FtsA has remained folded with the SMALP.

3.2.4.2 svAUC of FtsA

Sedimentation velocity analytical ultracentrifugation (svAUC) provides information on protein characterisation within its native state in the solution. The sedimentation velocity experiments exploit molecules sedimenting at different rates depending on their mass and shape. The sedimentation rate when processed correctly gives information about solution homogeneity, any potential protein oligomerisation states, protein mass and any potential protein aggregation (Serdyuk *et al*., 2007). SMALP-FtsA in 50 mM sodium phosphate, 150 mM NaCl, pH 8 was provided for AUC analysis. The raw data from the sedimentation velocity experiment was analysed using distribution plots (c(S) and c(M)) implemented within SEDFIT (Schuck, 2000). Variable parameters for FtsA (vbar and buffer density and viscosity) were formulated using SEDNTERP. The vbar for the SMALP is incalculable so is not included in the calculation. This c(M) distribution plot is illustrated in Figure 3-7.

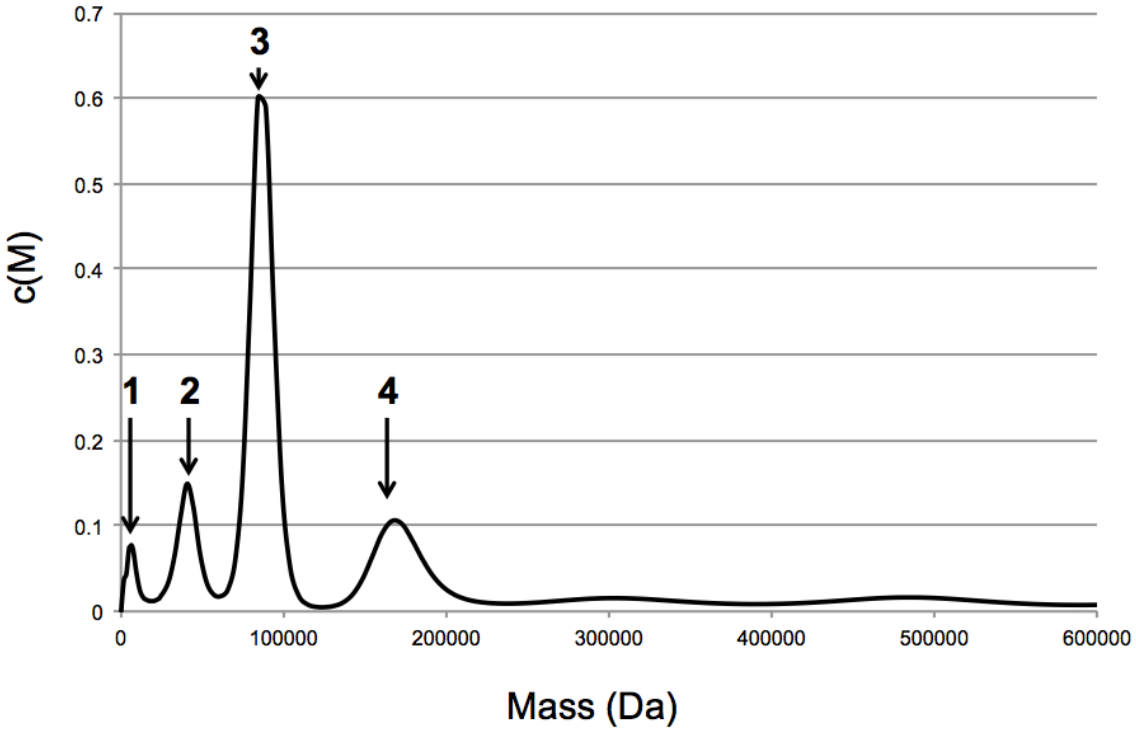


Figure 3-7. A SEDFIT c(M) deconvolution of sedimentation velocity analytical ultracentrifugation data collected on FtsA within a SMALP. Velocity experiments were executed in a Beckman Coulter XL-1 analytical ultracentrifuge, in a Ti50 rotor at 40,000 rpm. Protein concentration in the cell was observed by absorption at 280 nm. The gathered data was analysed using distribution plots (c(S) and c(M)) implemented within SEDFIT (Schuck, 2000). Variable parameters for FtsA (vbar and buffer density and viscosity) were formulated using SEDNTERP.

When analysing the c(M) distribution plot derived from the svAUC data, 4 peaks are visible. The first two peaks at 5 and 38 kDa are most consistent with other membrane proteins captured within the SMALP. It is most likely these correspond with SMA discs containing no protein (38 kDa) and free polymer (5 kDa) (Personal communication, Rosemary Parslow).

The strongest peak (3) at 83 kDa represents SMALP-FtsA. The mass of wild type FtsA is 45 kDa. When added to the mass of the observed peak of the SMALP disc (38 kDa) results in the theoretical protein mass at 83 kDa shown in the c(M) distribution plot. Peak 4 is at 165 kDa, and could represent dimerisation of the

SMALP-FtsA or potentially two FtsA protein molecules extracted by one SMALP. It has been discovered since the completion of this report that SMA lipid particles can dimerise which could also explain these observed results.

3.3 Discussion

The FtsA construct was synthesised with 6xHis tags at both terminals of the protein – facilitating protein purification. Generally it took four days of growing the *E. coli* cultures from transformation through to harvesting cells expressing the FtsA construct. It then took five days to solubilise the membrane protein with SMA and purify it *via* affinity and size exclusion chromatography. When the purified SMALP-FtsA was examined on an SDS-PAGE gel, it separated as if it had a mass of 58 kDa. Wild type FtsA is only 45 kDa, therefore FT-ICR mass spectrometry and Western blots probing for the His-tagged proteins were used to gather further evidence that the correct protein had been purified.

The disparity in the wild type mass of FtsA versus the observed running weight of the SMALP-FtsA on the SDS-PAGE gel can be attributed to membrane proteins running in a different manner to the soluble markers they are being compared to. Membrane proteins within the SMALP are often observed to be 25-30 kDa heavier than expected, supporting this theory (Personal communication, Mohammed Jamshad, Tim Dafforn). After examining the results of the FT-ICR mass spectrometry, six unique FtsA peptides were identified. These peptides are spread throughout the amino acid sequence of FtsA, with 31 % of the protein sequence being account for through these unique peptide sequences alone (131 of 420 amino acids are accounted for).

FtsA within the SMALP was biophysically characterised with CD and svAUC. CD data illustrated that the SMALP-FtsA protein contained α-helix regions and a section of protein that remained unfolded. Interpretation of the svAUC data illustrated that SMALP-FtsA are mostly monomeric. However, there is a less intense peak at double the mass (165 kDa) present on the c(M) distribution. This could indicate that some dimerisation of SMALP-FtsA particles may occur or that multiple FtsA copies can fit inside the SMALP and co-purify.

This solubilisation, purification and characterisation of *E. coli* FtsA within the SMALP is the first time SMA has been successfully applied to a peripheral membrane protein. No structural evidence exists for *E. coli* FtsA, however, the protein's structural homologues in *T. martima* and *S. aureus* provide some evidence to help us interpret the results from this experiment (van den Ent, 2000, Fujita *et al*., 2014). The domain 1C in FtsA has been identified as the membrane tether within the protein. We can then assume this section of the protein is completely folded due to the lateral pressures of the SMALP and the retention of annular lipids (van den Ent, 2000, Knowles *et al*., 2009). The N and C terminals of FtsA both fall within the same domain (1A) in *T. martima* FtsA (van den Ent, 2000). Within this construct, 6xHis-tags were added at both protein termini; the close tertiary proximity of these tags could be destabilising this protein domain and would account for the unfolded proteins region that the CD spectra identified.

Literature suggests that FtsA dimerises *in vivo* to function and bind the membrane, meaning the dimerisation identified in the svAUC data is a subject that will require further study. This phenomenon may be an artefact of the

overexpression and application of SMALP rather than the natural roles of FtsA within *E. coli*. Given the random process of SMALP formation in the membrane, the chances of two copies of a protein (being over expressed and therefore at a much higher concentration than normal) are high, as long as the two proteins combined are still below the overall size limitations of the SMALP particle. The recent discovery that SMA lipid particles can dimerise could also provide an explanation for the apparent multimeric nature of SMALP-FtsA

With the ambiguity regarding potential dimerisation and the need to prove whether SMALP-FtsA can to bind to FtsZ, there are many opportunities for additional work in this field that would contribute to our understanding of both *E. coli* FtsA and the process of applying SMALP to membrane proteins. This potential further work is detailed in section 7.2.1.

CHAPTER 4:

Expression, solubilisation, purification and characterisation of MurJ

4.1 Introduction

In chapter 3 we demonstrated that SMALP could be used to solubilise *E. coli* FtsA, allowing its purification and characterisation by traditional methods (affinity and size exclusion chromatography). FtsA is a peripheral membrane protein with a well documented role in cell division as a key initiator in the creation of the bacterial divisome (Sánchez *et al*., 1994). There are many other membrane proteins that have been identified in the bacterial divisome, however, little structural information is available on them due to the difficulties associated with studying membrane proteins. MurJ is an integral membrane protein that belongs to the multidrug/oligo-saccharidyl-lipid/polysaccharide (MOP) exporter family, which are secondary active antiporters that utilise the electrochemical potential created by Na^+ or H^+ gradients (Hvorup *et al*., 2003, Kuroda and Tsuchiya, 2009, Islam and Lam, 2013, Sham *et al.*, 2014). MurJ is a member of the bacterial divisome and is thought to be involved in the recycling and transport of Lipid II (a PG building block), however, limited structural information is available to test this theory (Personal communication, Dr D. Roper Warwick University).

In this chapter we express a MurJ-mfGFP coded on a plasmid transformed into OverExpress™ C43 (DE3) cells. We then solubilise the hybrid membrane protein construct with SMA and purify with traditional methods. Characterisation of the MurJ-mfGFP is then completed with CD and svAUC experiments.

4.2 Results

4.2.1 Expression screening of MurJ-mfGFP

The MurJ-mfGFP expression vector was a generous gift from the laboratory of Dr D. Roper of Warwick University. A large number of transformant colonies were observed after incubation at 37^{o}C overnight on an LB agar plate supplemented with 50 μg/mL kanamycin. An expression screen was completed to identify colonies producing the largest amount of protein. Target protein amount was estimated with a Western blot, using antibodies to the 8xHis tags at the C terminal of the MurJ-mfGFP sequence. The Western blots showed a band at 89 kDa. The mass of *E. coli* MurJ is 55 kDa and the mass of the mfGFP is 34 kDa, so the MurJ-mfGFP fusion protein is the band illuminated on the Western blot. An examination of MurJ-mfGFP expression by Western blotting using anti-His-tagged monoclonal antibody is shown in Figure 4-1.

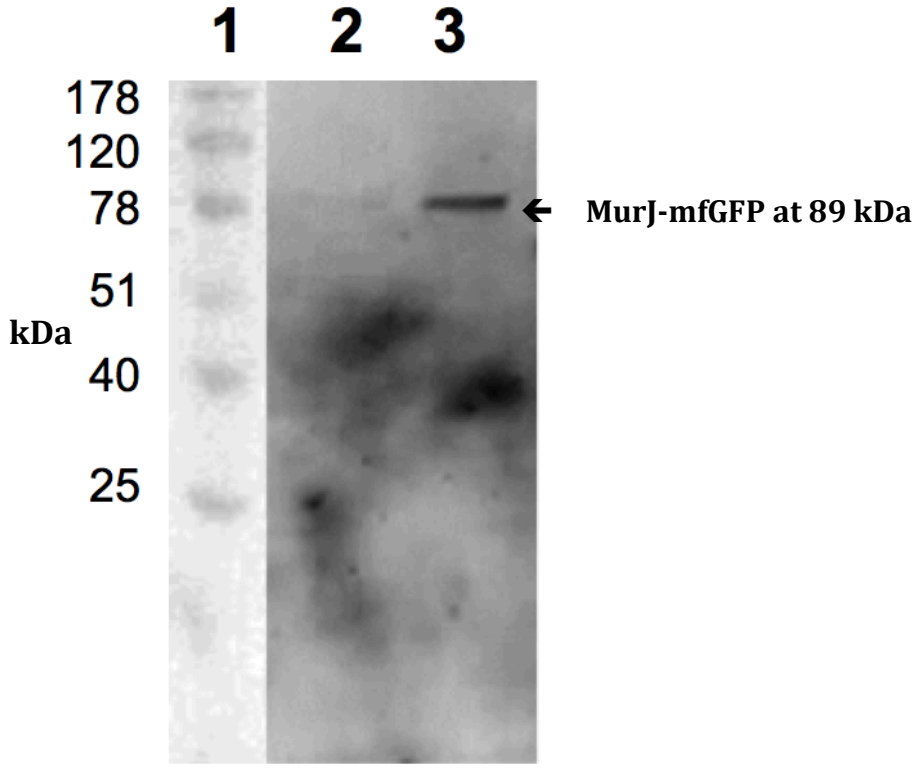


Figure 4-1. Western blotting using anti-His-tagged antibody to probe for MurJ-mfGFP fusion protein expression.
Lane 1 contains 5 µL of HyperPAGE prestained protein marker.
Lane 2 is an uninduced 1 mL cell culture pellet at and OD_{600} of 0.6.
Lane 3 is a 1 mL cell pellet after induction at an OD_{600} of 0.6 for 3 h.

After successfully expressing the MurJ-mfGFP at a small scale, we scaled up the experiments before attempting purification *via* the same 8xHis tag used to identify the protein on the Western blot.

4.2.2 Large-scale expression and purification of MurJ-mfGFP

Typically six flasks containing 4.8 L of *E. coli* culture between them were inoculated and induced to produce 10-15 g of dry cell pellet. The cells were broken and the membrane was separated by a series of centrifugations. These cell membranes were resuspended at a concentration of 40 mg/mL in SMA solution (2.5 % SMA, 50 mM Tris-HCl at pH 8) and incubated at room temperature with stirring, for 2 h.

Post incubation, the membrane that was not solublised by the SMALP was removed by another centrifugation step and the supernatant contained the solublised MurJ-mfGFP fusion protein. Using a Ni^{2+}-NTA resin, we then purified the solublised MurJ-mfGFP. A unique column elution protocol was used to purify MurJ-mfGFP, which displayed an unusually low affinity for the Ni^{2+}-NTA resin and consequently would start to elute from the column at any imidazole concentration higher than 20 mM. Two 15 mL column washes were completed with 50 mM Tris-HCl, 500 mM NaCl, pH 8. The target protein was then released in a 5 mL elution fraction (50 mM Tris-HCl, 500 mM NaCl, 200 mM imidazole, pH 8). A final elution from the column was then completed with 50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole at pH 8. A SDS-PAGE gel was completed running samples from the purification and it illustrated the batch release of an 89 kDa sized band in the desired elution fraction. Other proteins were also present in the sample but were removable with gel filtration. An SDS-PAGE gel illustrating the MurJ-mfGFP elution from Ni^{2+}-NTA resin is shown in Figure 4-2.

