UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING, SCIENCE & MATHEMATICS

School of Chemistry

Applications of Sortase A from *Staphylococcus aureus*

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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APPLICATIONS OF SORTASE A FROM *STAPHYLOCOCCUS AUREUS*

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Sortase A (SrtA) mediated ligation provides a mild and site-specific method for attaching a wide range of molecular probes to biological molecules, such as proteins and DNA. This method does not require any toxic reagents or harsh conditions to achieve high level of labelling, and the product could be easily isolated from the reaction mixture. This new labelling techniques could potentially improve exisiting technologies for studying molecular interations. A number of cloning and protein expression systems for the SrtA system were developed and the resulting proteins were very stable and in high purity. The use of this technique for site-specific protein labelling was investigated. A range of molecules, such as fluorecein derivitives and 25 bp double-stranded DNA, were successfully ligated to the target proteins in good yield. The use of the SrtA system in protein immobilisation was also thoroughly studied. A number of proteins with distintly different functionalties, such as fluorescent proteins (BFP, EGFP, DsRed), enzyme (Fpr) and DNA-binding protein (Tus), were successfully immobilised onto highly cross-linked polymeric beads (GMA), soft-polymer resin matrix (Affi-gel) and glass surfaces. The amount of non-specifically bound protein onto these surfaces was found to be neglectable and the activity of the attached protein was retained.

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DECLARATION OF AUTHORSHIP

I, Lok See Lilyan Chan, declare that the thesis entitled 'Applications of Sortase A from *Staphylococcus aureus*' and the work presented in it are my own. I confirm that:

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Abbreviations

BFP:	*Aequorea victoria* green fluorescent protein blue variant
BSA:	Bovine Serum Albumin
C:	Cysteine
CDDP:	c*is*-diamminedichloroplatinum (II)
Cy3:	Cyanine dye, ~550 nm excitation, ~570 nm emission
Cy5:	Cyanine dye, ~650 nm excitation, ~670 nm emission
DMF:	*N, N*-Dimethylformamide
DNA:	Deoxyribonucleic acid
dNTPs:	Deoxynucleotide triphosphates
DsRed:	*Discosoma sp.* red fluorescent protein variant
DTT:	Dithiothreitol
ECFP:	Enhanced Cyan Fluorescent Protein
E. coli:	Escherichia coli
EDC:	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA:	Ethylenedinitrilotetraacetic acid
EGFP:	Enhanced Green Fluorescent Proteins
FACS:	Fluorescence-activated cell sorter
FITC:	Fluorescein isothiocyanate
FPLC:	Fast protein liquid chromatography
FRET:	Fluorescence resonance energy transfer
GFP:	*Aequorea victoria* green fluorescent protein
GG-DNA:	Diglycine-DNA
GGF:	Diglycine-fluorescein
GGGF:	Triglycine-fluorescein

GGGGF:	Tetraglycine-fluorescein
GMA:	Glycidyl meracrylate
GST:	Glutathione S- transferase
His:	Histidine
His_6-tag:	Six Histidine tag
HOBt:	Hydroxybenzotriazole
HPLC:	High performance liquid chromatography
IC_{50}:	Half maximal inhibitory concentration
Intein:	Saccharomyces cerevisiae VMA1 intein
IPTG:	Isopropyl β- D -1-thiogalactopyranoside
K_d:	Equilibrium Dissociation constant
kDa:	Kilo Dalton
Km:	Michaelis constant
L:	Leucine
LB medium:	Luria broth
LC:	Liquid chromatography
LPNTA:	Leucine – Proline – Asparagine – Threonine – Alanine
LPXTG:	Leucine – Proline – any amino acid – Threonine – Glycine
MBP:	Myelin basic protein
MOPS:	4-Morpholinepropanesulfonic acid
MRSA:	Methicillin-resistant *S. aureus*
MS:	Mass spectrometry
MW:	Molecular weight
NADH:	Nicotinamide adenine dinucleotide
NADPH:	Nicotinamide adenine dinucleotide phosphate-oxidase
NMR:	Nuclear magnetic resonance

NPQTN:	Asparagine – Proline – Glutamine – Threonine – Asparagine
OD_{600}:	Optical density at 600 nm
p:	Plasmid
PBS:	Phosphate Buffered Saline
PCR:	Polymerase chain reaction
S:	Serine
S. aureus:	Staphylococcus aureus
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	SDS polyacrylamide gel electrophoresis
S. pneumoniae:	Staphylococcus pneumoniae
SrtA:	Sortase A
SSSS:	Staphylococcal scalded skin syndrome
T:	Threonine
TBB:	Tus binding buffer
TBTU:	*N,N,N′,N′*-Tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate
TCEP:	Tricarboxyethylphosphine
TEMED:	*N,N,N′,N′*-Tetramethylethylenediamine
Tris:	Tris(hydroxymethylethyl)aminomethane
UV:	Ultraviolet

Chapter 1: Introduction

Staphylococcus aureus (*S. aureus*) is a gram positive bacterium. It was first discovered by Sir Alexander Ogston from Scotland[1] and can be isolated from over 40% of the human population.[2,3] It causes abscesses in many different organ tissues, septicaemia and other diseases. It has a high occurrence of infections and death rate.[4,5] In infants, an infection of *S. aureus* can cause Staphylococcal scalded skin syndrome (SSSS).[6] *S. aureus* is also responsible for the most common postsurgical infections and nosocomial infections. Antibiotics such as β-lactam antibiotics (e.g. penicillin) and other anti-bacterial drugs like macrolides and fluoroquinolones are normally used for treating *S. aureus* infections.[7] However, under excessive use of antibiotic through out the past years, some strains of *S. aureus* have gained resistance to many, if not all, antibiotics treatments. Most notable antibiotic-resistant strains are Methicillin-resistant *S. aureus* (MRSA) and Vanomycin-resistant *S. Aureus* (VRSA, contain a vancomycin resistant gene, *VanA*) which are capable of carrying out normal bacterial functions, such as transpeptidation, even in the present of antibiotics.[8] The excessive use of antibiotic treatment has lead to an increase in MRSA infections in hospitals and other communities to young adults and new born babies.[9-13] Patients infected with such disease even under medical care could be fatal if it was not diagnosed early and treated aggressively.[14]

In gram positive bacteria, the cell wall is generally composed of a primary polymer, peptidoglycan, and a secondary polymer, such as proteins and carbohydrates immobilised in the peptidoglycan structure. In *S. aureus,* the most common surface protein is protein A, an immunoglobulin-binding protein,[15,16] which is covalently attached to the pentaglycine on a cell wall precursor, lipid II. Once lipid II is incorporated into the cell

wall, surface proteins are available to interact with the cell surface of host tissues and cause infections.[17] The covalent attachment of surface proteins to pentaglycine of lipid II is catalysed by sortase A (SrtA). It has been demonstrated that in the absence of SrtA gene (*srtA) S. aureus* failed to anchor surface proteins to the cell wall precursor and as a result reduced infectivity to the host cell dramatically.[18] This finding made SrtA a potential drug target for future anti-bacterial drug developments.

1.1) Sortase

Sortase, a transpeptidase, is a bacterial enzyme found in a wide range of gram-positive bacteria. It is responsible for the covalent attachment of bacterial surface proteins to peptidoglycan of the cell wall in bacteria. There are four main classes of sortase, namely class A, B, C and D.[19] Different bacterias have different numbers and types of sortases. For example, *S. aureus* has four different sortases, with SrtA and SrtB the most important ones;[20] *S. pneumoniae* has up to four different sortases[21] and *Corynebacterium effciens* up to ten.[22] Each class recognises different amino acid sequences of the motif. SrtA recognises LPXTG, where X can be any amino acid. SrtB recognises the C-terminal NPQTN motif; SrtC the LPNTA motif and SrtD recognises LPXTG motif in the pilin protein (BcpA) precursor.[23-27]

The structure of *S. aureus* SrtA has recently been solved by nuclear magnetic resonance (NMR) spectroscopy (**Figure 1.1**). The enzyme has a unique β-barrel structure with eight β-strands aligned in an antiparallel and parallel fashion.[28]

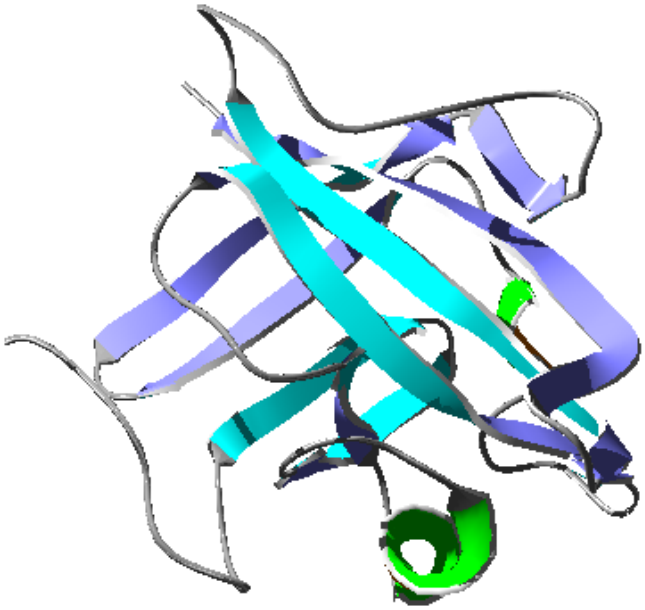


Figure 1.1 NMR structure of S. aureus SrtA protein.[28]

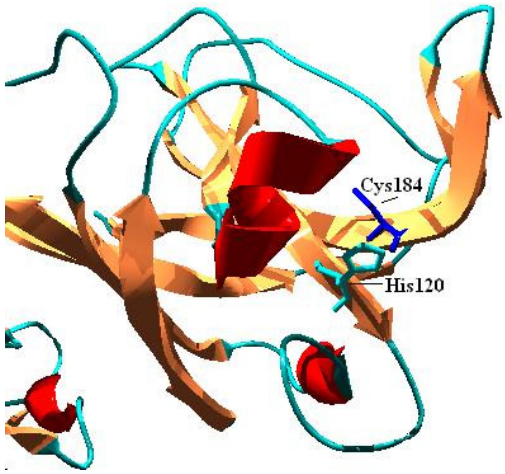


Figure 1.2 NMR structure of the catalytic pocket of S. aurues SrtA showing the side chain of His-120 and Cys-184 at the active site.[28]

At the active site the Cys-184 and His-120 ion pair[28,29] is located in the catalytic pocket which is formed by the residues in the loops at one end of the β-barrel (Figure 1.2). Cys-184 is activated by the imidazole ring of His-120[28,30] for mediating the

transpeptidation. It was found that in the present of Ca^{2+} the activity of SrtA increased.[28] Some suggested that the increase in enzymatic activity was due to modulation of the structure and dynamics of the active site loop by the cation.[31]

1.2) The action of SrtA in bacteria

At the N- terminus, a signal peptide directs the surface proteins to the secretory pathway on the cytoplasmic membrane. The signal peptide is then cleaved by signal peptidase after translocation of the surface protein across the cytoplasmic membrane. At the C-terminus there are three important features for the anchoring of surface protein to the bacterial cell wall:[29,32,33]

An LPXTG motif (Leucine, Proline, X, Threonine and Glycine. X: any amino acid)

A hydrophobic region

A tail of charged residues

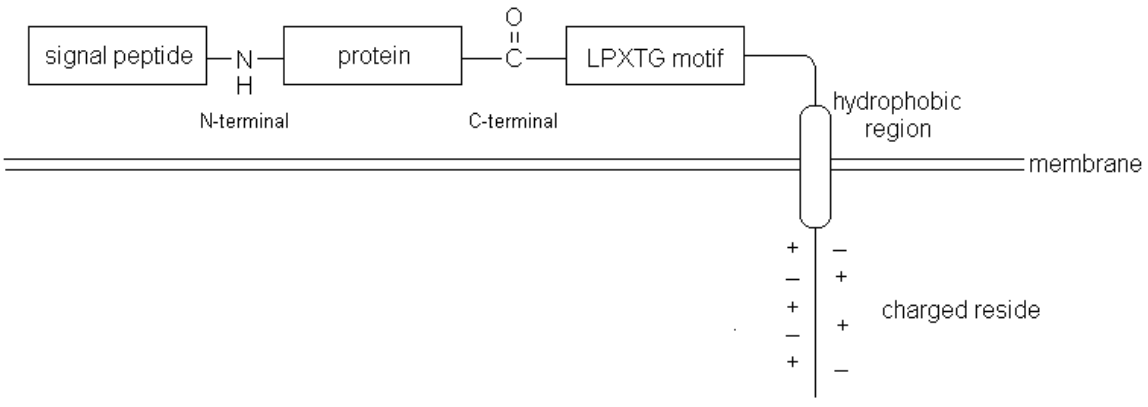


Figure 1.3 The secreted protein. The signal peptide directs the immatured-protein through the secretory pathway. The hydrophobic region and the charged residue lock the protein in position in the membrane.

The hydrophobic region and the charged residues help to position the protein substrate in

the cell membrane allowing SrtA to recognise the LPXTG motif.[34]

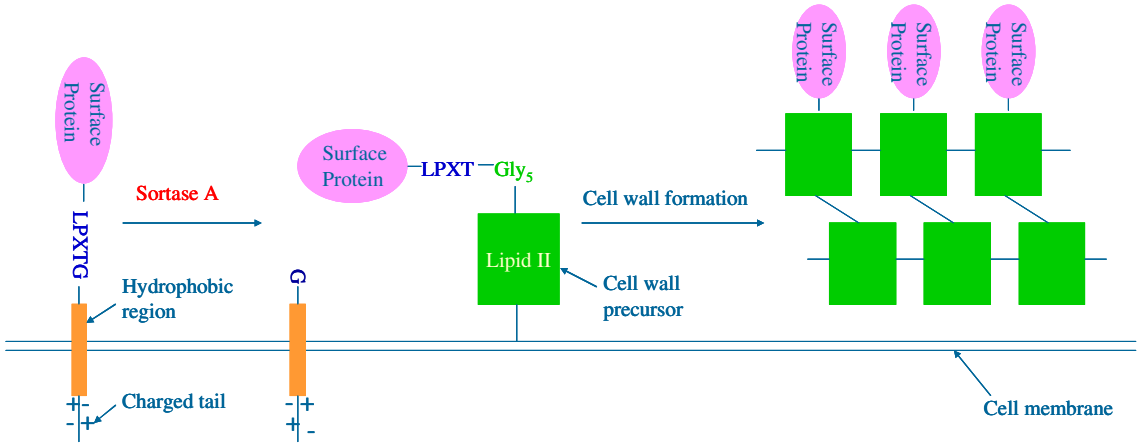


Figure 1.4 The SrtA-mediated cell surface protein anchoring. The signal peptide directs the target protein through the secretory pathway where the hydrophobic region and the charged residue hold the protein in position in the membrane. Afterwhich the signal peptide is cleaved by signal peptidase. SrtA recognises the LPXTG motif of the target protein and catalyses the tranpeptidation. Once the surface protein is incorporates with the cell wall precursor, the precursor is polymerisaed into a matured peptidoglycan.

Once the protein is held in position in the membrane, the sulfhydryl group of Cys-184 in SrtA carries out a nucleophilic attack on the amide bond between threonine and glycine of the LPXTG motif to form a thioester bond. The amine group of the pentaglycine unit of Lipid II then acts as a nucleophile, attacking the carbonyl group of thioester formed by SrtA to form an amide bond.[35-37]

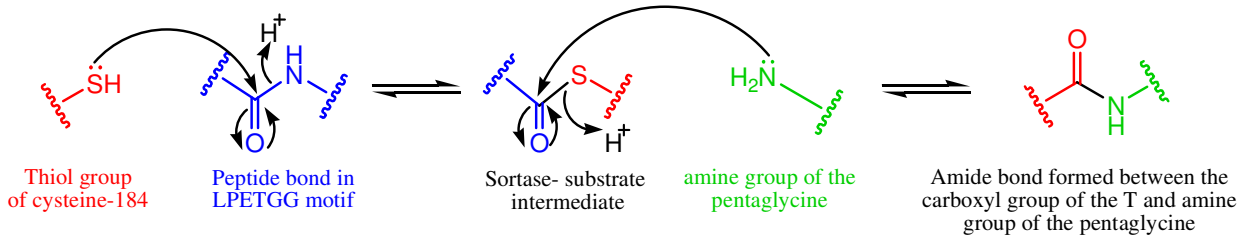


Figure 1.5 Schematic diagram of the SrtA-mediated transpeptidation.

Once the surface protein is covalently attached to lipid II, the precursor is polymerised into matured peptidoglycan and forms the bacterial cell wall[38-40] with covalently incorporated proteins on the surface.[41]

The mechanism of SrtA catalysed transpeptidation has been studied using a synthetic substrate Abz-LPXTG-Dap(Dnp)-OH by fluorescence measurement. In the presence of the fluorophore–quencher (Abz-Dnp pair) labelled LPXTG motif and a pentaglycine substrate, SrtA cleaved the amide bond between threonine and glycine of the LPXTG motif, removing the quencher from the synthetic substrate and covalently links threonine to the pentaglycine forming Abz-LPXTGGGGG-OH and NH_2-G-Dap(Dnp)-OH (Figure 1.6).

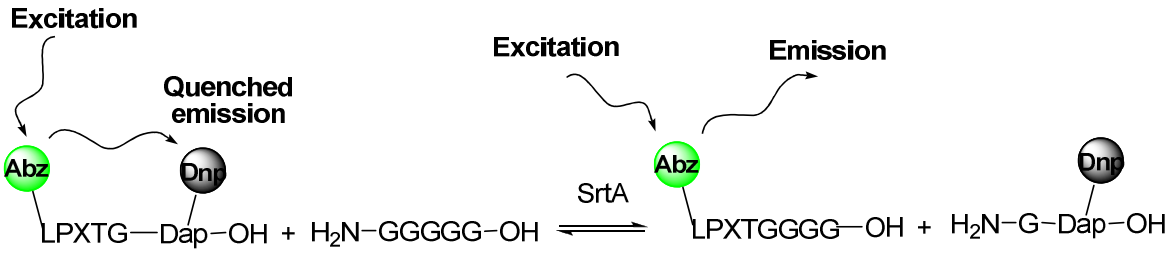


Figure 1.6 Schematic diagram of the SrtA substrate analysis.

As the Abz and Dnp pair were no longer in close proximity in the product, the emitted fluorescence by Abz was not quenched by Dnp. This in theory allows detection and quantification of the product by measuring the emitted fluorescence. However, in

practice the fluorophore attached to the LPXTG motif self-quenched at moderaetly high concentration and therefore affecting the measurement for kinetic analysis. An improved method which measured the formation of product by HPLC based on UV/Vis absorbance or fluorescence was used.[42] By using the this method, the estimated K_m and K_{cat} for SrtA using tetraglycine as substrate were determined to be 5.5 mM and 0.270 s^{-1} repectively, which is relatively fast.[42] The kinetic mechanism of different substrates has been studied using the same methodology.[35]

1.3) Sortase A inhibition

Sortase plays an important role in anchoring different surface proteins onto the cell wall precursor. In *S. aureus*, mutants that cannot express SrtA have shown dramatically reduced infectivity to the host cells.[17] There is a growing interest to inhibit SrtA and other similar proteins in the hope of treating bacterial infections, such as MRSA and VRSA, using novel anti-bacterial drugs. The chemical that inhibits sortase, which in turn prevents the cell wall anchoring reaction, was found even before the identification of sortase.[43] While later, Vancomycin and Moenomycin were also found to reduce the amount of surface proteins being anchored onto the cell wall precursor,[43] and compounds including sulfhydryl group, such as organomercurials and methanethiosulfonates, also show inhibitory of the surface protein anchoring reaction.