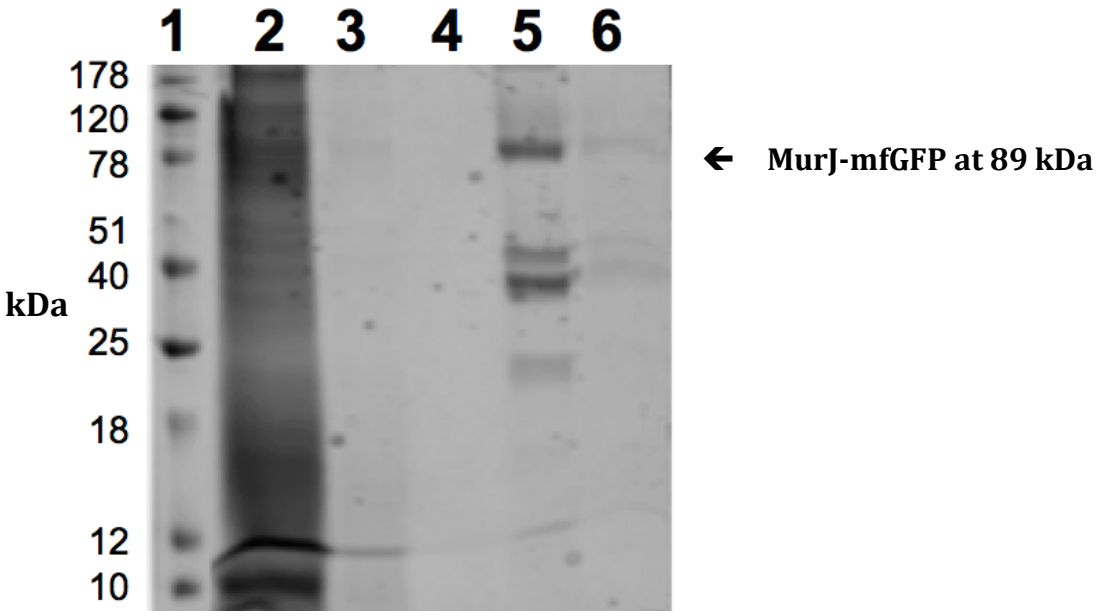


Figure 4-2. A SDS PAGE gel displaying the elution of MurJ-mfGFP from Ni^{2+}-NTA.
Lane 1 contains 5 µL of HyperPAGE prestained protein marker.
Lane 2 is the column run off containing proteins that didn't bind the resin.
Lane 3 contains a 15 mL wash (no imidazole) from the Ni^{2+}-NTA.
Lane 4 contains a second 15 mL wash (no imidazole) from the Ni^{2+}-NTA.
Lane 5 contains a 200 mM elution fraction from the Ni^{2+}-NTA.
Lane 6 contains a 500 mM elution fraction from the Ni^{2+}-NTA.

The 200 mM imidazole fraction that was eluted from the Ni^{2+}-NTA was concentrated before being loaded on to a Superdex 200 gel filtration column to remove the residual contaminant proteins. The gel filtration profile of MurJ-mfGFP produced an uncharacteristically broad peak across 8 elution fractions. To ensure the purity of the protein, all gel filtration fractions across the peaks were examined on a SDS PAGE gel. The gel filtration profile of MurJ-mfGFP and a SDS PAGE gel of the corresponding fractions are illustrated in Figure 4-3.

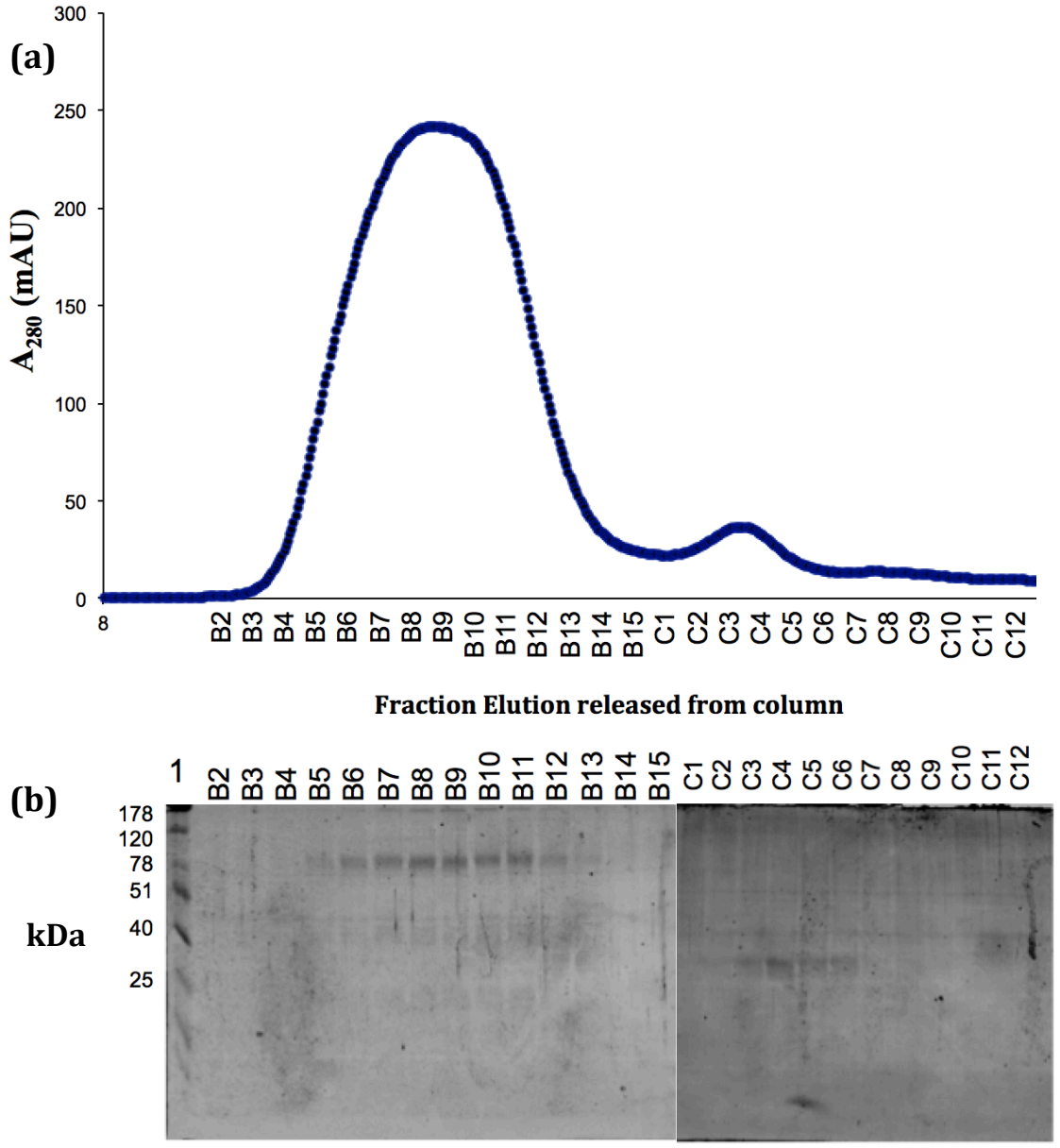


Figure 4-3 (a) A gel filtration profile of MurJ-mfGFP. The Ni^{2+}-NTA elution fractions containing FtsA were combined and concentrated before loading onto the gel filtration column. The progress was monitored by observing absorbance at 280 nm. The experiments were completed on a Superdex 200 10/300 GL column, which was operated by a ÄKTA™ purifier FPLC purification system (GE Healthcare). The fractions were eluted with 50 mM Tris-HCl, 150 mM NaCl, pH 8 and collected every 0.5 mL. The labeled x-axis of the profile displays the alpha numeric coding of subsequent 0.5 mL released fractions.

Figure 4-3 (b) A SDS PAGE gel displaying the 0.5 mL release fractions from the gel filtration of a Ni^{2+}-NTA purified MurJ-mfGFP. Lane 1 contains 5 µL of HyperPAGE prestained protein marker, to allow the size of proteins to be identified.

This gel filtration profile and the SDS PAGE gel examining the elutions from the column confirm that we have purified MurJ-mfGFP (89 kDa band) within the SMALP. Elution fractions B5-B12 were collected and concentrated ready for characterisation of the membrane protein. An 89 kDa band was extracted from the SDS-PAGE gel pictured in Figure 4-3 (b) to allow examination of the pure protein with FT-ICR mass spectrometry.

4.2.3 Protein identification *via* mass spectrometry

The band observed at 89 kDa on the SDS-PAGE gel display in Figure 4-3 (b) was excised with a scalpel and subjected to tryptic digestion for a FT-ICR mass spectrometry experiment. Peptides from the tryptic digest were cross-referenced with the *E. coli* proteome and the sequence of mfGFP, allowing the protein to be identified. Furthermore these peptide sequences show a relatively even spread throughout the 420 amino acid membrane protein. The peptides identified in the 89 kDa band through FT-ICR mass spectrometry for MurJ and mfGFP are listed in Table 4-1 and Table 4-2, respectively.

Table 4-1. MurJ-derived peptides present in the 89 kDa gel plug identified by FT-ICR mass spectrometry.

Peptide Fragments	Peptide Sequence	Peptide Location (amino acid)
1	MNLLKSLAAVSSMTMFSR	1-18
2	SLAAVSSMTMFSR	6-18
3	IFAEGAFSQAFVPILAEYKSK	54-74
4	FALTSQLLK	124-132
5	IGMLVLPRINFHDAGAMR	211-228
6	INFHDAGAMRVVK	219-231
7	SFASGNHDEYNR	294-305
8	FTAFDALMTQR	342-352
9	VLAPGFYSR	369-377
10	VLGFARDAIVAR	496-507

Table 4-2. mfGFP-derived peptides present in the 80 kDa gel plug identified by FT-ICR mass spectrometry.

Peptide Fragments	Peptide Sequence	Peptide Location (amino acid)
1	YPDHMKRHDFFK	77-88
2	RHDFFKSAMPEGYVQER	83-99
3	HDFFKSAMPEGYVQER	84-99
4	SAMPEGYVQER	89-99
5	TRAEVKFEGDTLVNR	111-125
6	AEVKFEGDTLVNRIELK	113-129
7	FEGDTLVNRIELK	117-129
8	IELKGIDFKEDGNILGHK	126-143
9	GIDFKEDGNILGHK	130-143

The peptide fragments derived from the 89 kDa band produce a high level of coverage of both the MurJ and mfGFP sections of the fusion protein. With this evidence we have proved that we expressed, solubilised and purified the MurJ-mfGFP within the SMALP. This chapter will now focus on characterisation of the SMALP-MurJ-mfGFP with CD and svAUC.

4.2.4 Characterisation of MurJ-mfGFP

4.2.4.1 Circular dichroism of MurJ-mfGFP

Circular dichroism (CD) spectroscopy has been widely applied to investigate the secondary structure makeup of proteins. Depending on which secondary structures are present, varying levels of absorbance occur at set wavelengths for right and left circularly polarised light – allowing conclusions to be drawn on the presence of secondary folds such as α-helices and β-sheets (Serdyuk *et al.*, 2007). In order to measure the CD spectrum of our SMALP-MurJ-mfGFP it was dialysed into a CD specific buffer (50 mM sodium phosphate, 150 mM NaCl, pH 8). The CD data was recorded 16 times and averaged to produce the spectra in Figure 4-4.

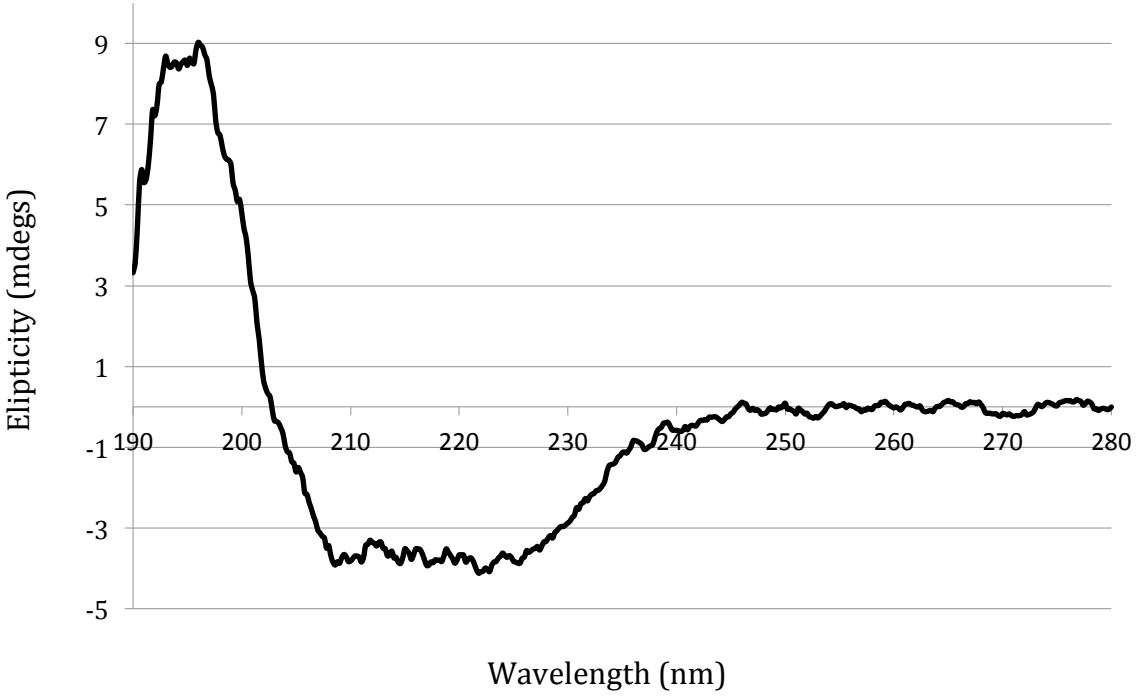


Figure 4-4. CD spectrum of purified SMALP-MurJ-mfGFP. The CD data was collected using the JASCO J-715 spectrophotometer using a 1 mm pathlength quartz cuvette. The concentration of the SMALP-MurJ-mfGFP was 0.05 mg/mL.

The CD spectrum of SMALP-MurJ-mfGFP presents a native folded protein. Strong evidence of α-helix structures exists in the presence of a double minimum at 208 nm and 222 nm and positive CD signals between 190 nm and 200 nm (Harding and Chowdhry, 2001). β-sheets elements also exist as evidence from the minimum between 216 nm and 220 nm and a positive around 195 nm (Serdyuk *et al*., 2007). It is also worth noting that fully folded proteins should present a positive signal between 195 and 200 nm, suggesting that the MurJ-mfGFP has remained folded with the SMALP.

4.2.4.2 svAUC of MurJ-mfGFP

SMALP-MurJ-mfGFP in 50 mM sodium phosphate, 150 mM NaCl, pH 8 was provided for AUC analysis. The raw data from the sedimentation velocity experiment was analysed using distribution plots (c(S) and c(M)) implemented within SEDFIT (Schuck, 2000). Variable parameters for the MurJ-mfGFP (vbar and buffer density and viscosity) were formulated using SEDNTERP. This c(M) distribution plot is illustrated in Figure 4-5.

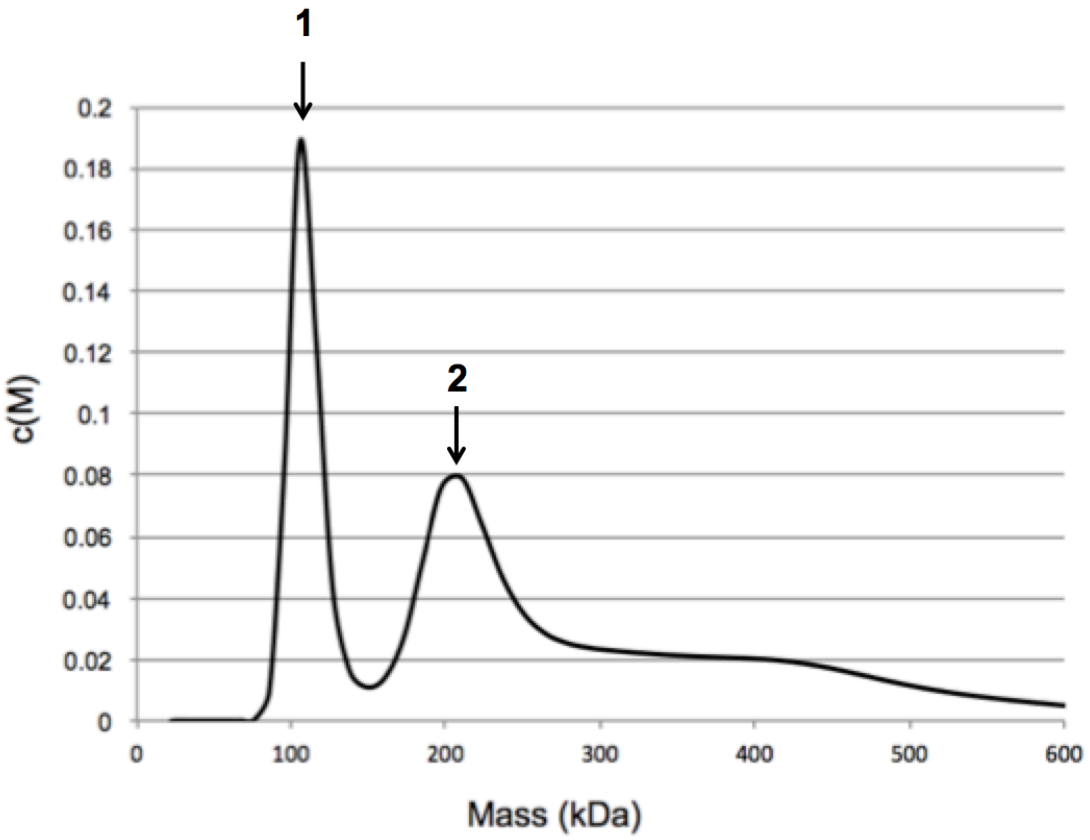


Figure 4-5. A SEDFIT c(M) deconvolution of sedimentation velocity analytical ultracentrifugation data collected on MurJ-mfGFP within a SMALP. Velocity experiments were executed in a Beckman Coulter XL-1 analytical ultracentrifuge, in a Ti50 rotor at 40,000 rpm. Protein concentration in the cell was observed by absorption at 280 nm. The gathered data was analysed using distribution plots (c(S) and c(M)) implemented within SEDFIT (Schuck, 2000). Variable parameters for MurJ-mfGFP (vbar and buffer density and viscosity) were formulated using SEDNTERP.

When analysing the c(M) distribution plot derived from the svAUC data, 2 peaks are visible. The strongest peak (1) at 102 kDa represents SMALP-MurJ-mfGFP. Peak 2 is at 208 kDa, and could represent dimerisation of the SMALP-MurJ-mfGFP or potentially two MurJ-mfGFP protein molecules extracted by one SMALP.

4.3 Discussion

The MurJ-mfGFP fusion construct was synthesised with an 8xHis tag in order to facilitate protein purification. Generally it took four days of growing the *E. coli* cultures from transformation through to harvesting cells expressing the MurJ-mfGFP construct. It then took five days to solubilise the membrane protein with SMA and purify it with affinity and size exclusion chromatography. When examined on a SDS-PAGE gel the purified SMALP-MurJ-mfGFP appeared 89 kDa in size. FT-ICR mass spectrometry was used to ensure the 89 kDa band contained the MurJ-mfGFP.

After examining the results of the FT-ICR mass spectrometry, ten unique MurJ peptides and nine peptides that are unique to mfGFP were identified. These peptides are spread throughout the amino acid sequence of MurJ-mfGFP, with 27 % of the protein sequence being account for through these unique peptide sequences alone (224 of 820 amino acids are accounted for).

MurJ-mfGFP within the SMALP was biophysically characterised with CD and svAUC. CD data illustrated that the SMALP-MurJ-mfGFP protein contained α-helix, β-sheets and a section of protein that remain unfolded. Interpretation of the svAUC data illustrated that MurJ-mfGFP are mostly monomeric. However, there is a less intense peak at double the mass (208 kDa) present on the c(M) distribution. This could indicate that some dimerisation of SMALP-MurJ-mfGFP particles may occur or that multiple MurJ-mfGFP copies can fit inside the SMALP and co-purify.

This solubilisation, purification and characterisation of *E. coli* MurJ-mfGFP within the SMALP is the first time SMA has been successfully applied to a membrane protein fused to a fluorescent protein. No evidence has been found to suggest that MurJ dimerises *in vivo* to function, meaning the potential dimerisation identified in the svAUC data is an unexpected discovery. As discussed in chapter 3, further study is required to investigate potential dimerisations or co-extractions within the SMALP's.

With the possible dimerisation to explore and the many potential applications of the mfGFP protein fused to MurJ, there are many opportunities for additional work in this field that would contribute to our understanding of both *E. coli* MurJ and the process of applying SMALP to membrane proteins. This potential further work is detailed in section 7.2.1.

CHAPTER 5:

Expression, solubilisation and purification of FtsW

5.1 Introduction

In chapter 3 we demonstrated that SMALP could be used to solubilise *E. coli* FtsA, allowing its purification and characterisation by traditional methods (affinity and size exclusion chromatography). In chapter 4 we demonstrated that SMALP could be used to solubilise *E. coli* MurJ (fused to mfGFP), allowing its purification and characterisation by traditional methods (affinity and size exclusion chromatography). There are many other membrane proteins that have been identified in the bacterial divisome which require further study to completely understand their roles within the process. FtsW is an integral membrane that spans the cytoplasmic membrane which accommodates the transport of Lipid II (a peptidoglycan building block) through the bacterial cytoplasmic membrane (Mohammadi *et al*., 2014). FtsW is highly conserved amongst all bacteria that synthesise peptidoglycan-containing cell walls and has no counterpart in the human genome (Mercer and Weiss, 2002).

In this chapter, we express a FtsW-mfGFP coded on a plasmid transformed into OverExpress™ C43 (DE3) cells. We then solubilise the hybrid membrane protein construct with SMA and purify with conventional methods. The FtsW-mfGFP is then investigated with FT-ICR mass spectrometry.

5.2 Results

5.2.1 Expression screening of FtsW-mfGFP

The FtsW-mfGFP expression vector was a generous gift from the laboratory of Dr D. Roper of Warwick University. A large number of transformant colonies were

observed after incubation at 37°C overnight on an LB agar plate supplemented with 50 µg/mL kanamycin. An expression screen was completed to identify colonies producing the largest amount of protein. Target protein amount was estimated with a Western blot, using antibodies to the 8xHis tags at the C terminal of the FtsW-mfGFP sequence. The Western blots showed a band at 80 kDa. The mass of *E. coli* FtsW is 46 kDa and the mass of the mfGFP is 34 kDa, so the FtsW-mfGFP fusion protein is the band illuminated on the Western blot. An examination of FtsW-mfGFP expression by Western blotting using anti-His-tagged monoclonal antibody is shown in Figure 5-1.

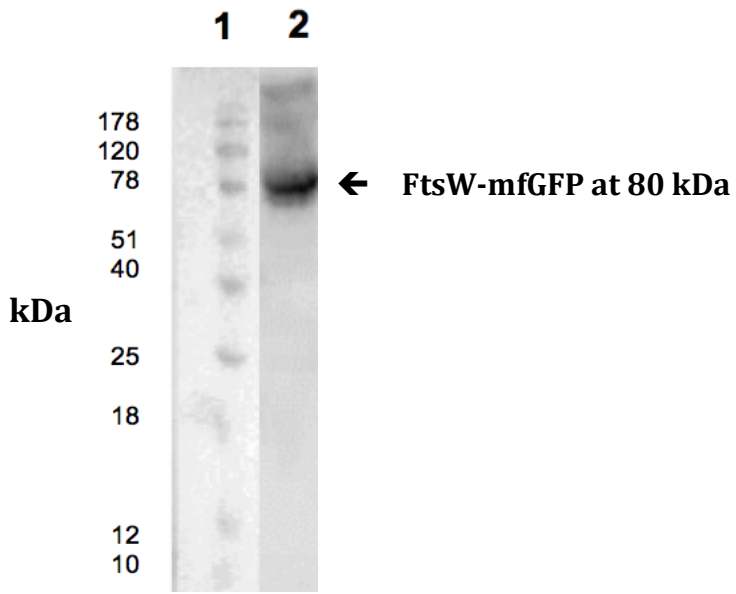


Figure 5-1. Western blotting using anti-His-tagged antibody to probe for FtsW-mfGFP protein expression.
Lane 1 contains 5 µL of HyperPAGE prestained protein marker.
Lane 2 is a 1 mL cell pellet after induction at an OD_{600} of 0.6 for 3 h.

After successfully expressing the FtsW-mfGFP at a small scale, we scaled up the experiments before attempting purification *via* the same 8xHis tag used to identify the protein on the Western blot.

5.2.2 Large-scale expression and purification of FtsW-mfGFP

Typically six flasks containing 4.8 L of *E. coli* culture between them were inoculated and induced to produce 10-15 g of dry cell pellet. The cells were broken and the membrane was separated by a series of centrifugations. These cell membranes were resuspended at a concentration of 40 mg/mL in SMA solution (2.5 % SMA, 50 mM Tris-HCl at pH 8) and incubated at room temperature with stirring, for 2 h.

Post incubation, the membrane that was not solublised by the SMALP was removed by another centrifugation step and the supernatant contained the solublised FtsW-mfGFP fusion protein. Using a Ni^{2+}-NTA resin, we then purified the solublised FtsW-mfGFP. Five milliliter elution volumes with increasing concentrations of imidazole were used to elute the His tagged protein from the resin. A SDS-PAGE gel was completed running samples from the purification and it illustrated the gradual release of an 80 kDa sized band as imidazole concentration increased. The elution fractions were notably dilute, making them barely visible using Coomassie blue staining; this can be observed in Figure 5-2. The same elution fractions were observed *via* a Western blot, which better illustrates the elution profile given the low protein concentration of the 5 mL elution fractions. This Western blot is annotated in Figure 5-3.

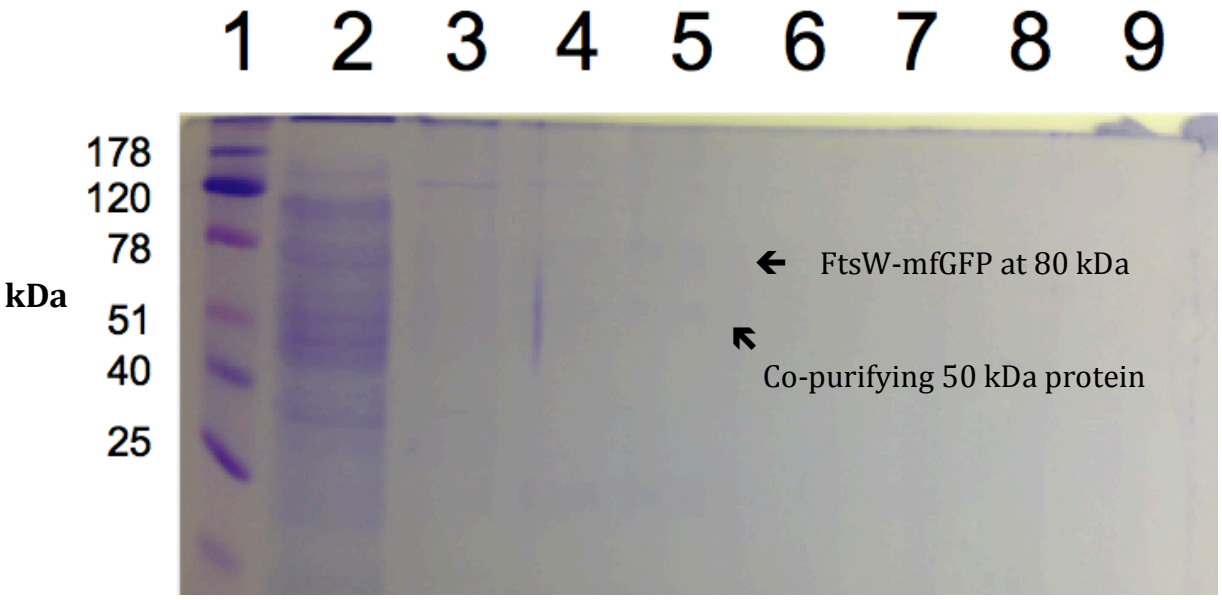


Figure 5-2. A SDS PAGE gel displaying the elution profile of FtsW-mfGFP from Ni^{2+}-NTA with increasing concentrations of imidazole.
Lane 1 contains 5 µL of HyperPAGE prestained protein marker.
Lane 2 is the column run off containing proteins that didn't bind to the resin.
Lane 3 contains a 25 mM elution fraction from the Ni^{2+}-NTA.
Lane 4 contains a 50 mM elution fraction from the Ni^{2+}-NTA.
Lane 5 contains a 100 mM elution fraction from the Ni^{2+}-NTA.
Lane 6 contains a 200 mM elution fraction from the Ni^{2+}-NTA.
Lane 7 contains a 300 mM elution fraction from the Ni^{2+}-NTA.
Lane 8 contains a 400 mM elution fraction from the Ni^{2+}-NTA.
Lane 9 contains a 500 mM elution fraction from the Ni^{2+}-NTA.