Not only synthetic compounds have been tested for the inhibition of SrtA, natural products extracted from plants and marine invertebrate also found to inhibit the enzyme. Compounds from eighty medicinal plants from Korea were tested for the inhibitory effects on sortase.[41] A specific compound, β-sitosterol-3-*O*-glucopyranoside, isolated from *Fritillaria verticillata* was found to have an inhibitory effect on sortase with an IC_{50} of

18.3 μg/mL.[44] Another example of natural compounds with inhibitory effect on sortase was Isoquinoline alkaloids, isolated from the rhizomes of *Coptis chinensis*. It was determined to have an IC_{50} of 8.7 μg/mL.[45] Extracts of a marine invertebrate, *Topsentai genitrix*, which is known to have antimicrobial activity was also tested for the inhibition of sortase. A compound was found in the extract, identified as bis(indole) alkaloids with an IC_{50} of ~15 μg/mL.[46]

1.4) Protein microarrays

With its unique property in catalysing specific substitution reactions, sortase has many potential applications, such as in biological molecule labelling and site-specific immobilisation. These will be discussed in more details in Chapter 3 and 4.

One of the promising technology to study protein–molecule interaction is protein microarrays. It has been demonstrated in a wide range of fields, *in vitro* diagnostic immunoassays and drug discovery. This method was first demonstrated by MacBeath *et al.* in 2000,[63] where the methodology was "borrowed" from then exisiting DNA microarrays. In this demonstration, proteins, such as BSA and protein G, were spotted directly on to aldehyde-functionalised glass-slide using a commercially available DNA arrayer. The immobilised proteins showed some degree of functionality. Modified methodologies were developed to immobilise different proteins. For instance, in another example, glass slides coated with avidin were used to capture biotinylated protein probes, namely EGFP, MBP and GST.[47,48] Afterwhich, the slide was immersed in a solution of Cy3-labelled glutathione, a ligand for GST.[47,49] The result showed that only area spotted

with GST reacted with the Cy3-labelled substrate, whereas other areas had little reactions.

Figure 1.7 The protein probes were spotted onto the avidin plate after which a Cy3-labelled substrate for GST was added. Only the area spotted with GST reacted with the substrate.

This powerful technique provides many solutions to the ever growing protein analysis problems. The glass slides can accommodate an ultra-high density of proteins, each with nanoliter volume. With the on-going development of automated instrumentations, a high number of protein-based analysis could be carried out in a highly parallel manner.

1.5) Suspension Arrays

Three-dimentional micron-sized solid supports have many applications, such as chromatography and biological assays in suspension arrays. One application is the purification of nuclear proteins that binds to cisplatin (*cis*-diamminedichloroplatinum (II), CDDP),[50] a chemotherapy agent, which is used to treat different kinds of caner. At the point of contact between the CDDP and DNA molecule , it causes the DNA to unwind and bend. The CDDP binds to the cancer cell DNA and causes different effects, including phosphorylation of tumor suppressor protein p53, which causes apoptosis[51] and DNA repair.[52] The protein was being purified using affinity chromatography on cellulose, but

the efficiency was very low as it was hard to make the protein to bind to the column.[53] Noval submicron-sized latex beads were synthesised from the co-polymerisation of styrene and GMA in soap free aqueous solution[54] for the attachment of CDDP-DNA.

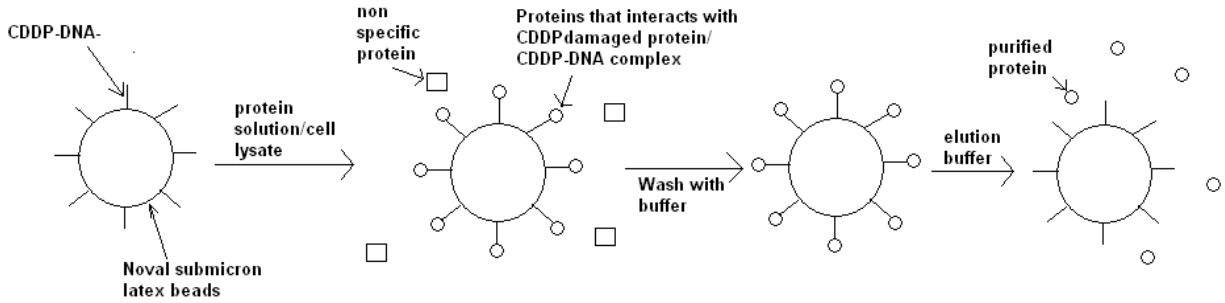


Figure 1.8 The pathway of the CDDP-DNA binding protein purification. The CDDP-DNA-bead was loaded into the protein solution, the CDDP-DNA binding protein or proteins that recognise the CDDP-damaged DNA will attach to the beads. The beads were washed with buffer and washed with elution buffer to elute the protein out from the beads.

The beads were then incubated in the protein solution for the attachment of specific proteins that interact with CDDP-DNA complex or CDDP-damaged DNA. The purification affinity of the CDDP-DNA-beads has a higher level of protein purified than the DNA-beads. The immobolisation of proteins onto surfaces has been done by using reactive groups, such as carboxyl-, amino- or sulfide groups on the protein and covalently linked to appropriate functional groups on the solid surface.[55]

However, the lack of specificity results in proteins attached on the surface in random orientation. As a result, the protein probe might not retain its activity and substrates may not be able to access to the binding site. In addition, the protein solution has to be extremely pure in order to prevent contamination during the coupling reaction. These

disadvantages could potentially be solved by affinity binding where a synthetic tag on the protein probe is captured by its counterpart on the suface.[56] Examples include the His-tag/Ni-affinity system and protein A/G for immunoglobulin capturing.[57,58]

We would like to use the SrtA to provide a new, mild and site-specific method for labelling biological molecules such as proteins or DNA. In addition, we would hope to use SrtA-mediated ligation for immobilising proteins onto a range of solid supports and surfaces.[59-62]

1.6) Aim of this project

In this project, we aimed to develop novel applications of SrtA. In particular, new methodologies for *in vitro* labelling of biological molecules and novel protein immobilisation techniques.

To this end, we wanted to clone, express and purify a number of proteins, such as His_6-SrtA, BFP-, EGFP-, DsRed- and Fpr-LPETGG-His_6 proteins. These proteins would then be used for a number of experiments to demonstrate the SrtA-mediated reactions.

For protein immobilisation, we also wanted to study the functionality of the immobilised protein probe molecules.

1.7) References

(1) Ogston. *Rev Infect Dis* **1984**, *6*, 122-8.

(2) Lowy, F. D. *N Engl J Med* **1998**, *339*, 520-32.

(3) Peacock, S. J.; de Silva, I.; Lowy, F. D. *Trends Microbiol* **2001**, *9*, 605-10.

(4) Jonsson, I. M.; Mazmanian, S. K.; Schneewind, O.; Verdrengh, M.; Bremell, T.; Tarkowski, A. *J Infect Dis* **2002**, *185*, 1417-24.

(5) Weiss, W. J.; Lenoy, E.; Murphy, T.; Tardio, L.; Burgio, P.; Projan, S. J.; Schneewind, O.; Alksne, L. *J Antimicrob Chemother* **2004**, *53*, 480-6.

(6) Curran, J. P.; Al-Salihi, F. L. *Pediatrics* **1980**, *66*, 285-90.

(7) Lowy, F. D. *J Clin Invest* **2003**, *111*, 1265-73.

(8) Jevons, M. P.; Coe, A. W.; Parker, M. T. *Lancet* **1963**, *1*, 904-7.

(9) Fridkin, S. K.; Hageman, J. C.; Morrison, M.; Sanza, L. T.; Como-Sabetti, K.; Jernigan, J. A.; Harriman, K.; Harrison, L. H.; Lynfield, R.; Farley, M. M. *N Engl J Med* **2005**, *352*, 1436-44.

(10) Noskin, G. A.; Rubin, R. J.; Schentag, J. J.; Kluytmans, J.; Hedblom, E. C.; Jacobson, C.; Smulders, M.; Gemmen, E.; Bharmal, M. *Clin Infect Dis* **2007**, *45*, 1132-40.

(11) Klevens, R. M.; Morrison, M. A.; Fridkin, S. K.; Reingold, A.; Petit, S.; Gershman, K.; Ray, S.; Harrison, L. H.; Lynfield, R.; Dumyati, G.; Townes, J. M.; Craig, A. S.; Fosheim, G.; McDougal, L. K.; Tenover, F. C. *Emerg Infect Dis* **2006**, *12*, 1991-3.

(12) Klevens, R. M.; Edwards, J. R.; Tenover, F. C.; McDonald, L. C.; Horan, T.; Gaynes, R. *Clin Infect Dis* **2006**, *42*, 389-91.

(13) Klevens, R. M.; Morrison, M. A.; Nadle, J.; Petit, S.; Gershman, K.; Ray, S.; Harrison, L. H.; Lynfield, R.; Dumyati, G.; Townes, J. M.; Craig, A. S.; Zell, E. R.; Fosheim, G. E.; McDougal, L. K.; Carey, R. B.; Fridkin, S. K. *JAMA* **2007**, *298*, 1763-71.

(14) Gillet, Y.; Issartel, B.; Vanhems, P.; Fournet, J. C.; Lina, G.; Bes, M.; Vandenesch, F.;

Piemont, Y.; Brousse, N.; Floret, D.; Etienne, J. *Lancet* **2002**, *359*, 753-9.

(15) Cossart, P.; Jonquieres, R. *Proc Natl Acad Sci U S A* **2000**, *97*, 5013-5.

(16) Uhlen, M.; Guss, B.; Nilsson, B.; Gotz, F.; Lindberg, M. *J Bacteriol* **1984**, *159*, 713-9.