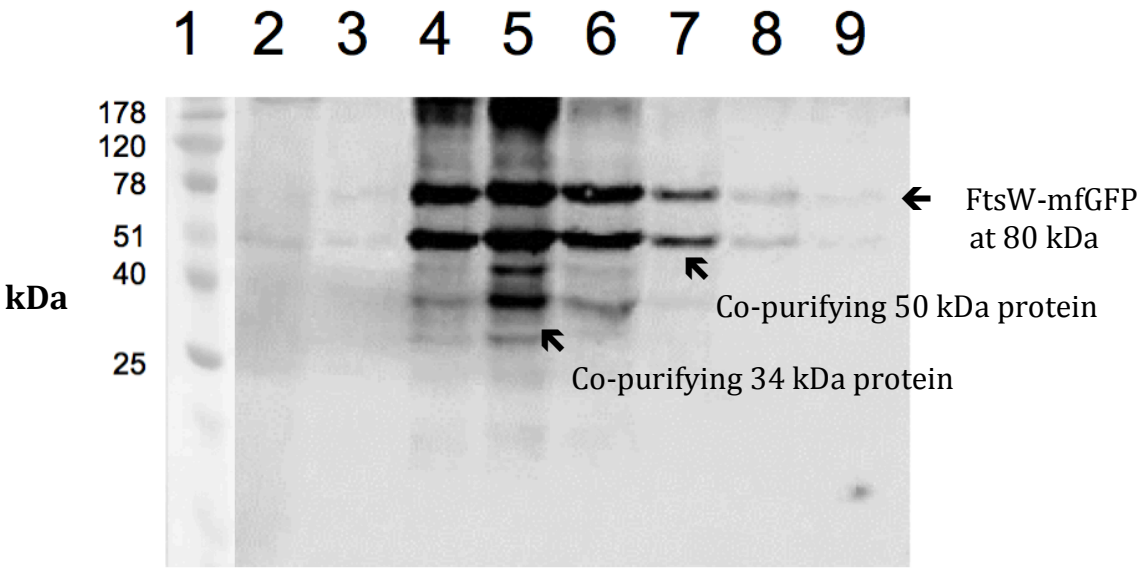


Figure 5-3. Western blotting using anti-His-tagged antibody to probe for the FtsW-mfGFP elution profile from Ni^{2+}-NTA resin.
Lane 1 contains 5 µL of HyperPAGE prestained protein marker.
Lane 2 is the column run off containing proteins that didn't bind to the resin.
Lane 3 contains a 25 mM elution fraction from the Ni^{2+}-NTA.
Lane 4 contains a 50 mM elution fraction from the Ni^{2+}-NTA.
Lane 5 contains a 100 mM elution fraction from the Ni^{2+}-NTA.
Lane 6 contains a 200 mM elution fraction from the Ni^{2+}-NTA.
Lane 7 contains a 300 mM elution fraction from the Ni^{2+}-NTA.
Lane 8 contains a 400 mM elution fraction from the Ni^{2+}-NTA.
Lane 9 contains a 500 mM elution fraction from the Ni^{2+}-NTA.

Although not at a high enough concentration to be visualised clearly on a Coomassie blue stained SDS-PAGE gel, FtsW-mfGFP was eluted from the column successfully. However, two other proteins appear to have co-purified alongside the FtsW-mfGFP; one runs at 50 kDa and the other at 34 kDa. The three co-purified proteins proved to be separable by gel filtration. Elution fractions containing FtsW-mfGFP (50 mM – 400 mM elution fractions) were collected and concentrated before being loaded on to a gel filtration column to remove the co-purified contaminant proteins. The gel filtration profile of FtsW-mfGFP is illustrated in Figure 5-4.

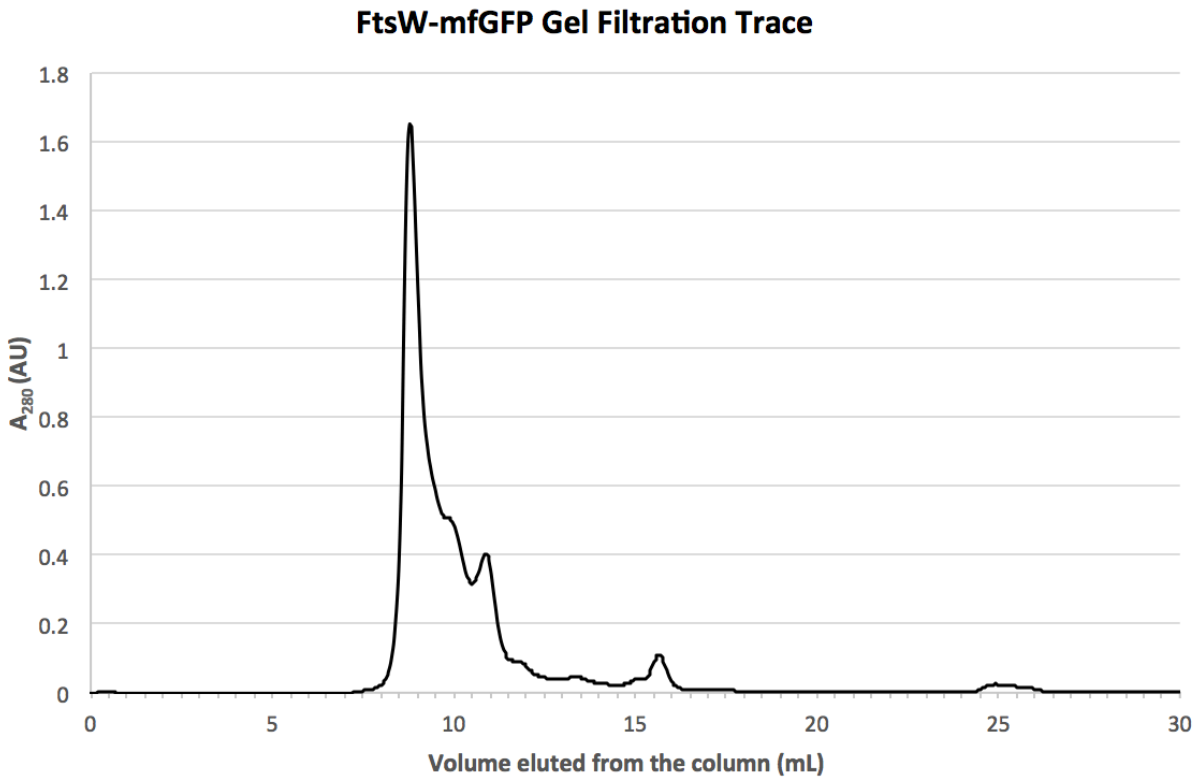


Figure 5-4. A Gel filtration profile of FtsW-mfGFP. The Ni^{2+}-NTA elution fractions containing FtsW-mfGFP were combined and concentrated before loading onto the gel filtration column. The progress was monitored by observing absorbance at 280 nm. The experiments were completed on a Superdex 200 10/300 GL column, which was operated by a ÄKTA™ purifier FPLC purification system (GE Healthcare). The fractions were eluted with 50 mM Tris-HCl, 150 mM NaCl, pH 8 and collected every 0.5 mL.

The gel filtration fractions collected across the peak were examined on a SDS-PAGE gel to identify which of them contained pure FtsW-mfGFP. Those that did were collected, concentrated and examined on the SDS-PAGE gel in Figure 5-5.

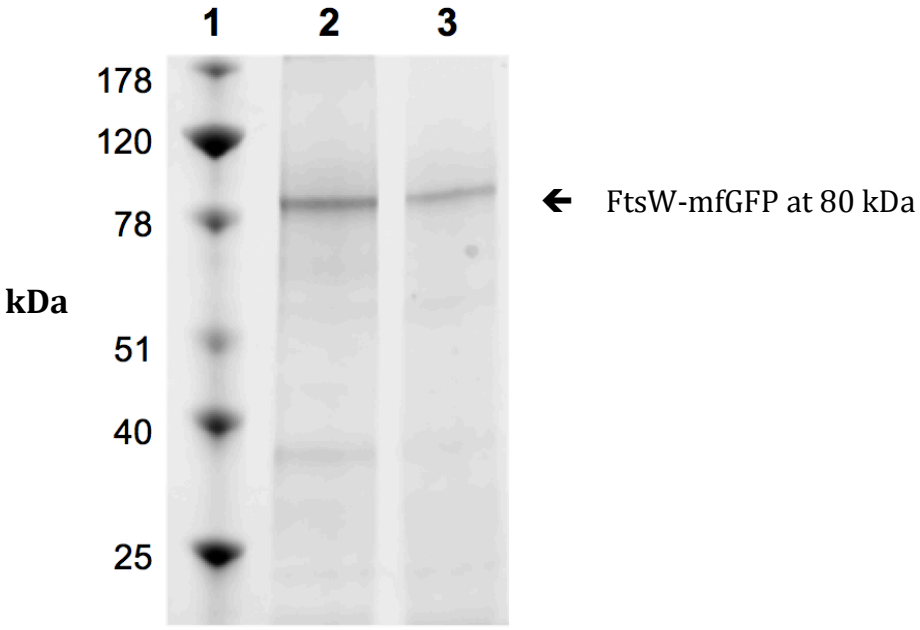


Figure 5-5. A SDS PAGE gel displaying FtsW-mfGFP purified by Ni^{2+}-NTA affinity chromatography and size exclusion chromatography through gel filtration. Lane 1 contains 5 µL of HyperPAGE prestained protein marker.
Lanes 2 and 3 are the pure FtsW-mfGFP fusion protein, lane 2 is twice the concentration of lane 3.

All evidence to this point suggests that we successfully solublised the FtsW-mfGFP fusion protein with SMA before purifying the protein with affinity and size exclusion chromatography. In order to ensure the band at 80 kDa was the FtsW-mfGFP fusion protein, we completed FT-ICR mass spectrometry on a gel plug extracted from the gel pictured in Figure 5.5.

5.2.3 Mass spectrometry of FtsW-mfGFP and co-purifying proteins

To ensure the 80 kDa band observed on the SDS-PAGE gel displayed in Figure 5-5 is the FtsW-mfGFP fusion protein, mass spectrometry experiments were completed. The 80 kDa band was excised from the SDS-PAGE gel with a scalpel and subjected to tryptic digestion for FT-ICR mass spectrometry experiments. The peptides identified were cross-referenced with the *E. coli* proteome and the protein sequence of mfGFP. The peptides identified through FT-ICR mass spectrometry throughout the structure of FtsW and mfGFP are listed in Table 5-1 and Table 5-2, respectively.

Table 5-1. FtsW-derived peptides present in the 80 kDa gel plug identified by FT-ICR mass spectrometry.

Peptide Fragments	Peptide Sequence	Peptide Location (amino acid)
1	LPGFSILVWISTALK	13-27
2	EKDTNSLIMYDR	35-46
3	LTNDPFFFAK	74-83
4	WIDLGLLR	137-144
5	LSLFCYIANYLVR	153-165
6	VTAFWNPWEDPFGSGYQLTQSLMAFGR	246-272
7	GELWGQGLGNSVQK	283-296
8	GLTLPLISYGGSSLLIMSTAIMMLLR	370-395

Table 5-2. mfGFP-derived peptides present in the 80 kDa gel plug identified by FT-ICR mass spectrometry.

Peptide Fragments	Peptide Sequence	Peptide Location (amino acid)
1	GEELFTGVVPILVELDGDVNGHK	5-27
2	FSVSGEGEGDATYGK	28-42
3	LPVPWPTLVTTLTYGVQCFSR	56-76
4	SAMPEGYVQER	89-99
5	TIFFKDDGNYK	100-110
6	AEVKFEGDTLVNR	113-125
7	FEGDTLVNR	117-125
8	GIDFKEDGNILGHK	130-143
9	LEYNYNSHNVYIMADK	144-159
10	DHMVLLEFVTAAGITLGMDELYK	286-308

Given the large peptide coverage across both the FtsW and mfGFP sections of the expressed fusion protein within the SMALP, it can be concluded that the band running at 80 kDa on SDS-PAGE gels contains the SMALP-FtsW-mfGFP.

Gel plugs from a SDS-PAGE gel corresponding to the co-purified 34 kDa and 50 kDa proteins were also subjected to FT-ICR mass spectrometry. The 50 kDa band produced no evidence of any particular protein. It displayed insignificant coverage of any proteins it detected. However, the 34 kDa band was proven to be mfGFP without FtsW. This data is displayed below in Table 5-3.

Table 5-3. mfGFP-derived peptides present in the 34 kDa gel plug identified by FT-ICR mass spectrometry.

Peptide Fragments	Peptide Sequence	Peptide Location (amino acid)
1	GEELFTGVVPILVELDGDVNGHK	5-27
2	FSVSGEGEGDATYGKLTLK	28-46
3	FICTTGK	49-55
4	LPVPWPTLVTTLTYGVQCFSR	56-76
5	SAMPEGYVQER	89-99
6	TIFFKDDGNYK	100-110
7	AEVKFEGDTLVNR	113-125
8	GIDFKEDGNILGHK	133-146
9	LEYNYNSHNVYIMADK	144-159
10	GGHVVEGLAGELEQLR	199-214

The results presented in this chapter prove we have expressed and solubilised the integral *E. coli* membrane protein FtsW fused to mfGFP (FtsW-mfGFP) with SMA. We then purified the SMALP-FtsW-mfGFP with affinity and size exclusion chromatography. FT-ICR mass spectrometry was exploited to analyse the pure FtsW-mfGFP and the two proteins that co-purified with it.

5.3 Discussion

The FtsW-mfGFP fusion construct was synthesised with 8xHis tags in order to facilitate protein purification. Generally it took four days of growing the *E. coli* cultures from transformation through to harvesting cells expressing the FtsW-mfGFP construct. It then took five days to solubilise the membrane protein with SMA and purify it. The fusion protein was initially purified with affinity chromatography, however, three distinct bands were visible when the elution fractions were studied on a SDS PAGE gel. The purified SMALP-FtsW-mfGFP (80 kDa band) co-purified with proteins with masses of 50 kDa and 34 kDa.

FT-ICR mass spectrometry was used to obtain extra information about the three bands. When we analysed the 80 kDa protein we confirmed that it contained the target fusion protein. Eight unique FtsW peptides and ten peptides that are unique to mfGFP were identified. These peptides are spread throughout the amino acid sequence of FtsW-mfGFP, with 35 % of the protein sequence being accounted for through these unique peptide sequences alone (251 of 723 amino acids). The data collected for the 34 kDa protein allowed us to conclude that it was the mfGFP separated from fusion protein, whilst the 50 kDa band could not be identified as a specific protein.

The three proteins present in the elution from the Ni^{2+}-NTA proved separable with the application of size exclusion chromatography. Pure FtsW-mfGFP was obtained from the gel filtration, however, it was at insufficient concentrations to complete svAUC and CD experiments that were successfully completed with SMA-FtsA and SMA-MurJ-sfGFP.

Considering MurJ and FtsW were cloned into the same expression plasmid, the large variation in expression level is unexpected. Another contributory factor in the low target protein concentration is the evidence of its breakdown before affinity chromatography was completed. The presence of mfGFP without FtsW in the Ni^{2+}-NTA elution fractions proves this to be the case. There is no 34 kDa band illuminated on the Western blot of protein expression. Therefore the cleavage of the fusion protein is occurring either during cell storage at -70^{0}C, in the breaking of the cell pellet or is due to the solubilisation of the FtsW-mfGFP with SMA. Without re-engineering the plasmid, increasing culture volumes or further optimising the purification conditions are potential options that may circumvent the low protein expression levels.

CHAPTER 6:

Exploring SMA as a

periplasmic release agent

6.1 Introduction

Periplasmic targeting of recombinant proteins in *E.* coli offers many advantages over cytoplasmic production – these include folding benefits, increased purity and protein stability (Mergulhão *et al*., 2005). The periplasmic contents can be selectively released from the cell whilst retaining the integrity of the cytoplasmic membrane. A multitude of different methods have been shown to do this to some extent, the most popular of which is a Tris-HCl, EDTA (TE) based buffer at various ingredient ratios and conditions (Quan *et al*., 2013). The issue with many available methods that exhibit limited success is that no universal method exists for selective periplasmic release. This is holding back the potential of the periplasmic targeting of recombinant proteins.

Styrene maleic acid copolymer (SMA) has demonstrated the ability to produce SMA lipid particles (SMALP) by removing 9-11 nm discs of membrane bilayer and any attached proteins (Knowles *et al*., 2009). We hypothesise SMA could be exploited to compromise the outer membrane of gram-negative bacteria, causing leakage of the periplasmic contents whilst leaving the cytoplasm of the bacteria intact. In order to investigate this theory, two periplasm-targeted fluorescent proteins are expressed and their selective release is initiated with a traditional TE release method and the styrene maleic acid copolymer.

6.2 Results

6.2.1 Peri-sfGFP and Peri-mCherry strain establishment

Both the Peri-sfGFP and Peri-mCherry plasmids were transformed into *E. coli* BL21 Codon Plus (DE3). A large number of colonies were observed after the overnight incubation on the LB-amp agar plate (ampicillin at 100 μg/mL). These 10 cultures were then incubated overnight at 37^{o}C, 200 RPM. 100 μL of these overnight cultures were used to inoculate 10 more 5 mL aliquots of LB without antibiotic (as per the manufacturer's protocol) which were incubated for 2 h at 37^{o}C, 200 RPM. The cultures were then induced with a 1 mM final concentration of IPTG. Post induction the cultures were separated by centrifugation at 8000 x g at 4^{o}C for 600 s.

Upon inspection of the cell pellets, expression was evident due to the colour change of the cell pellets. The cells expressing Peri-sfGFP had a green cell pellet, whilst the cells expressing peri-mCherry were red in colour. Furthermore if these cell pellets were observed in blue light through an orange filter, the presence of the target protein was even more obvious as their fluorescent abilities caused them to illuminate. Sedimented cell pellets expressing both peri-sfGFP, and peri-mCherry, are shown in white light and in blue light through an orange filter in Figure 6-1.

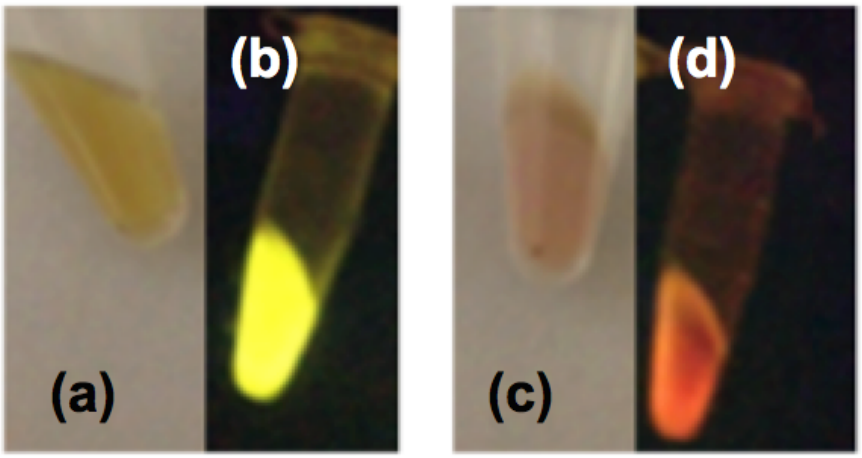


Figure 6-1. Cell pellets expressing perisplasmic-targeted fluorescent proteins.

Figure 6-1. (a) and (b) display a cell pellet expressing Peri-sfGFP. Figure 6-1. (a) displays a cell pellet expressing Peri-sfGFP in white light. Figure 6-1. (b) displays a cell pellet expressing Peri-sfGFP in blue light through an orange filter.

Figure 6-1. (c) and (d) display a cell pellet expressing Peri-mCherry. Figure 6-1. (c) displays a cell pellet expressing Peri-mCherry in white light. Figure 6-1. (d) displays a cell pellet expressing Peri-mCherry in blue light through an orange filter.

This notable colour change and fluorescent output illustrate a successful expression of the fluorescent target proteins in question. Further proof of successful protein induction is evident from growth curves recorded throughout cell proliferation. Samples were taken during growth and induction phases of the Peri-sfGFP and Peri-mCherry strains; their absorbance was immediately measured at OD_{600} to allow the monitoring of cellular density and growth rate over time. The growth curves of two separate cultures containing Peri-sfGFP and Peri-mCherry are shown in Figure 6-2.

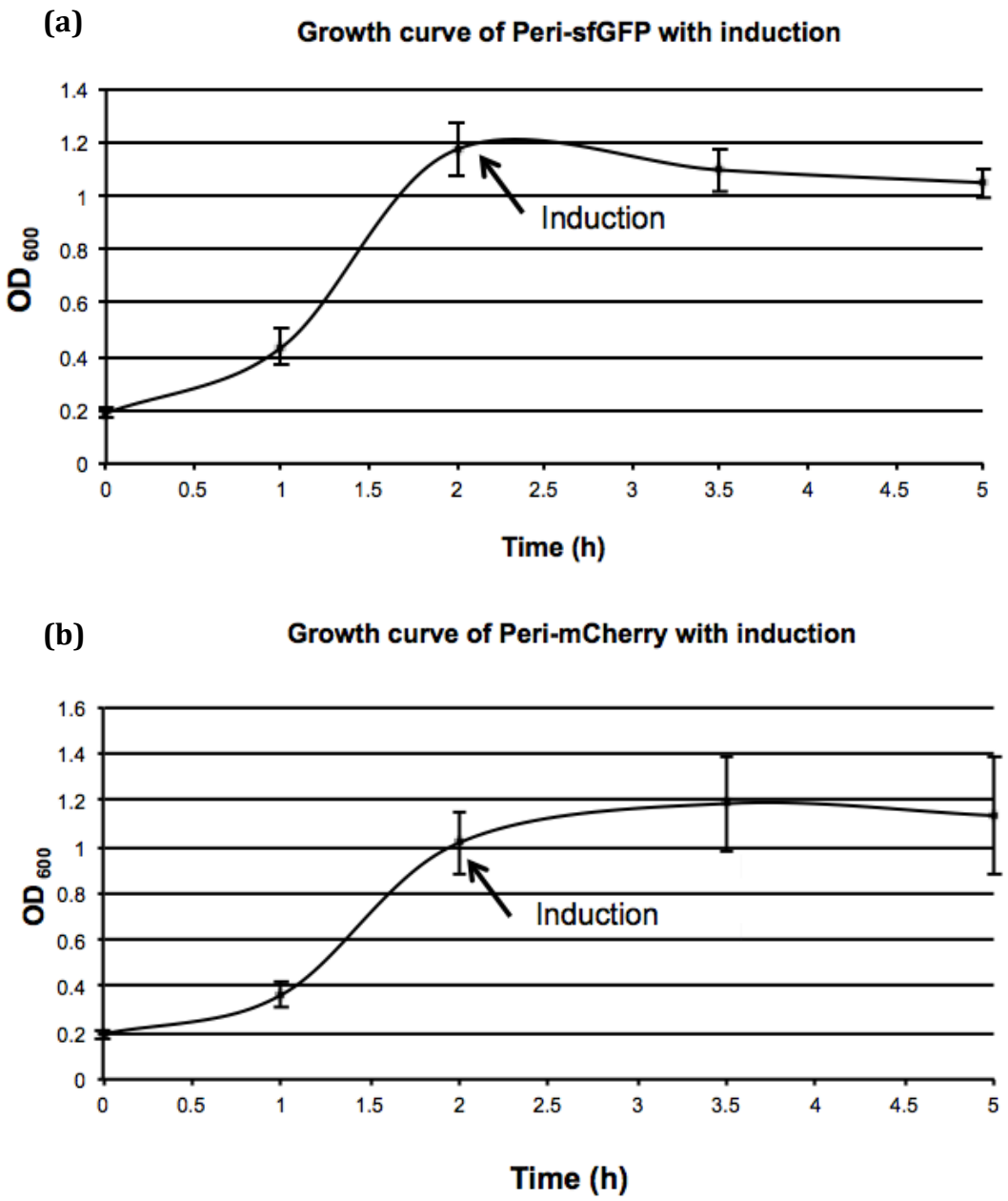


Figure 6-2. Growth curves across five separate colonies of (a) Peri-sfGFP and (b) Peri-mCherry. Cultures were induced 2 h post inoculation with 1 mM IPTG, the growth variation from colony to colony is indicated by the error bars.

Both these curves show that growth rates slow post-induction. This is caused by cellular resources being directed towards protein synthesis instead of cell division. After ensuring both the periplasm-targeted proteins are being expressed, the next stage is to apply periplasmic release methods and observe for the fluorescent products post treatment.

6.2.2 Larger scale Peri-sfGFP and Peri-mCherry expression

In order to have enough fluorescent product to test for target protein production, the expression protocols must be scaled-up. Two successful transformant colonies were selected from the LB-antibiotic plate and each was used to inoculate 100 mL of LB with 100 µg/mL ampicillin and 50 µg/mL chloramphenicol (as per manufacturer's protocol). These cultures were grown overnight at 37^{o}C, 200 rpm. 25 mL of the overnight culture was added to 500 mL of LB containing no selection antibiotics (as per manufacturers protocol). The inoculated LB was grown for 2 h at 37^{o}C and 200 rpm. After 2 h, gene expression was induced by the addition of IPTG to a final concentration of 1 mM. The induced culture was incubated for 3 h at 37^{o}C and 200 rpm before 500 mL of the induced culture was separated by centrifugation at 12,000 x g, 4^{o}C for 0.25 h.

The supernatant was discarded and the pellets of the Peri-sfGFP and Peri-mCherry were gently resuspended in 1 mL of TE buffer (100 mM Tris-HCl, 10 mM EDTA at pH 7.5) to investigate whether an existing periplasmic release protocol would release the fluorescent perisplasmic contents from the cell. The cell pellets in TE buffer were incubated at 37^{o}C, 200 RPM for 2 h before being separated by centrifugation at 12,000 x g, 4^{o}C for 0.25 h. The supernatant at this stage was collected and stored at 4^{o}C for future experiments.

If the TE buffer has released the periplasmic contents of the cells that overexpressed Peri-sfGFP and Peri-mCherry the stored supernatant will fluoresce at the correct conditions. Fluorescence studies were successfully completed under the conditions listed in Table 2-10. Initial fluorescence spectra for Peri-sfGFP and Peri-mCherry are illustrated in Figure 6-3.

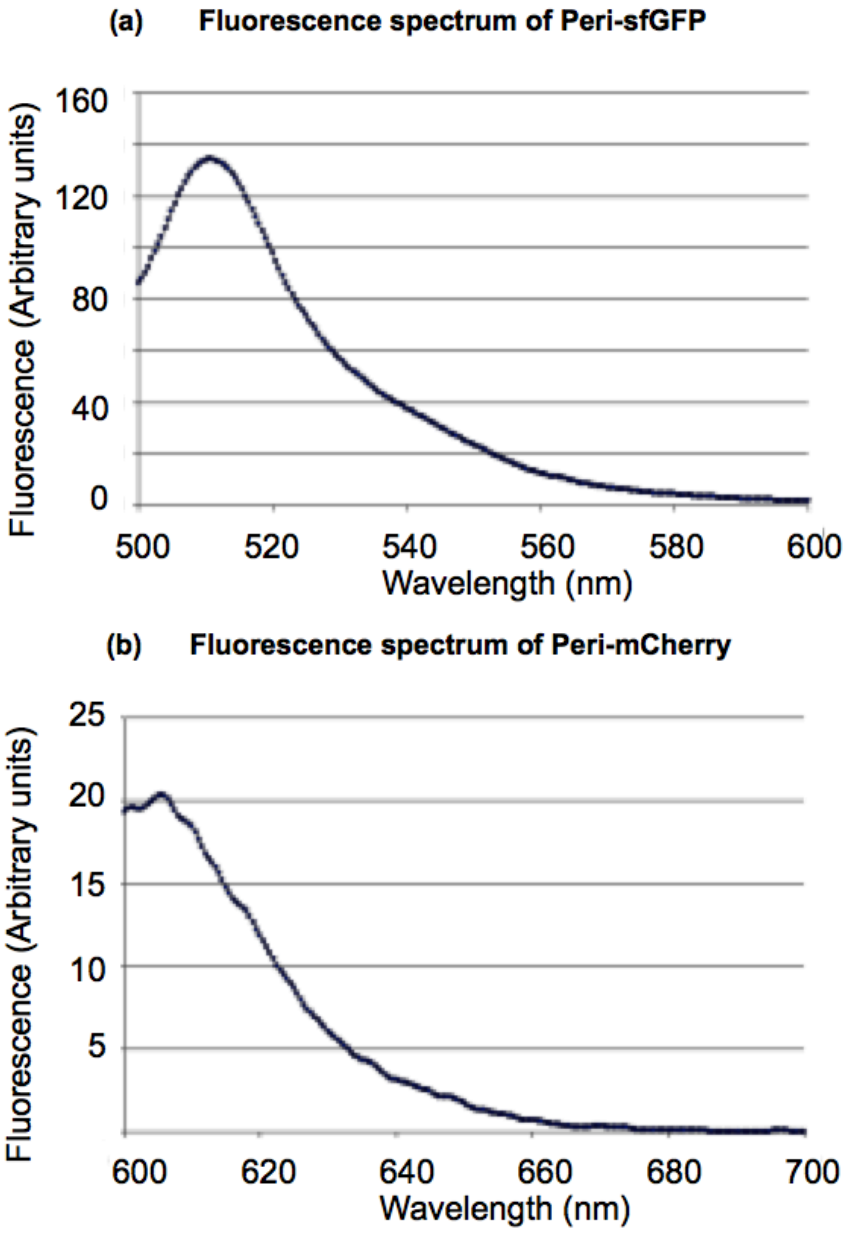


Figure 6-3. Initial fluorescence spectra of Peri-sfGFP and Peri-mCherry, which were completed under the conditions detailed in Table 2-10. The spectra were measured 5 times and averaged to account for instrumental error.

The fluorescence spectrum for Peri-sfGFP produces a smooth curve with a fluorescence peak at 512 nm. The fluorescence spectrum for peri-mCherry also produces a distinct peak, this time at 608 nm, however, the curve is not as smooth as the curve produced by Peri-sfGFP. The units of fluorescence are arbitrary, therefore direct comparisons across the two strains cannot be made. The fact repeatable fluorescence spectra are visible in the samples tested suggests that periplasmic release was initiated with the TE buffer over a 2 h period. These findings act as further evidence confirming the successful expression and subsequent secretion of the sfGFP and mCherry proteins to the periplasm.

After proving unequivocally that Peri-sfGFP and Peri-mCherry can be released *via* existing proven methods for periplasmic extraction (TE) we focused on examining SMA as a periplasmic release agent compared to TE.

6.2.3 Comparing the periplasmic release reagents (TE and SMA)

In order to compare SMA to TE as a periplasmic release agent, equivalent volumes of each buffer were applied to the same sized cell culture pellets. Fluorescence profiles were recorded and relative intensities of their spectra peaks can be compared (Peri-sfGFP – 512 nm, Peri-mCherry – 608 nm). Furthermore we wanted to ensure the periplasmic protein is being released by the buffer application over the incubation period, to rule out the initial resuspension of the cell pellet was causing periplasmic leakage into the extracellular milieu. To ensure the resuspension was not compromising the

integrity of the cells expressing the periplasmic protein, post resuspension they were immediately separated by centrifugation at 8000 x g at 4°C for 300 s. In order to obtain the most comparable data possible, three fresh transformant colonies were picked for each protein and these three colonies were used in triplicate for each set of experiments. To facilitate processing, the scale of each release experiment was decreased, so the extra replicates could be obtained and all culture could be grown and induced in the same flask.