(17) Bierne, H.; Mazmanian, S. K.; Trost, M.; Pucciarelli, M. G.; Liu, G.; Dehoux, P.; Jansch, L.; Garcia-del Portillo, F.; Schneewind, O.; Cossart, P. *Mol Microbiol* **2002**, *43*, 869-81.

(18) Mazmanian, S. K.; Liu, G.; Jensen, E. R.; Lenoy, E.; Schneewind, O. *Proc Natl Acad Sci U S A* **2000**, *97*, 5510-5.

(19) Dramsi, S.; Trieu-Cuot, P.; Bierne, H. *Res Microbiol* **2005**, *156*, 289-97.

(20) Mazmanian, S. K.; Ton-That, H.; Schneewind, O. *Mol Microbiol* **2001**, *40*, 1049-57.

(21) Tettelin, H.; Nelson, K. E.; Paulsen, I. T.; Eisen, J. A.; Read, T. D.; Peterson, S.; Heidelberg, J.; DeBoy, R. T.; Haft, D. H.; Dodson, R. J.; Durkin, A. S.; Gwinn, M.; Kolonay, J. F.; Nelson, W. C.; Peterson, J. D.; Umayam, L. A.; White, O.; Salzberg, S. L.; Lewis, M. R.; Radune, D.; Holtzapple, E.; Khouri, H.; Wolf, A. M.; Utterback, T. R.; Hansen, C. L.; McDonald, L. A.; Feldblyum, T. V.; Angiuoli, S.; Dickinson, T.; Hickey, E. K.; Holt, I. E.; Loftus, B. J.; Yang, F.; Smith, H. O.; Venter, J. C.; Dougherty, B. A.; Morrison, D. A.; Hollingshead, S. K.; Fraser, C. M. *Science* **2001**, *293*, 498-506.

(22) Paterson, G. K.; Mitchell, T. J. *Trends Microbiol* **2004**, *12*, 89-95.

(23) Maresso, A. W.; Chapa, T. J.; Schneewind, O. *J Bacteriol* **2006**, *188*, 8145-52.

(24) Mazmanian, S. K.; Skaar, E. P.; Gaspar, A. H.; Humayun, M.; Gornicki, P.; Jelenska, J.; Joachmiak, A.; Missiakas, D. M.; Schneewind, O. *Science* **2003**, *299*, 906-9.

(25) Mazmanian, S. K.; Liu, G.; Ton-That, H.; Schneewind, O. *Science* **1999**, *285*, 760-3.

(26) Ton-That, H.; Liu, G.; Mazmanian, S. K.; Faull, K. F.; Schneewind, O. *Proc Natl Acad Sci U S A* **1999**, *96*, 12424-9.

(27) Budzik, J. M.; Marraffini, L. A.; Souda, P.; Whitelegge, J. P.; Faull, K. F.; Schneewind, O. *Proc Natl Acad Sci U S A* **2008**, *105*, 10215-20.

(28) Ilangovan, U.; Ton-That, H.; Iwahara, J.; Schneewind, O.; Clubb, R. T. *Proc Natl*

Acad Sci U S A **2001**, *98*, 6056-61.

(29) Marraffini, L. A.; Ton-That, H.; Zong, Y.; Narayana, S. V.; Schneewind, O. *J Biol Chem* **2004**, *279*, 37763-70.

(30) Ton-That, H.; Mazmanian, S. K.; Alksne, L.; Schneewind, O. *J Biol Chem* **2002**, *277*, 7447-52.

(31) Naik, M. T.; Suree, N.; Ilangovan, U.; Liew, C. K.; Thieu, W.; Campbell, D. O.; Clemens, J. J.; Jung, M. E.; Clubb, R. T. *J Biol Chem* **2006**, *281*, 1817-26.

(32) Lee, S. F.; McGavin, M. K. *Infect Immun* **2004**, *72*, 4314-7.

(33) Liew, C. K.; Smith, B. T.; Pilpa, R.; Suree, N.; Ilangovan, U.; Connolly, K. M.; Jung, M. E.; Clubb, R. T. *FEBS Lett* **2004**, *571*, 221-6.

(34) Strominger, J. L. *Harvey Lect* **1968**, *64*, 179-213.

(35) Huang, X.; Aulabaugh, A.; Ding, W.; Kapoor, B.; Alksne, L.; Tabei, K.; Ellestad, G. *Biochemistry* **2003**, *42*, 11307-15.

(36) Novick, R. P. *Trends Microbiol* **2000**, *8*, 148-51.

(37) Ruzin, A.; Severin, A.; Ritacco, F.; Tabei, K.; Singh, G.; Bradford, P. A.; Siegel, M. M.; Projan, S. J.; Shlaes, D. M. *J Bacteriol* **2002**, *184*, 2141-7.

(38) Frankel, B. A.; Kruger, R. G.; Robinson, D. E.; Kelleher, N. L.; McCafferty, D. G. *Biochemistry* **2005**, *44*, 11188-200.

(39) Perry, A. M.; Ton-That, H.; Mazmanian, S. K.; Schneewind, O. *J Biol Chem* **2002**, *277*, 16241-8.

(40) Ton-That, H.; Marraffini, L. A.; Schneewind, O. *Biochim Biophys Acta* **2004**, *1694*, 269-78.

(41) Kim, S. W.; Chang, I. M.; Oh, K. B. *Biosci Biotechnol Biochem* **2002**, *66*, 2751-4.

(42) Kruger, R. G.; Dostal, P.; McCafferty, D. G. *Anal Biochem* **2004**, *326*, 42-8.

(43) Ton-That, H.; Schneewind, O. *J Biol Chem* **1999**, *274*, 24316-20.

(44) Kim, S. H.; Shin, D. S.; Oh, M. N.; Chung, S. C.; Lee, J. S.; Chang, I. M.; Oh, K. B. *Biosci Biotechnol Biochem* **2003**, *67*, 2477-9.

(45) Kim, S. H.; Shin, D. S.; Oh, M. N.; Chung, S. C.; Lee, J. S.; Oh, K. B. *Biosci Biotechnol Biochem* **2004**, *68*, 421-4.

(46) Oh, K. B.; Mar, W.; Kim, S.; Kim, J. Y.; Oh, M. N.; Kim, J. G.; Shin, D.; Sim, C. J.; Shin, J. *Bioorg Med Chem Lett* **2005**, *15*, 4927-31.

(47) Lesaicherre, M. L.; Lue, R. Y.; Chen, G. Y.; Zhu, Q.; Yao, S. Q. *J Am Chem Soc* **2002**, *124*, 8768-9.

(48) Paborsky, L. R.; Dunn, K. E.; Gibbs, C. S.; Dougherty, J. P. *Anal Biochem* **1996**, *234*, 60-5.

(49) Yin, J.; Liu, F.; Li, X.; Walsh, C. T. *J Am Chem Soc* **2004**, *126*, 7754-5.

(50) Tomohiro, T.; Sawada Ji, J.; Sawa, C.; Nakura, H.; Yoshida, S.; Kodaka, M.; Hatakeyama, M.; Kawaguchi, H.; Handa, H.; Okuno, H. *Bioconjug Chem* **2002**, *13*, 163-6.

(51)Oda, K.; Arakawa, H.; Tanaka, T.; Matsuda, K.; Tanikawa, C.; Mori, T.; Nishimori, H.; Tamai, K.; Tokino, T.; Nakamura, Y.; Taya, Y. *Cell* **2000**, *102*, 849-62.

(52) Tanaka, H.; Arakawa, H.; Yamaguchi, T.; Shiraishi, K.; Fukuda, S.; Matsui, K.; Takei, Y.; Nakamura, Y. *Nature* **2000**, *404*, 42-9.

(53) Hughes, E. N.; Engelsberg, B. N.; Billings, P. C. *J Biol Chem* **1992**, *267*, 13520-7.

(54) Wada, T.; Watanabe, H.; Kawaguchi, H.; Handa, H. *Methods Enzymol* **1995**, *254*, 595-604.

(55) Tomizaki, K. Y.; Usui, K.; Mihara, H. *Chembiochem* **2005**, *6*, 782-99.

(56) Schmidbauer, S. B.; Strobel, O. K. *Front Biosci* **1997**, *2*, c6-8.

(57) Wang, Z.; Jin, G. *J Biochem Biophys Methods* **2003**, *57*, 203-11.

(58) Rusmini, F.; Zhong, Z.; Feijen, J. *Biomacromolecules* **2007**, *8*, 1775-89.

(59) Chan, L.; Cross, H. F.; She, J. K.; Cavalli, G.; Martins, H. F.; Neylon, C. *PLoS ONE* **2007**, *2*, e1164.

(60) Clow, F.; Fraser, J. D.; Proft, T. *Biotechnol Lett* **2008**, *30*, 1603-7.

(61) Nguyen, H. D.; Schumann, W. *J Biotechnol* **2006**, *122*, 473-82.

(62) Parthasarathy, R.; Subramanian, S.; Boder, E. T. *Bioconjug Chem* **2007**, *18*, 469-76.

(63) MacBeath, G.; Schreiber, S. L. Science 2000, 289, 1760-3.

Chapter 2: Production of proteins

2.1) Introduction

Sortase-mediated ligation[1, 2] could potentially provide an improved method for protein immobilisation. There are three major methods for attaching biological molecules on surfaces: 1) passive adsorption; 2) affinity binding and 3) covalent linkage. The attachment of protein molecules on surfaces by these methods are either non-permanent or in random oreientation. These undesired properties have great impacts on reducing the activity of the immobilised proteins. Instead of directing the protein molecules to bind to the surface, porous gel matrix can be formed around the protein molecules, providing a gentle approach for trapping biological molecules and preserving the delicate protein three-dimentional structures.[3-5] The immobilisation of proteins onto surfaces will be further discussed in chapter 4.

For protein labelling, most methods involve the formation of covalent linkage between the target protein and the label. Most commercially available fluorescent dyes are pre-functionalised with reactive functional groups, such as aldehyde, NHS-ester and isothiocyanate, to simplify the attachment process. These functional groups reacts with any available free amines of the protein surface and so the reactionis non site-specific and highly uncontrollable. Another method called intein-mediated ligation which provides site-specific ligation to the target protein. In this method, a target protein is "activated" by expressing with intein (~50 kDa) as a fusion protein. The thioester linkage between the two proteins can be nucleopililly attacked by thio-containing compound, replacing the

intein portion. However, according to the three-dimentional structure of the target protein, the expression of the target-intein fusion could be difficult. The thioester linkage between the two protins can be easily hydrolysed *in vivo,* resulting in immature cleavage.[5]

Unlike intein, sortase-mediated ligation does not activate the target protein unless the enzyme is present in the reaction. This prevents immature cleavage by hydrolysis during storage. The environment for protein ligation with sortase is mild (50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.5),[6] which is suitable for most proteins.

We were interested in using the SrtA-mediated ligation for 1) labelling biological molecules for visualising molecular interactions and 2) attaching proteins onto solid surfaces for functional analysis. To this end, we selected a range of functionally different proteins for the labelling and attachment experiments (see Chapter 3 and 4 for more details).

2.1.1) Fluorescent proteins

Fluorescent proteins are extremely useful as biomolecular tags for visualising biological interactions in both *in vitro* and *in vivo* studies. The most well-known fluorescent protein is Green fluorescent protein (GFP) which is isolated from the jellyfish *Aequorea Victoria*.[7] Figure 2.1 shows the crystal structure on a monomeric GFP molecule. It has a β-barrel structure (Figure 2.1) with the matured chromophore situated inside the barrel. The chromophore is formed *in situ* using three internal amino acid residues of the peptide chain: Ser-65, Tyr-66 and Gly-67 (Figure 2.1)[8-10] Matured GFP shows maximum excitation and emission wavelengths of 488 nm and 509 nm repectively.

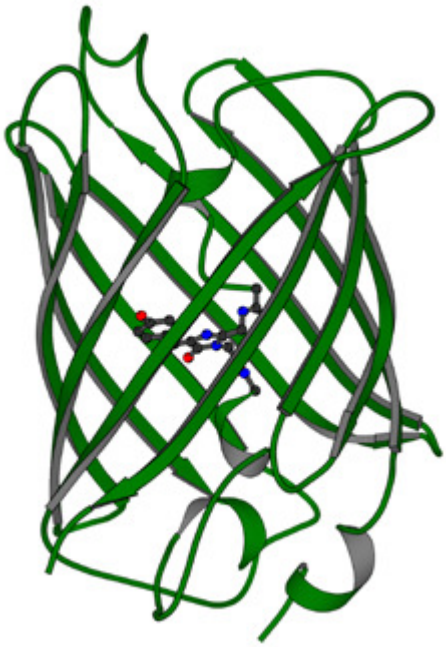


Figure 2.1 Crystal structure of monomeric GFP with the matured chromophore situated inside the β-barrel.

Many other fluorescent proteins routinely used in biological assays are mutants of GFP.[8-11] For example, Blue Fluorescent Protein (BFP) is mutated from GFP by substituting Tyr-66 with His-66.[10, 12-14] This changes the fluorescence properties of the matured chromophore where the excitation emission wavelength are shifted to 382 nm and 459 nm resepectively.

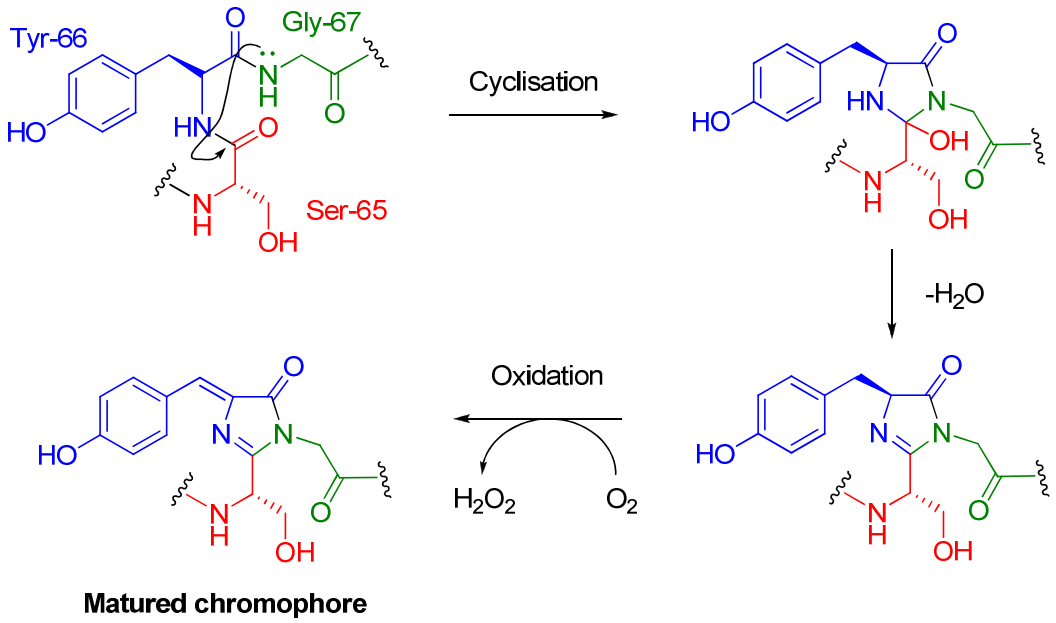


Figure 2.2 Biosynthesis of the matured GFP chromophore.

Other fluorescent proteins, such as Yellow Fluorescent Protein (YFP) and Cyan Fluorescent Protein (CFP) are prepared by a similar method. Another natural fluorescent protein, DsRed, is isolated from *Discosoma sp.*[15-17] The amino acid sequence of this protein is very different from GFP but they are structurally very similar. The maximum excitation and emission wavelengths are 545 nm and 620 nm respectively.

Dur to their excellent fluorescence properties, fluorescent proteins are extensively used in molecular biology for visualising molecular interactions. They can be easily incorporated at the N- or C-terminus of the target protein by genetic engineering and express as a fusion protein.

2.1.2) Tus

Tus is a DNA-binding protein that forms a complex with the chromosomal *ter* DNA sequence and inhibit the progression of the DNA replication fork.[18] It also inhibits the DNA replication of plasmid R6K.[19, 20] The binding of Tus to several *ter* sites was shown by footprinting with copper-phenanthroline,[21] DNase I,[22,23] and hydroxyl radicals.[24,25] Tus binds to the *terB* site with an equilibrium dissociation constant (K_d) of 3.4 X 10^{-13} M and a dissociation half-life of about 550 min at pH 7.5 *in vitro*.[24] Its binding to R6K *terR2* under the same conditions was weaker due to a higher dissociation rate.[24] Interesingly, Tus bind to *terB* as a monomer, which is unusual for a DNA-binding protein but this is consistent with the asymmetry of the *ter* sites and replication fork arrest.[26] We were interested in immobilising this DNA-binding protein to solid supports using the SrtA system and studying the protein activity after the attachment. This will be discussed in more details in Chapter 4.

2.1.3) Flavodoxin reductase

Flavodoxin (ferredoxin) reductase is found in *E. coli*[27-31] and is responsible for transporting electrons between flavodoxin and NADPH.[30, 32] It is involved in the reductive activation of pyruvate formate lyase,[33] cobalamin-independent methionine synthase,[34] and anaerobic ribonucleotide reductase.[35] It also protects against superoxide radicals due to methyl viologen in the presence of oxygen.[31] In order to understand the enzymatic activity, Wood *et al.* attempted to label Fpr with fluorescein derivities using the intein labelling system. The first step was to express Fpr-intein fusion protein. Unfortuately, the fusion protein was found to be unstable and the linkage between Fpr and the intein was hydrolysed.[5] We were interested to access the possibility of labelling such challenging protein with the novel SrtA system. This will be discussed in more details in Chapter 3.

2.2) Plasmid construction

For SrtA to recongise the target protein for labelling reactions, a LPETGG tag was added to the C-terminus of the protein, followed by a His_6 tag to assits protein purification by Ni-affinity chromatography. For the convenience for future plasmid constructions, restriction site *Nde*I was situated before the target gene, and *Xho*I was inserted by PCR between the target gene and LPETGG-His_6 tag. The target gene can be replaced by any desired gene to express different proteins tagged with LPETGG-His_6. For example, the plasmid pLLC066 (BFP-LPETGG-His_6) was constructed by introducing LPETGG-His_6 into pJS100 plasmid using PCR with lc001 and SrtAf primers.

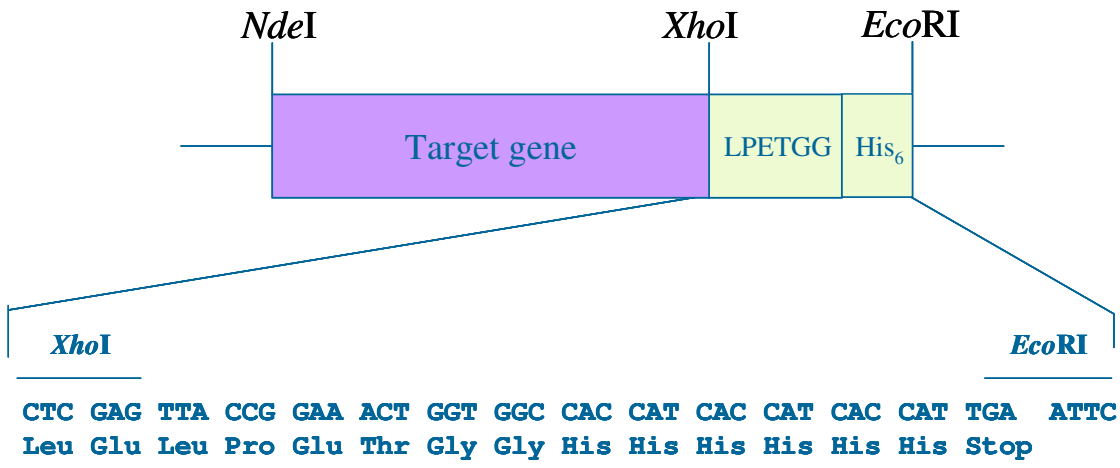


Figure 2.3 Construct design for the expression of LPETGG-His_6 tagged proteins.