Three transformant colonies of both periplasm-targeted proteins were selected from the freshly transformed plate and independently grown up and induced to produce 525 mL of either Peri-sfGFP or Peri-mCherry culture. The culture was then separated into 50 mL aliquots to be separated by centrifugation at 8000 x g at 4°C for 300 s. These 50 mL culture cell pellets (approximately 0.3 g dry cell mass) are the starting point for the experiment depicted in Figure 2-5. Six cell pellets for both proteins were gently resuspended with both periplasmic release agents. Three of the six experimental replicates were immediately separated again and the supernatant tested for fluorescence. The remaining replicates were incubated for 2 h at 37°C and 200 RPM before having their supernatants extracted to be tested for fluorescent properties. Fluorescence spectra for these 12 samples were recorded and the arbitrary fluorescence units of the spectrums peak were calculated. Replicate results were then averaged so comparisons can be made between the different release agents for both the Peri-sfGFP and Peri-mCherry.

This chapter will first focus on how effective TE and SMA are at releasing Peri-sfGFP before shifting focus onto work completed with the Peri-mCherry strain.

6.2.3.1 Comparing TE and SMA mediated release of Peri-sfGFP

Relative fluorescent peaks (completed in replicate following the release of Peri-sfGFP) under different release conditions are displayed in Figure 6-4.

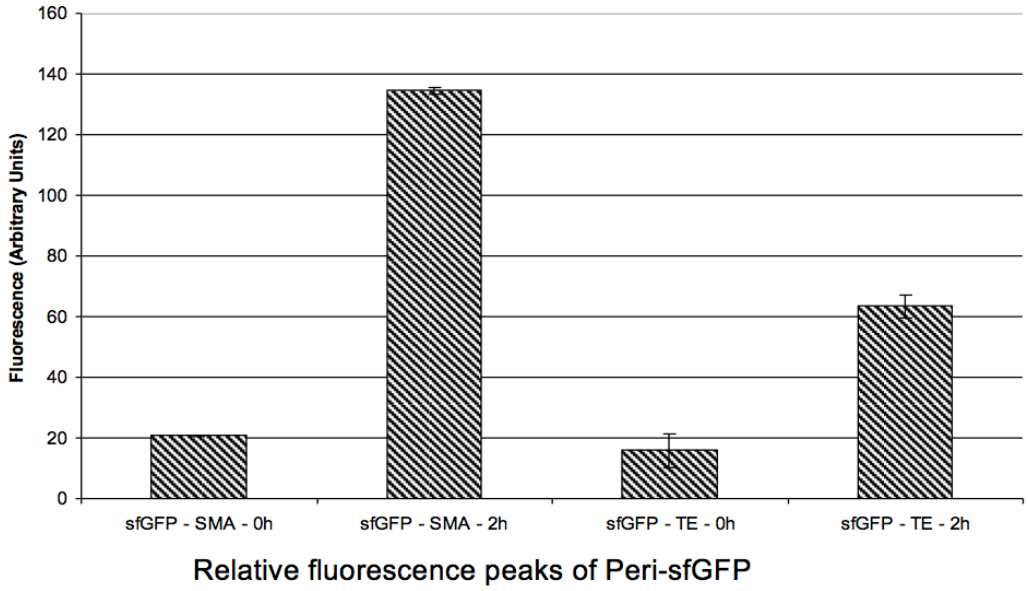


Figure 6-4 A histogram illustrating the relative peak fluorescence of Peri-sfGFP under different release conditions. SMA – 0h sample subjected the Peri-sfGFP cell pellet to resuspension in SMA before being immediately separated and tested for fluorescent properties. SMA – 2h sample subjected the Peri-sfGFP cell pellet to a 2 h incubation in SMA before the supernatant was separated from insoluble debris and tested for fluorescent properties. TE – 0h sample subjected the Peri-sfGFP cell pellet to resuspension in TE before being immediately separated and tested for fluorescent properties. TE – 2h sample subjected the Peri-sfGFP cell pellet to a 2 h incubation in TE before the supernatant was separated from insoluble debris and tested for fluorescent properties.

When assessing this data, we can assume an increase in fluorescence is caused directly by an increase in concentration of the successfully released Peri-sfGFP from the periplasm. When the cell culture pellet is gently resuspended in either SMA or TE, and then immediately separated by centrifugation, there is only a very small difference in SMA compared to TE (21 fluorescence units for SMA - 0h

versus 16 for TE - 0h). Furthermore this small difference is even less significant given the relatively large error bars from the data spread shown in the TE experiments. The similarity in these two figures and the increase in the amount of Peri-sfGFP after a 2 h incubation in both buffers suggests that the initial resuspension is not causing cellular leakage and that both buffers are successfully causing periplasmic discharge from the *E. coli*.

When observing Peri-sfGFP release over a 2 h period with SMA compared to TE, a larger amount of sfGFP is released with the SMA polymer: 135 fluorescence units compared with 63. TE release over a 2 h period is the traditional method for periplasmic extraction. Therefore we expressed all values as a comparative percentage of the figure corresponding to TE-mediated release over the 2 h incubation period (TE - 2h). This data is illustrated in Figure 6-5.

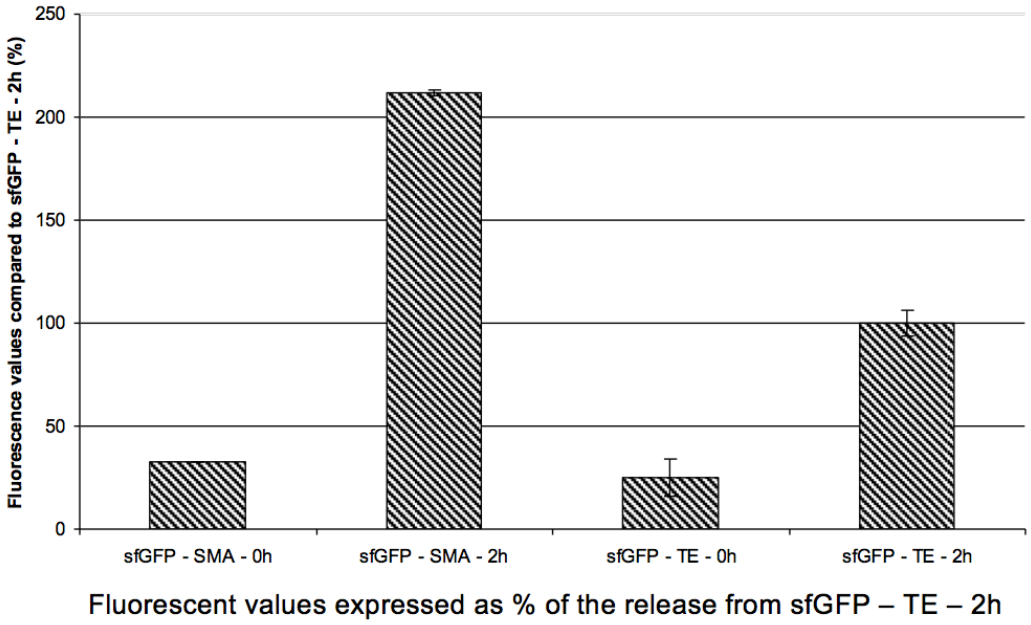


Figure 6-5. The fluorescent values from Figure 6-4 expressed as a percentage of the Peri-sfGFP released by TE over the 2 h incubation.

Upon examination, there is a 214 % increase in sfGFP release by SMA when compared to TE over a 2 h period at 37°C. This suggests it has great potential to release proteins from *E. coli*. In order to ensure that SMA and TE are releasing just periplasmic contents, the values in Figure 6-5 need to be examined with respect to total protein concentration within the samples tested for their fluorescence spectra. This comparison is known as the specific activity of a reagent. To calculate total protein concentration of the samples, a BCA assay was completed in a 96-well plate format. All samples were run in triplicate at multiple concentrations to ensure experimental accuracy. These results are displayed in Figure 6-6.

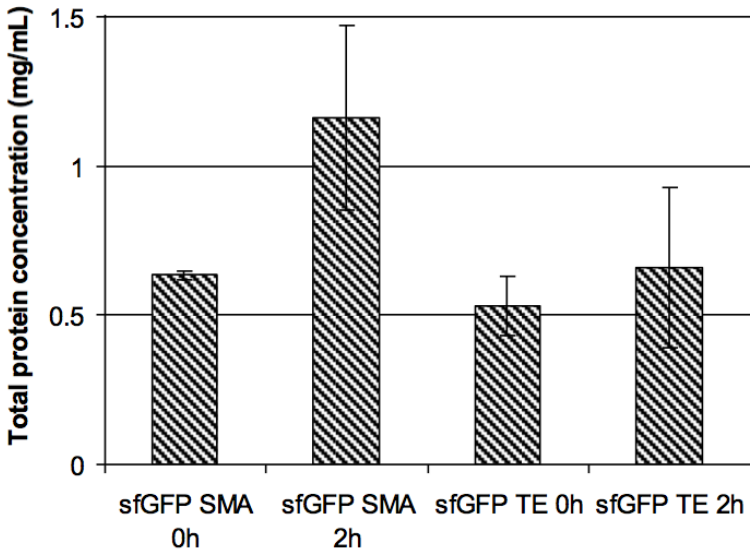


Figure 6-6. A histogram illustrating the total protein concentration (mg/mL) in the samples containing released Peri-sfGFP, determined by using a BCA assay.

To calculate the specific activity for each release method, the value for the arbitrary fluorescence units from Figure 6-4 must be divided by the total protein concentration (mg/mL) calculated in Figure 6-6. The specific activities of all four experiments are displayed in Figure 6-7.

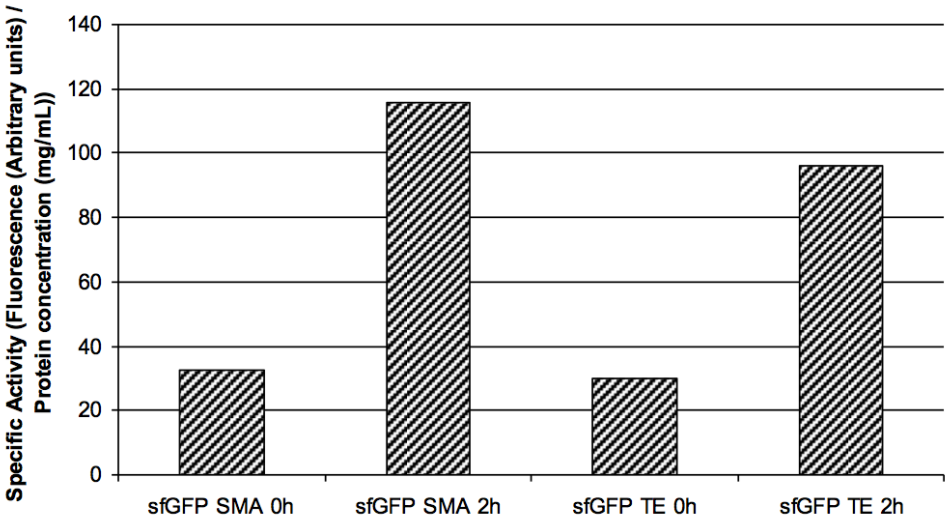


Figure 6-7. A histogram depicting the specific activity for selectively releasing Peri-sfGFP compared to other cellular proteins. This data was calculated by dividing the fluorescence values in Figure 6-4 by the corresponding total protein concentration values in Figure 6-6. The units for this specific activity is fluorescence (Arbitrary units)/total protein concentration (mg/mL).

This data shows Peri-sfGFP is released with the highest specific activity when treated with SMA buffer for a 2 h incubation. This release method resulted in 116 units compared to 96 units produced by a TE-mediated release over a 2 h incubation period. This data shows SMA-mediated periplasmic release displays a 21 % increase in specific activity over TE over a 2 h period at 37^{o}C. Culture pellet treatment with SMA and TE followed by immediate separation by centrifugation results in specific activity units for SMA and TE of 33 and 30, respectively.

This thesis will now display the data from this same experimental protocol for the Peri-mCherry strain to allow comparisons between the specific activity of SMA and TE across the release of different periplasm-targeted proteins.

6.2.3.2 Comparing TE and SMA mediated release of Peri-mCherry

Relative fluorescent peaks (completed in replicate following the release of Peri-mCherry) under different release conditions are displayed in Figure 6-8.

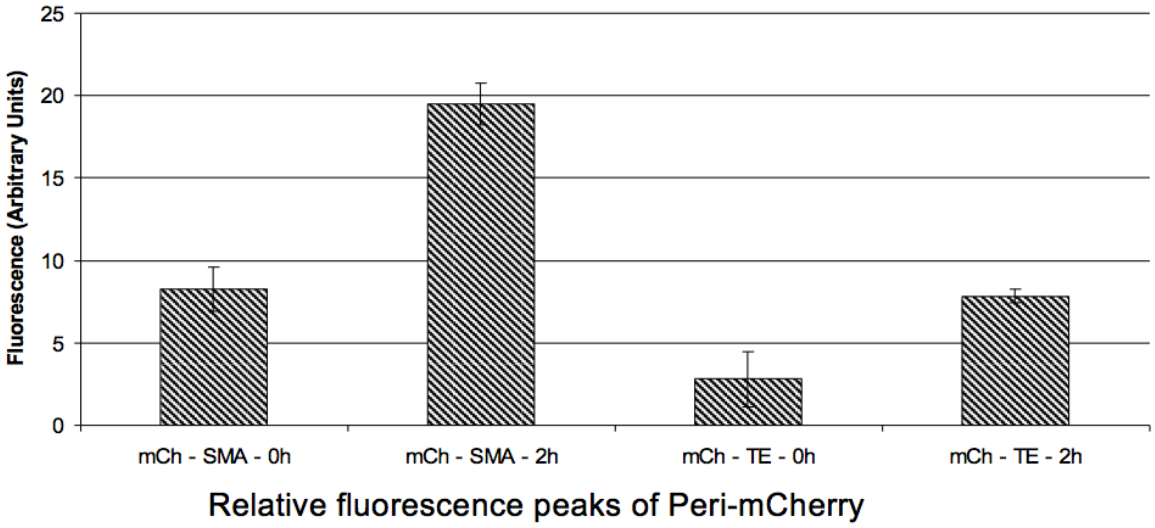


Figure 6-8 A histogram illustrating the relative peak fluorescence of Peri-mCherry under different release conditions. SMA – 0h sample subjected the Peri-mCherry cell pellet to resuspension in SMA before being immediately separated and tested for fluorescent properties. SMA – 2h sample subjected the Peri-mCherry cell pellet to a 2 h incubation in SMA before the supernatant was separated from insoluble debris and tested for fluorescent properties. TE – 0h sample subjected the Peri-mCherry cell pellet to resuspension in TE before being immediately separated and tested for fluorescent properties. TE – 2h sample subjected the Peri-mCherry cell pellet to a 2 h incubation in TE before the supernatant was separated from insoluble debris and tested for fluorescent properties.