2.3) Results and Discussion

2.3.1) Amplification and subcloning of *srtA*

The gene *srtA* was amplified by PCR from *S. aureus* subsp. genomic DNA (ATCC 35556) with primers *SrtA*f and *SrtA*b (section 2.5.1) which inserted a His_6 tag at the C-terminus of protein. The PCR product was digested with *Nde*I and *EcoR*I for analysis and then gel purified. The purified *his_6-srtA* gene was inserted into pETMCSIII subcloning vector (SOT012) forming pLLC064. Plasmid was transformed into *E. coli* XL-1 Blue competent cells (Clontech, CA, USA). Ampicillin-resistant colonies were selected and the desired plasmid isolated using commercially available miniprep kit according to manufacturor's instruction (Promega, Southampton, U.K.). The purified plasmid was digested by *Nde*I and *Eco*RI restriction enzymes and then analysed by gel electrophoresis. From the 1% agarose gel shown below (**Figure 2.4**) the linearlised plasmid and the *Nde*I-*Eco*RI fragments were in agreement with the theoretical value of the plasmid (5221 bp), *srtA* (555 bp) and the plasmid backbone (4666 bp).

Figure 2.4 Gel electrophoreisis analysis of the pLLC064. 1% agarose gel was stained

with ethidium bromide. Lane 1: DNA marker; lane 2: plasmid with no digestion; lane 3: plasmid linearlised with *Nde*I; lane 4: plasmid digested with *Nde*I and *Eco*RI.

2.3.2) Amplification and subcloning of *bfp-lpetgg-his$_6$*

The gene *bfp-lpetgg-his$_6$* was amplified from pKLC065 by PCR using primers Lc01 and SOT015 (section 2.5.1). The purified PCR product was digested with *Nde*I and *Eco*RI and then gel purified. Similarly, pETMCSI was digested with *Nde*I and *Eco*RI and the plasmid backbone was gel purified. The *bfp-lpetgg-his$_6$* gene was inserted into the pETMCSI backbone forming pLLC066. Plasmid was transformed into XL-1 Blue competent cells for replication. Ampicilin-resistant colonies were selected and the plasmid extracted by miniprep. The extracted plasmid was digested by *Nde*I and *Xho*I restriction enzymes and then analysed by gel electrophoresis. The linearlised plasmid and the *Nde*I-*Xho*I fragments were in agreement with the theorectical value of the plasmid (5415 bp), *bfp-lpetgg-his$_6$* (771 bp) and the plasmid backbone (4644 bp).

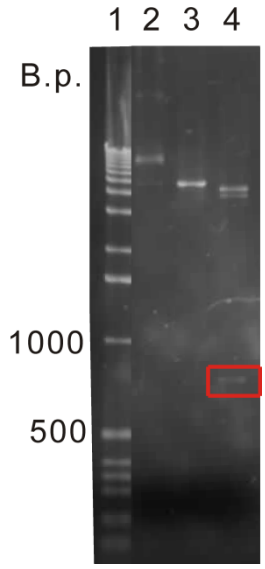


Figure 2.5 Gel enectrophoresis analysis of pLLC066. 1% agarose gel was stained with ethidium bromide. Lane 1: DNA marker; lane 2: plasmid with no digestion; lane 3: plasmid linearlised with *Nde*I; lane 4: plasmid digested with *Nde*I and *Xho*I.

2.3.3) Amplification and subcloning of *egfp-lpetgg-his$_6$*

The construction of pLLC146 (*egfp-lpetgg-his$_6$*) was different from pLLC066 (*bfp-lpetgg-his$_6$*). The gene *egfp* was extracted from pJS110 (provided by Joseph She) by digestion with *Nde*I and *Xho*I restriction enzymes and then gel purified. The backbone was obtained by digesting pLLC066 with *Nde*I and *Xho*I restriction enzymes. The gene was inserted inframe to the backbone to form pLLC146. The plasmid was transformed into *E. coli* XL-1 Blue and extracted using a miniprep kit as descriped previously. The extracted plasmid was digested by *Nde*I and *Xho*I restriction enzymes and then analysed by gel electrophoresis. The linearlised plasmid and the *Nde*I-*Xho*I fragments were in agreement with the theorectical value of the plasmid (5415 bp), *egfp-lpetgg-his$_6$* (771 bp) and the plasmid backbone (4644 bp).

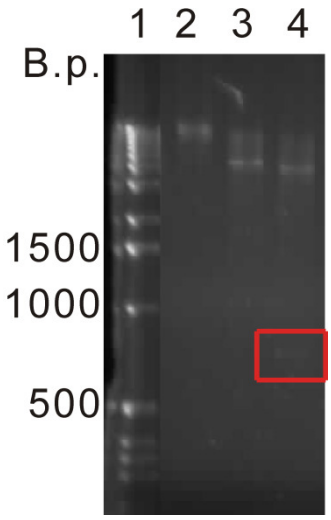


Figure 2.6 Gel electrophoresis analysis of pLLC146. 1% agarose gel was stained with ethidium bromide. Lane 1: DNA marker; lane 2: plasmid with no digestion; lane 3: plasmid linearlised with *Nde*I; lane 4: plasmid digested with *Nde*I and *Xho*I.

2.3.4) Amplification and subcloning of *dsred-lpetgg-his$_6$*

The construction of pLLC152 (DsRed-LPETGG-His$_6$) plasmid was similar to pLLC146. The *dsred* gene was extracted from pJS120 (provoded by Joseph She) by digestion with *Nde*I and *Xho*I restriction enzymes and then gel purified. The backbone was obtained by digesting pLLC066 with *Nde*I and *Xho*I restriction enzymes. The purified *dsred* gene was ligated to the backbone containing LPETGG-His$_6$ to form pLLC152 in pETMCSI subcloning vector. The plasmid was transformed into *E. coli* XL-1 Blue and extracted using miniprep. The extracted plasmid was redigested by different sets of restriction enzymes and analysed by 1% agarose gel to check the correct plasmid was formed.

Figure 2.7 Gel electrophoresis analysis of pLLC152. 1% agarose gel was stained with ethidium bromide. Lane 1: DNA marker; lane 2: plasmid with no digestion; lane 3: plasmid linearlised with *Nde*I; lane 4: plasmid digested with *Nde*I and *Xho*I.

2.3.5) Amplification and subcloning of *fpr-lpetgg-his$_6$*

The construction of pLLC155 (*fpr-lpetgg-his$_6$*) plasmid was different from the others. Since there was a natural *Nde*I site situated witin the *fpr* gene, a *Pci*I site was introduced at the 5' end of the gene instead. *fpr* was extracted from pRJW/2960/88 by digestion with *Pci*I and *Xho*I and then gel purified. The pBAD vector backbone was obtained by digesting pLLC066 with the same set of restriction enzymes. The purified *fpr* was inserted into the backbone containing the *lpetff-his$_6$* tag to form pLLC155. The plasmid was transformed into XL-1 Blue competent cells and the desired plasmid extracted by miniprep as decribed above. The extracted plasmid was digested by two different sets of restriction enzymes, set 1: *Nde*I, and produced two fragments with 2300 bp and 2683 bp respectively; set 2: *Pci*I and *Nco*I restriction enzymes and produced two fragments with 1756 bp and 3227 bp respectively. The redigestion was analysed by gel electrophoresis on 1% agarose gel.

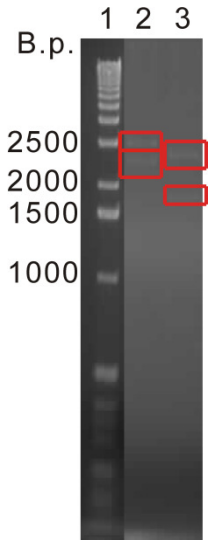


Figure 2.8 Gel electrophoresis analysis of pLLC155. 1% agarose gel was stained with ethidium bromide. Lane 1: DNA marker, lane 2: set 1 of redigestion, lane 3: set 2 of redigestion.

2.3.6) Protein expression studies

For the BFP-, EGFP-, DsRed-, Fpr-LPETGG-His_6 and His_6-SrtA proteins, the corresponding plasmids were individually transformed into *E coli.* BL21(DE3) competent cells to express the desired proteins.

A small scale expression study was carried out and the expressed proteins were purified using a small Ni-affinity column. In this purification method, proteins with a His_6 tag were complexed strongly to the immobilised Ni^{2+} cations on the resin. Most of the other proteins were washed away from the column during the washing step. After which, the proteins were eluted with eluting buffer containing low (50 mM) or high (500 mM) concentration on imidazole. Proteins non-specifcally bound to the resin were eluted in the presence of low concentration of imidazole, while desired proteins were eluted at higher concentration. Protein fractions were collected during the elution and analysed by

SDS-PAGE.

2.3.7) Expression of His_6-SrtA

The plasmid containing the *his_6-srtA* sequence (pLLC064) was transformed into *E. coli* strain BL21(DE3) and then plated onto a LB-argar gel supplemented with ampicillin. Antibiotic-resistant colonies were selected to inoculate 100 mL of LB medium overnight. After overnight incubation, 1 mL of the overnight culture was transferred to 100 mL of sterile LB medium and incubated at 37 ℃ until the OD_{600} reach ~0.6. The culture was then induced with 0.1% w/v of IPTG for expressing the desired proteins for a further 3 hours at 37 ℃. The cells were pelleted by centrifugation and the supernatant discarded. The pellet was resuspended in lysing buffer and treated with lysozyme and sonication. The desired protein was purified from the clear lysate using a Ni-affinity spin column. The flow through, wash through and elution samples were analysed by 15% SDS-PAGE gel stained with Coomassie blue (**Figure 2.9**). According to the gel, a strong band at ~30 kDa was observed in the elution samples, which is higher than the expected value of His_6-SrtA (~21 kDa). In order to confirm the identy of this protein, a plasmid with a similar construct (a gift from Prof. Bailey) was transformed into *E. coli* strain BL21(DE3) and the clear lysate from the two expressions were compared. The standard His_6-SrtA also appears to have a higher molecular weight at ~30 kDa. This phenomenon might be due to incomplete unfolding of the protein during gel electrophoresis. A large scale expression (5 L) and purification of this protein was carried out using pLLC064. The purified protein was stored at -80 ℃ for future use.

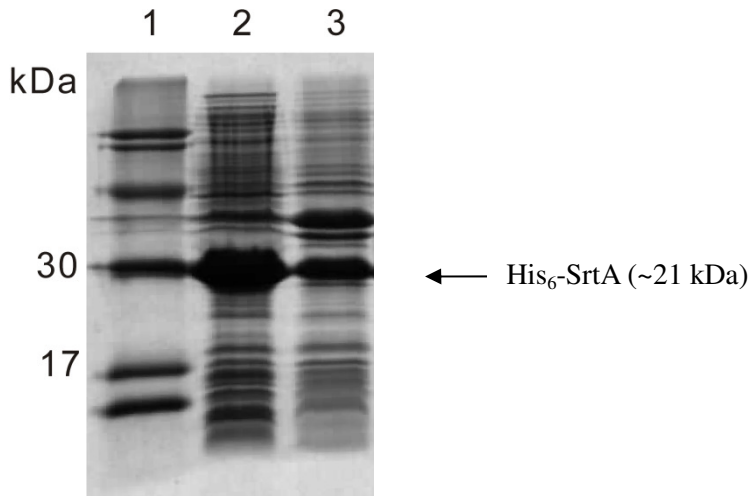


Figure 2.9 SDS-PAGE analysis of the expressed His_6-SrtA. 15% acrylamide gel was stained with Coomassie blue. Lane 1: molecular weight marker; lane 2: whole cell from SrtA expression in BL21(DE3), lane 3: cell pellet from SrtA expression in BL21(DE3).

2.3.8) Expression of BFP-LPETGG-His_6

The plasmid containing the *bfp-lpetgg-his*$_6$ sequence (pLLC066) was transformed into *E. coli* strain BL21(DE3) and the BFP-LPETGG-His_6 protein was expressed and purified by Ni-NTA affinity chromatography (50 mL). The supernatant and cell pellet from the expression were analysed by 15% SDS-PAGE gel with Coomassie blue staining (**Figure 2.10**). The purified protein was excited by UV light and the resulting fluorescence was observed visually. From the gel, a strong band ~29 kDa coorespond to BFP-LPETGG-His_6 was found. A large scale expression (5 L) and purification of BFP-LPETGG-His_6 protein was carried out and the purified fluorescent protein was stored at -80 ℃ for future use.

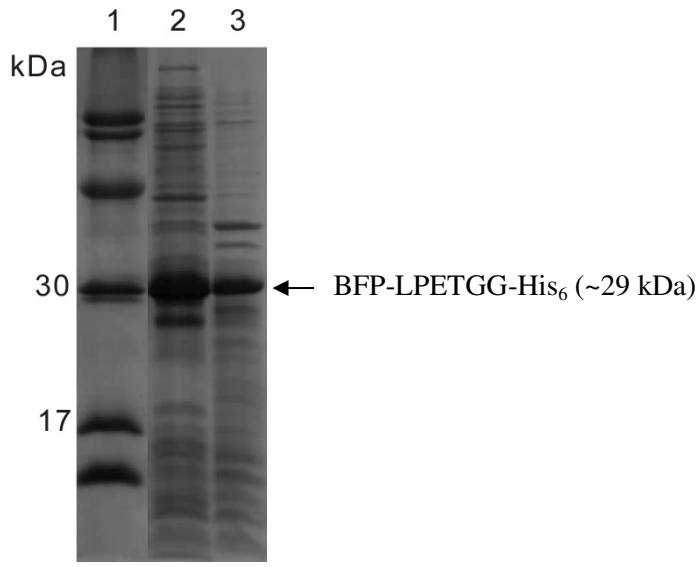


Figure 2.10 SDS-PAGE analysis of the expressed BFP-LPETGG-His_6. 15% gel was stained with Coomassie blue. Lane 1: molecular weight marker, lane 2: supernatant from expression in BL21(DE3), lane 3: cell pellet from expression in BL21(DE3).

2.3.9) Expression of EGFP-LPETGG-His_6

The plasmid containing the *egfp-lpetgg-his*$_6$ sequence (pLLC146) was transformed into *E. coli* strain BL21(DE3) and the resulting EGFP-LPETGG-His_6 protein was expressed and purified by Ni-affinity chromatography as decibed above. The supernatant and cell pellet were analysed by 15% SDS-PAGE with Coomassie blue staining (**Figure 2.11**). The purified protein was exicited by UV and the fluorescence was observed visually. A strong band ~29 kDa corresponding to the desired protein was observed. A large scale expression (5 L) and purification were carried out and the purified fluorescent protein at -80 ℃ for future use.

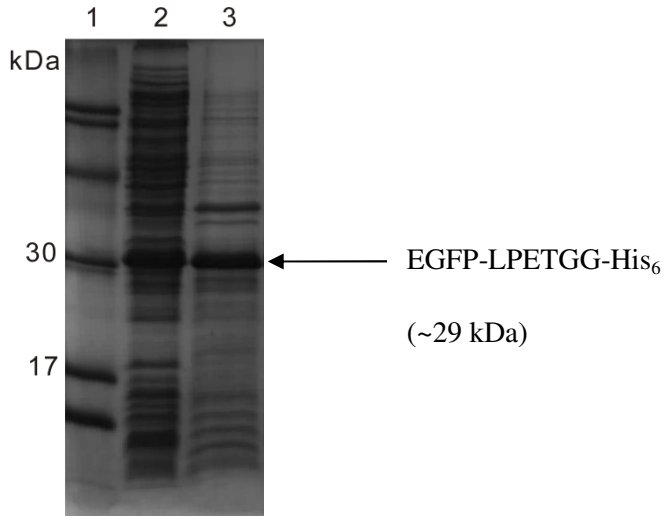


Figure 2.11 SDS-PAGE analysis of the expressed EGFP-LPETGG-His_6. 15% gel was stained with Coomassie blue. Lane 1: molecular weight marker, lane 2: supernatant from expression in BL21(DE3), lane 3: cell pellet from expression in BL21(DE3).

2.3.10) Expression of DsRed-LPETGG-His_6

The plasmid containing the *dsred-lpetgg-his*$_6$ sequence (pLLC152) was transformed into *E. coli* strain BL21(DE3) and the resulting DsRed-LPETGG-His_6 was expressed and purified by Ni-affinity chromatography as described above. The supermatamt amd cell pellet were analysed by 15% SDS-PAGE gel stained with Coomassie blue (**Figure 2.12**). Unlike BFP and EGFP, the red fluorescence was observed visually under laboratory white light. A strong band ~29 kDa corresponding to the desired protein was observed. A large scale expression (5 L) and purification were carried out and the purified fluorescent protein was stored at -80 ℃ for future use.

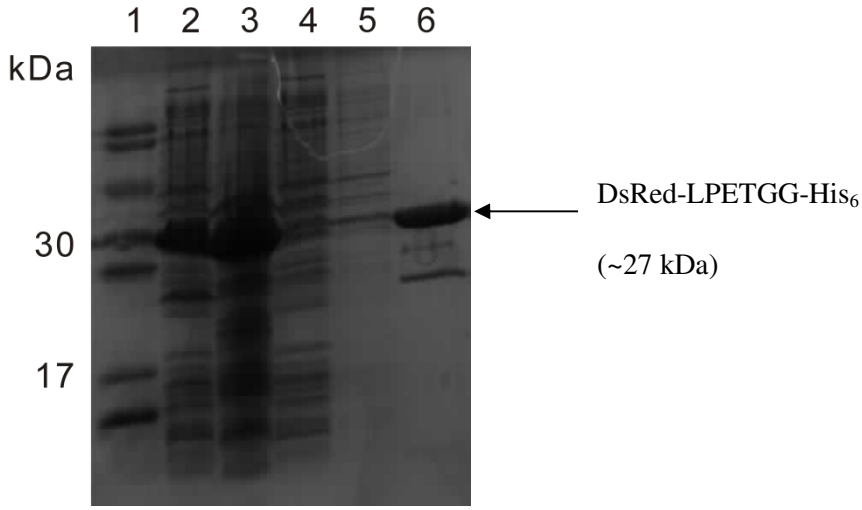


Figure 2.12 SDS-PAGE analysis of the expressed EGFP-LPETGG-His_6. 15% gel was stained with Coomassie blue. Lane 1: molecular weight marker, lane 2: cell pellet from expression in BL21(DE3); lane 3: supernatant from expression in BL21(DE3); lane 4: flow through from the small Ni affinity column purification; lane 5: wash through from the small Ni affinity column purification; lane 6: elution from the small Ni affinity column purification.

2.3.11) Expression of Fpr-LPETGG-His_6

The plasmid containing the *fpr-lpetgg-his*$_6$ sequencnce (pLLC155) was transformed into *E. coli* strain BL21(DE3) and the resulting protein was small scale Ni-affinity chromatography. The purified protein was analysed by 15% SDS-PAGE gel stained with Coomassie blue (**Figure 2.13**). From previous reports,[5] immatured cleavage was observed from the expression of Fpr-intein fusion proteins. From the SDS-PAGE analysis, there was no evidence that Fpr-LPETGG-His_6 underwent desired cleavage. This finding further confirmed the cleavage of Fpr-intein fusion was due to the instability of the thioester linkage between the two proteins. In our case, no modification to the peptide sequence was done until the enzyme was added. This had a great advantange in terms of

stability of the target tagged proteins.