When assessing this data, we can assume an increase in fluorescence is caused directly by an increase in concentration of the successfully released Peri-mCherry from the periplasm. When the cell culture pellet is gently resuspended in either SMA or TE, and then immediately separated by centrifugation, there is only a very small difference in SMA compared to TE (8 fluorescence units for

SMA - 0h versus 3 for TE - 0h). Furthermore this small difference is even less significant given the relatively large error bars from the data spread shown in the TE experiments. The similarity in these two figures and the increase in the amount of Peri-mCherry after a 2 h incubation in both buffers suggests that the initial resuspension is not causing cellular leakage and that both buffers are successfully causing periplasmic discharge from the *E. coli*.

When observing Peri-mCherry release over a 2 h period with SMA compared to TE, a larger amount of mCherry is released with the SMA polymer – 20 fluorescence units compared with 8. TE release over a 2 h period is the traditional method for periplasmic extraction. Therefore we expressed all values as a comparative percentage of the figure corresponding to TE-mediated release over the 2 h incubation period (TE - 2h). This data is illustrated in Figure 6-9.

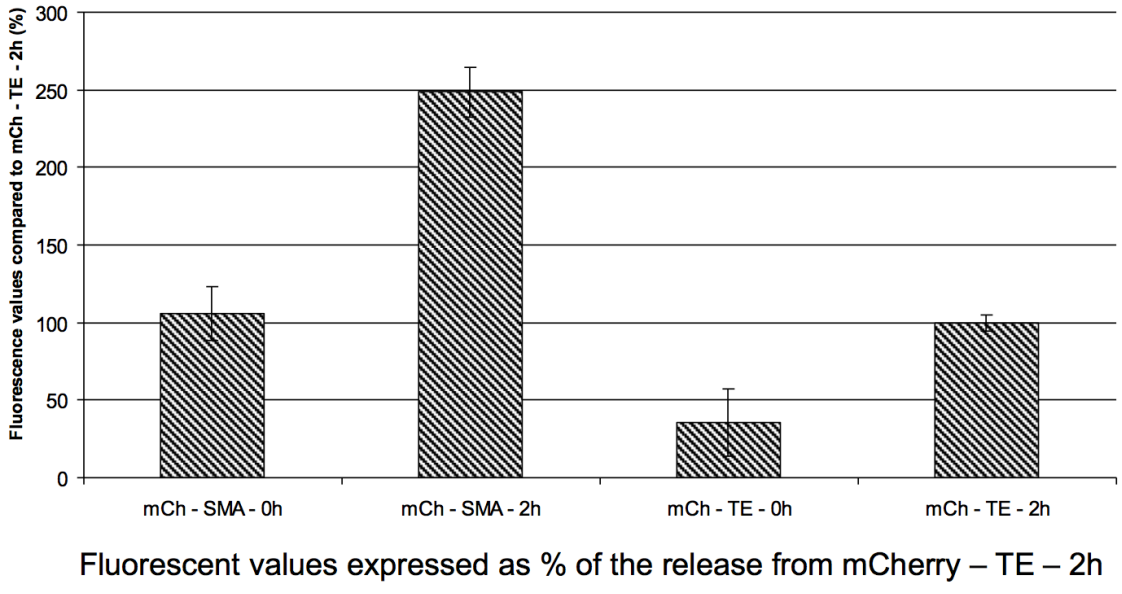


Figure 6-9. The fluorescent values from Figure 6-4 expressed as a percentage of the Peri-mCherry released by TE over the 2 h incubation.

Upon examination, there is a 250 % increase in mCherry release by SMA when compared to TE over a 2 h period at 37°C. This suggests it has great potential to release proteins from *E. coli*. In order to ensure that SMA and TE are releasing just periplasmic contents, the values in Figure 6-9 need to be examined with respect to total protein concentration within the samples tested for their fluorescence spectra. This comparison is known as the specific activity of a reagent. To calculate total proteins concentration of the samples a BCA assay was completed in a 96-well plate format. All samples were run in triplicate at multiple concentrations to ensure experimental accuracy. These results are displayed in Figure 6-10.

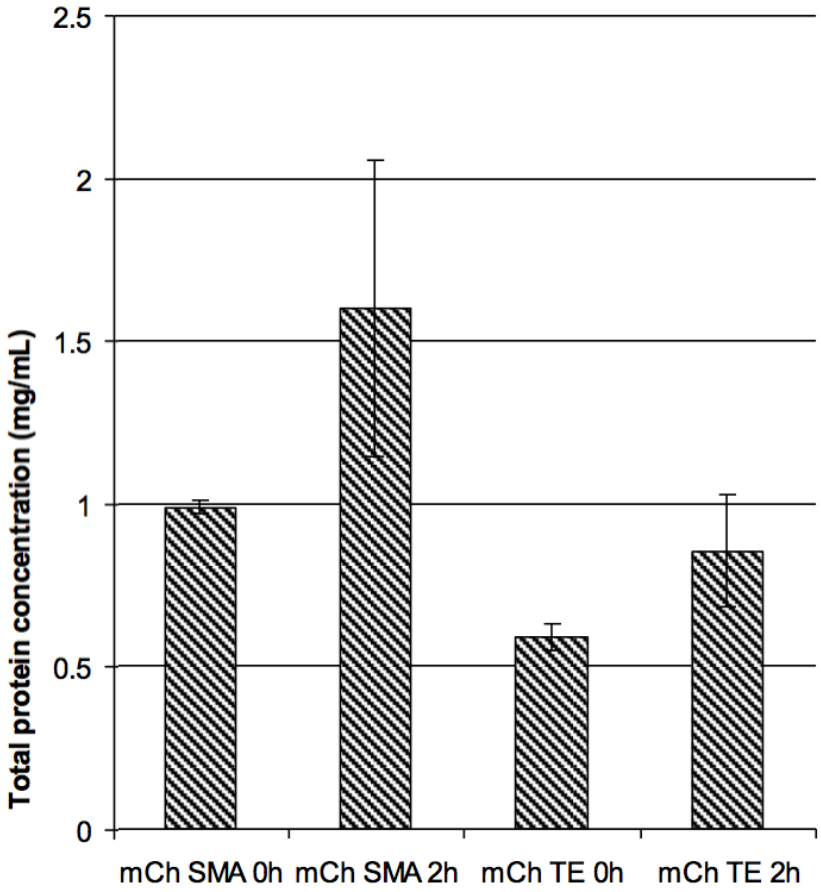


Figure 6-10. A histogram illustrating the total protein concentration (mg/mL) in the samples containing released Peri-mCherry, determined by using a BCA assay.

To calculate the specific activity for each release method, the value for the arbitrary fluorescence units from Figure 6-8 must be divided by the total protein concentration (mg/mL) calculated in Figure 6-10. The specific activities of all four experiments are displayed in Figure 6-11.

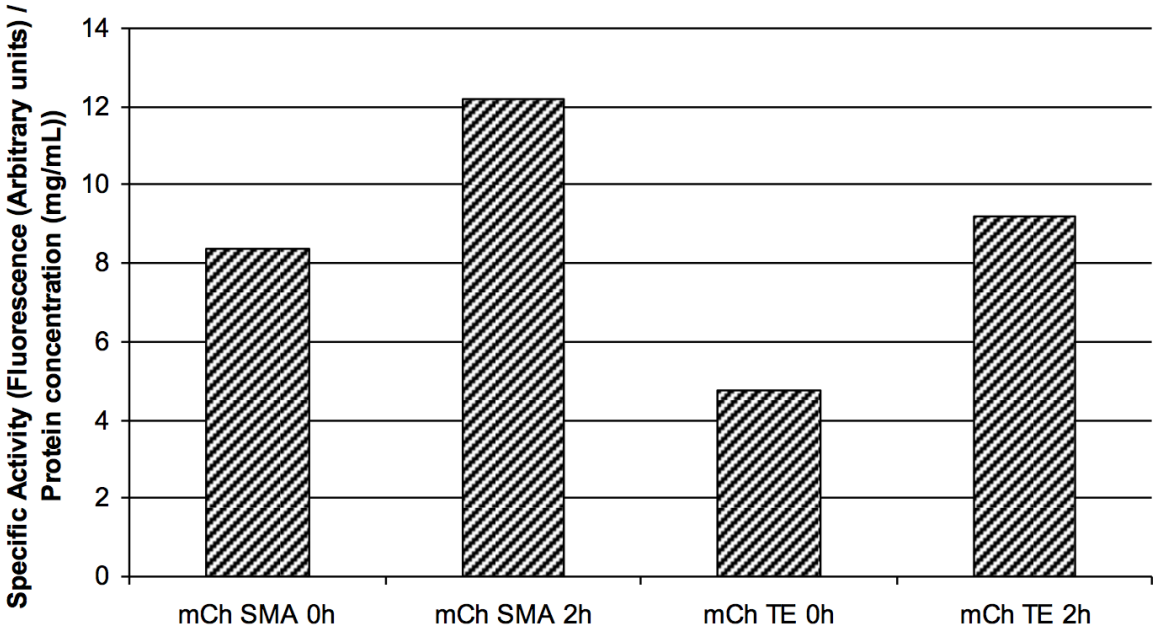


Figure 6-11. A histogram depicting the specific activity for selectively releasing Peri-mCherry compared to other cellular proteins. This data was calculated by dividing the fluorescence values in Figure 6-8 by the corresponding total protein concentration values in Figure 6-10. The units for this specific activity is fluorescence (Arbitrary units)/total protein concentration (mg/mL).

This data shows Peri-mCherry is released with the highest specific activity when treated with SMA buffer for a 2 h incubation. This release method resulted in 12 units compared to 9 units produced by a TE-mediated release over a 2 h incubation period. This data shows SMA-mediated periplasmic release displays a 33 % increase in specific activity over TE over a 2 h period at 37 ˚C. Culture pellet treatment with SMA and TE followed by immediate separation by centrifugation results in specific activity units for SMA and TE of 8 and 5 respectively.

6.3 Discussion

The Peri-sfGFP and Peri-mCherry strains were designed to target fluorescent proteins to the periplasm to facilitate tracking of the cells periplasmic contents. It was necessary to work from fresh transformant colonies on every occasion. If a previously transformed glycerol stock (stored correctly at -70^{o}C) was plated to select a single colony, lower levels of target protein expression were observed. There was also a reduction in protein expression if a transformed colony was used for inoculation after being left on an LB plate (with selection antibiotic) for more than 3 days. *E. coli* expressing the periplasm-targeting strain were also very sensitive, for example the pellet resuspensions needed to be extremely gentle. Aggressive pipetting to resuspend the pellet involved the release of a dark, high viscosity substance; this is most likely DNA. This compromising of the cytoplasmic membrane is something that should obviously be avoided. With all these concerns taken into account, each run of the experiment took, on average, five days to complete from transformation through to the capturing of all fluorescence spectra.

Proof of protein expression (sfGFP and mCherry) was assured through the collaborative evidence identified. After induction of protein expression, coloured, fluorescing dry cell pellets studied under white light and blue light through an orange filter illustrated that the proteins were being expressed. Furthermore, the growth curves illustrate a decline in cell proliferation post induction, which is a typical reaction for cells successfully overexpressing a recombinant protein. Finally, the fluorescence spectrum captured for both the sfGFP and mCherry expressing *E. coli* demonstrated the presence of the proteins. In order to confirm

where in the cell the protein is being expressed, the specific activity results after immediate separation (0 h) must be compared to the corresponding readings after the two hour incubation (2 h) across both strains and release agents. In each of the four circumstances (sfGFP and mCherry release by SMA and TE methods) there is always an increase in specific activity over the course of the 2 h incubation. Therefore it is clear that the fluorescent product (periplasmic contents) is being released at a higher rate compared to the increase in total protein concentration over the 2 h period. This fact illustrates that the target proteins have both been successfully expressed in the periplasm. If they were being expressed in the cytoplasm incorrectly and the periplasmic release method was functional, the specific activity would not increase over the 2 h period. If the proteins were expressed in the cytoplasm, and the periplasmic release methods were accidently lysing the whole cell, then the specific activity would remain the same over the 2 h period.

SMA proved to be more effective releasing both Peri-sfGFP and Peri-mCherry strains when compared to the existing TE method (214 % and 250% respectively). This extra release of protein was specific to the cellular periplasmic contents, since across both strains the specific activity of SMA-mediated release compares favorably to that of TE-mediated release.

Direct comparisons across different fluorescent proteins are difficult to quantify as each fluorescent protein has a unique quantum yield. A higher quantum yield means the fluorescent proteins will fluoresce with more intensity for each photon of light (Wall *et al*., 2014). The quantum yield of sfGFP and mCherry are 0.65 and 0.22 respectively (Wall *et al*., 2014). This disparity in quantum yield

(with sfGFP being the more efficient fluorescer) goes some way to explain the much lower levels of fluorescence by mCherry compared with sfGFP. Even with a larger total protein concentration across all comparable results between sfGFP and mCherry, the fluorescence was notably less intense with mCherry, meaning other methods should be exploited to monitor mCherry release more effectively.

Investigating this trend of SMA proving itself as an effective and selective periplasmic release reagent should act as the focus of all future work in this area. Furthermore other experiments clarifying the release and correct expression and secretion of the proteins should be completed.

CHAPTER 7:

General discussion, conclusions

and future work

7.1 General discussion and conclusions

7.1.1 Membrane proteins

There is a well-documented disparity in the number of structurally characterised soluble proteins versus membrane proteins. A large number of factors combine to complicate study of membrane proteins: their insolubility in water, complex native environment and lower native expression levels make their study a challenging task (Hunte *et al*., 2003). Despite these limitations, membrane protein study remains an important field due to their key roles within cells. Membrane proteins have crucial roles in cellular human biology; playing pivotal roles in processes such as cellular signaling and transport (Musnier *et al*., 2010), respiration and cell division (Egan and Vollmer, 2013). The major obstruction to studying membrane proteins is their insolubility in water; this issue has been circumvented with the use of detergents, amphipols and protein scaffold containing nanodiscs (Jamshad *et al*., 2011). These methods have been applied with moderate success due to all their inherent limitations, resulting in the need for a single, universal method to solubilise membrane proteins, facilitating downstream study. Styrene maleic acid (SMA) has demonstrated potential to be this breakthrough method. SMA has the ability to spontaneously insert into the cell membrane, excising 9-11 nm discs of lipid and any associated membrane proteins with SMA lipid particles (SMALP) (Knowles *et al*., 2009). In the first three results chapters in this thesis, three membrane proteins with different transmembrane membrane topologies were solubilised with SMALP and subsequently purified with traditional methods.

FtsA is a peripheral membrane protein in the actin fold that demonstrates the ability to bind ATP (Fujita *et al*., 2013). The bacterial cell division protein is highly conserved throughout prokaryotes (Pichoff and Lutkenhaus, 2007). FtsA homologues from *T. martima* and *S. aureus* have been purified and crystal structures are available, however, no structural data of *E. coli* FtsA, which we study here, is available (van den Ent, 2000, Fujita *et al*., 2013). In chapter 3, we over expressed *E. coli* FtsA and solubilised the peripheral membrane protein with SMA, before purifying the protein with conventional methods (affinity and size exclusion chromatography). The pure protein was characterised with FT-ICR mass spectrometry and CD.

MurJ is an integral membrane protein that belongs to the multidrug/oligo-saccharidyl-lipid/polysaccharide (MOP) exporter family, which are secondary active antiporters that utilise the electrochemical potential created by Na^+ or H^+ gradients (Hvorup *et al*., 2003, Kuroda and Tsuchiya, 2009, Islam and Lam, 2013, Sham *et al.*, 2014). MurJ is thought to be involved in the recycling and transport of Lipid II (a PG building block), however, limited structural information is available to test this theory. In chapter 4, we over expressed *E. coli* MurJ fused to mfGFP (MurJ-mfGFP) and solubilised the integral membrane protein with SMA, before purifying the protein with conventional methods (affinity and size exclusion chromatography). The pure protein was characterised with FT-ICR mass spectrometry and CD.

FtsW is an integral membrane that spans the cytoplasmic membrane which accommodates the transport of Lipid II (a peptidoglycan building block) through

the bacterial cytoplasmic membrane (Mohammadi *et al*., 2014). FtsW is highly conserved amongst all bacteria that synthesise peptidoglycan-containing cell walls and has no counterpart in the human genome (Mercer and Weiss, 2002). In chapter 5, we over expressed *E. coli* FtsW fused to mfGFP (FtsW-mfGFP) and solubilised the integral membrane protein with SMA, before purifying the protein with conventional methods (affinity and size exclusion chromatography).

The successful application of SMALP to purify three more membrane proteins further confirms the huge potential of the technology. The same SMALP protocol was used across all the proteins; highlighting its versatility as a solubilisation system. A simple, universal protocol allowing membrane protein solubilisation and purification has the potential to unlock a plethora of previously unattainable structural information that will have massive implications in drug development and our understanding of cellular processes.

7.1.2 Periplasmic release

Periplasmic targeting of recombinant proteins in *E. coli* offers many advantages over cytoplasmic production, – including folding benefits, increased purity and protein stability (Mergulhão *et al*., 2005). The periplasmic contents can be selectively released from the cell whilst retaining the integrity of the cytoplasmic membrane. A multitude of different methods have been shown to do this to some extent, the most popular of which is a Tris-HCl EDTA (TE) based buffer at various ingredient ratios and conditions (Quan *et al*., 2013). The issue with many available methods that exhibit limited success is that no universal method exists

for selective periplasmic release. This is holding back the potential of the periplasmic targeting of recombinant proteins.

Styrene maleic acid copolymer (SMA) has demonstrated the ability to produce SMA lipid particles (SMALP) by removing 9-11 nm discs of membrane bilayer and any attached proteins (Knowles *et al*., 2009). The results presented in chapter 6 investigate the theory that SMA could be exploited to compromise the outer membrane of gram-negative bacteria, causing leakage of the periplasmic contents whilst leaving the cytoplasm of the bacteria intact. Two fluorescent, periplasm-targeted proteins (Peri-sfGFP and Peri-mCherry) were exploited to identify how SMA compares to TE - a traditional periplasmic release method.

We proved that SMA is more effective releasing both Peri-sfGFP and Peri-mCherry strains when compared to the existing TE method (214 % and 250 %, respectively). This extra release in protein was specific to the cellular periplasmic contents, since across both strains the specific activity of SMA-mediated release compares favorably to that of TE-mediated release. A cheap, versatile protocol for periplasmic release would go a long way to helping the perisplasmic expression of proteins reach its potential.

7.2 Future work

7.2.1 Membrane proteins

The solubilising and subsequent purification of three more membrane proteins with the application of SMALP suggests that the technology will be applicable to a large number of membrane proteins, making it an extremely valuable tool for biochemists. Structural data from active membrane proteins within their native environment remains a long term goal for work with SMA, however, in the short term this report acts as a great foundation for future work with the three purified membrane proteins – FtsA, MurJ and FtsW.

In chapter 3, we reported an unfolded section of FtsA that we hypothesised was due to the C-terminal 6xHis purification tag. To gather more information about how this 6xHis tag and the N-terminal 6xHis tag are affecting the function of FtsA within the SMALP, the two tags can be systematically removed to assess the impact they have on the amount of unfolded protein (identifiable using CD). Removing one of the purification tags will likely affect the ease in which the protein can be purified with Ni^{2+}-NTA, as such, changes will have to be made to the affinity chromatography stage. Furthermore, the C-terminal 6xHis-tag will need to be removed before any binding studies can be completed with FtsZ. FtsA contains a highly conserved 10-13 amino acid C-terminal motif that is key to binding FtsZ (Gayda *et al*., 1992, Löwe and van den Ent, 2001).

In chapter 4, we purified an *E. coli* MurJ-mfGFP fusion protein. The fluorescent properties of mfGFP will allow colorimetric assays of the pure protein, which may add insight to the proteins structure and the function of MurJ *in vivo*.

Similarly, in chapter 5 we purified an *E. coli* FtsW-mfGFP fusion protein. Although, the next step with this protein is to optimise expression levels to create more protein for downstream study, the same colorimetric assays can be completed with the FtsW-mfGFP. Literature suggests that the roles of FtsW and MurJ in the cell are heavily linked to each other, and the movement and recycling of Lipid II. Experimenting with Lipid II across both proteins would add great insight to the roles of these two proteins in peptidoglycan synthesis and cell division.

7.2.2 Periplasmic release

The results from chapter 6 examining SMA as a potential periplasmic release agent are extremely encouraging. The research from this report will act as a great foundation for investigating the potential of the styrene maleic acid copolymer (SMA) as a periplasmic release reagent. Extra testing for cytoplasmic markers could be included in further work to further corroborate the specific activity results that suggest the release of periplasmic contents is specific. Another way to extend this study would be to increase the scale of the experiments detailed in this report. Up-scaling could easily be easily achieved with the correct equipment and would result in more dry cell pellet that can be added to the same buffer volume, increasing the relative concentration of the Peri-sfGFP and Peri-mCherry. This could potentially increase the mCherry concentration to such a level that its release and subsequent study would produce a smoother fluorescence spectrum with less error between replicates. This experimental protocol could also be expanded to incorporate more existing

periplasmic release buffers and methods (in terms of incubation times and temperatures).

Another way to expand our understanding of the process would be to complete further experimentation on samples created with the existing protocol documented in this report. Expression levels could be more accurately estimated and compared with Western blots if the correct antibodies (Peri-sfGFP and Peri-mCherry contain a C-terminal 6xHN purification tag) are sourced and the samples are prepared correctly. Furthermore, if the proteins are at a sufficient concentration, uv/vis spectroscopy could be used to provide complementary evidence to the fluorescence data. Absorbance is less sensitive than fluorescence, however, the results output are not dependent on quantum yield, meaning it could be an ideal method to study the release of Peri-mCherry.

To summarise, in this chapter we have demonstrated that SMA has great potential as a periplasmic release reagent. Across both the Peri-sfGFP and Peri-mCherry strains SMA proved more effective at releasing the periplasmic contents.

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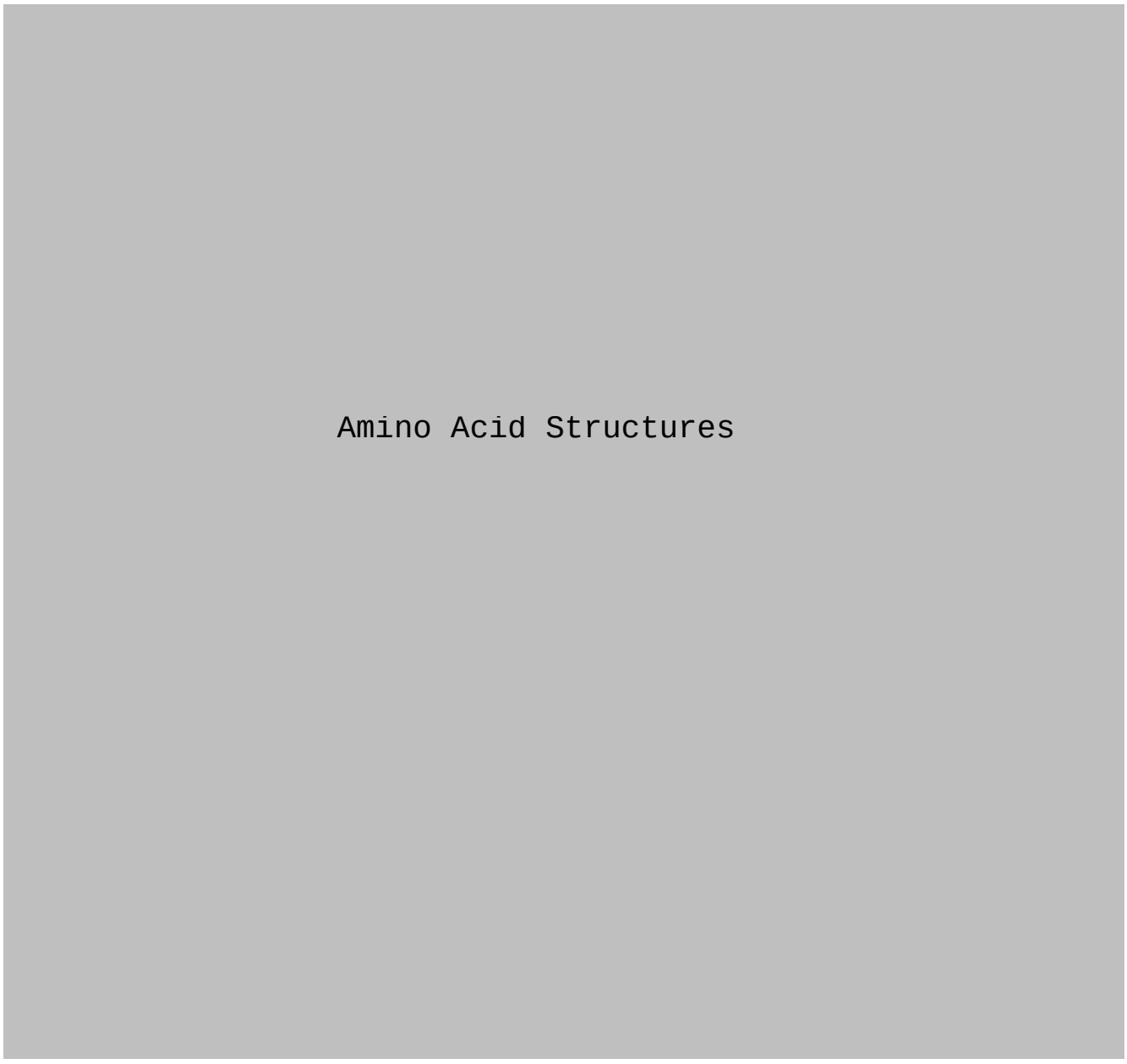
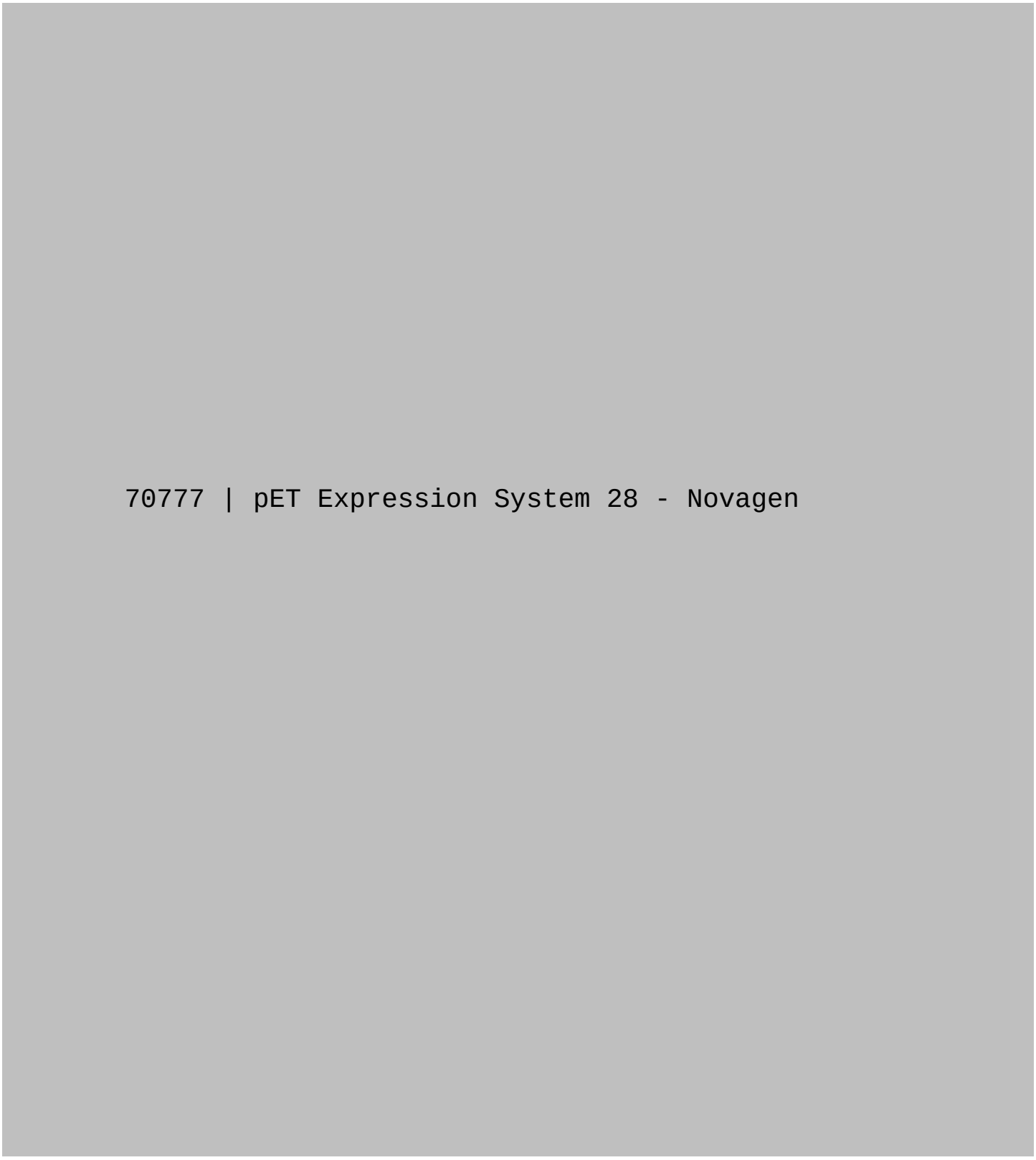
Appendix

Standard 3 and 1 letter amino acid abbreviations and structures



The *E. coli* expression vector pET-32 Xa/LIC (FtsA)

The *E. coli* expression vector pET-28a(+) (FtsW-mfGFP and MurJ-mfGFP)



The *E. coli* expression vector pEcoli-6xHN-GFPuv (Peri-sfGFP and Peri-mCherry)

pEcoli-6xHN-GFPuv Vector Information

Cat. Nos. 631417 & 631418