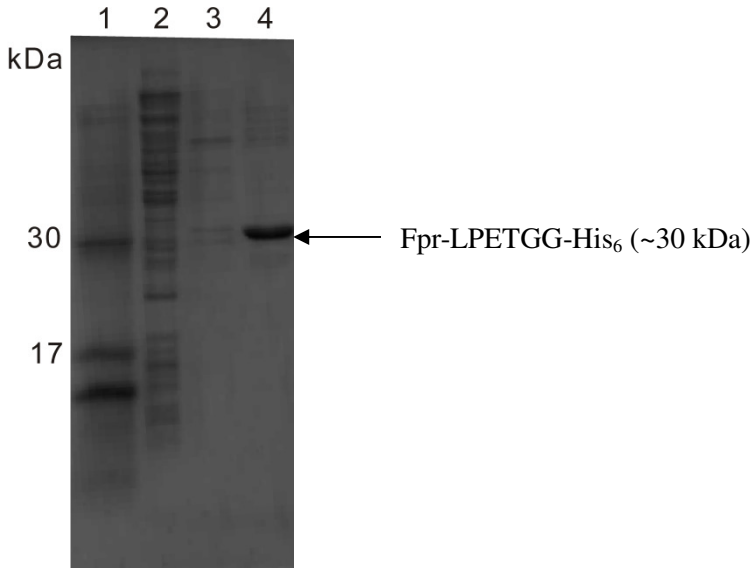


Figure 2.13 SDS-PAGE analysis of Fpr-LPETGG-His_6 expression. 15% SDS-PAGE gel was stained with Coomassie blue for visulisation. Lane 1: molecular weight marker, lane 2: flow through from the small Ni affinity column purification, lane 3: wash through from the small Ni affinity column purification, lane 4: elution from the small Ni affinity column purification.

A large scale expression (5 L) and purification were carried out and the purified protein was stored at -80 ℃ for future use.

2.3.12) Protein purification

The clear lysate extracted from large scale expression was loaded onto a Ni-affinity column (50 mL) (Prepared in-house see 5.2.17) which was prequilibrated with washing buffer. The desired protein was purified from other proteins in the present of high

concentration of imidazole (500 mM). After which the eluted protein fractions were gel purified to remove excess imidazole to prevent protein precipitation. The fractions from the gel filtration were analysed by SDS-PAGE.

2.3.13) Purification of His_6-SrtA

His_6-SrtA (molecular weight: 23028) was purified from clear lysate by Ni-affinity chromatography as described above. The flow through, wash through and elution were analysed by SDS-PAGE. The eluted protein fractions were over 95% pure. Instead of gel filfration, the protein was dialysed overnight to remove excess imidazole. The final concentration of SrtA was 123 μM, 2.8 mg/mL, as determined by Bradford assay, and was stored at -80 ℃.

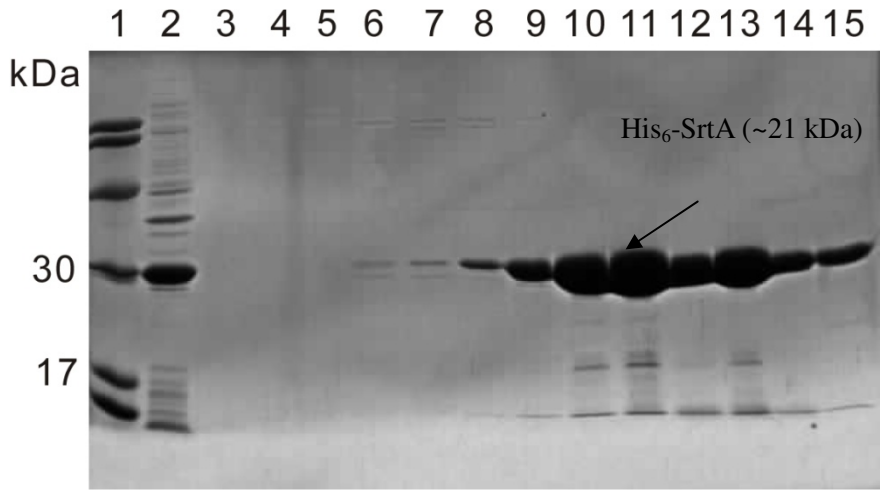


Figure 2.14 SDS-PAGE analysis of the purified protein fractions. 15% gel was stained with Coomassie blue. Lane 1: molecular weight marker, lane 2: supernatant, lane 3: water, lane 4: fraction 8, lane 5: fraction 10, lane 6: fraction 12, lane 7: fraction 14, lane 8: fraction 16, lane 9: fraction 18, lane 10: fraction 20, lane 11: fraction 22, lane 12: fraction 24, lane 13: fraction 26, lane 14: fraction 28, lane 15: fraction 30.

2.3.14) Purification of BFP-LPETGG-His_6

BFP-LPETGG-His_6 was purified from clear lysate by Ni-affinity chromatography as decribed above. The clear lysate extracted after large scale expression was loaded onto a Ni affinity column. The eluted fractions containing were collected and analysed by SDS-PAGE (Fig. 2.13). The fractions containing the purest protein (11 to 21) were pooled and concentrated. To remove excess inidazole, the concentrated protein was loaded onto a S200 gel filtration column. Collected fractions were again analysed by a SDS-PAGE. Fractions with the purest proteins were pooled and concentrated to a final concentration of 930 μM, as determined by Bradford assay, and stored at -80 ℃.

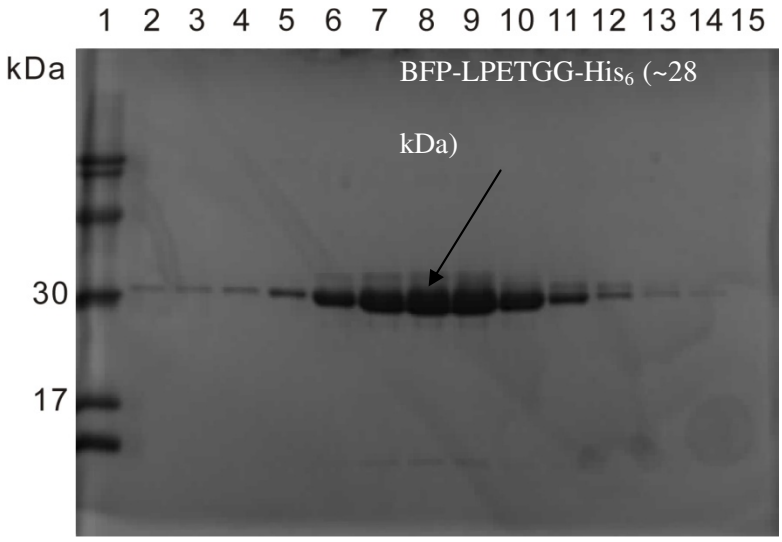


Figure 2.15 SDS-PAGE analysis of the eluted protein fractions from Ni-affinity column. 15% gel was stained with Coomassie blue. Lane 1: molecular weight marker, lane 2: fraction 1, lane 3: fraction 3, lane 4: fraction 5, lane 5: fraction 7, lane 6: fraction 11, lane 7: fraction 13, lane 8: fraction 15, lane 9: fraction 17, lane 10: fraction 19, lane 11: fraction 21, lane 12: fraction 23, lane 13: fraction 25, lane 14: fraction 27, lane 15: fraction 29.

2.3.15) Purification of EGFP-LPETGG-His_6

Similar to BFP-LPETGG-His_6, EGFP-LPETFF-His_6 was purified from clear lysate by Ni-affinity chromatography. The eluted protein fractions were analysed by SDS-PAGE (Fig. 2.14). Fractions containing the purest protein (7-19) were pooled and concentrated. The green protein solution was loaded onto a S200 gel filtration column to remove excess imidazole. Due to the high purity of the protein after Ni affinity purifitcation, the fractions were not analysed by SDS-PAGE after gel filtration. Fractions with bright green colour were pooled together and concentrated to 940 μM, as determined by Bradford assay, and stored at -80 ℃.

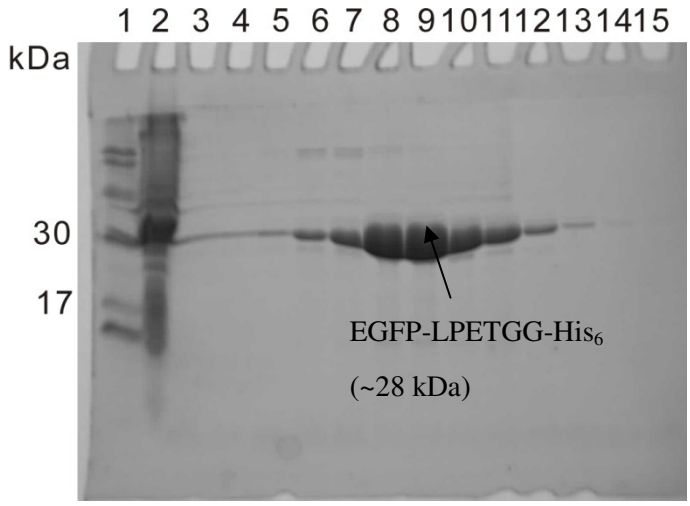


Figure 2.16 SDS-PAGE analysis of the eluted protein fractions from Ni-affinity column. 15% gel was stained with Coomassie blue for visualisation. Lane 1: molecular weight marker, lane 2: supernatant from expression in BL21(DE3), lane 3: fraction 1, lane 4: fraction 3, lane 5: fraction5, lane 6: fraction 7, lane 7: fraction 9, lane 8: fraction 11, lane 9: fraction 13, lane 10: fraction 15, lane 11: fraction 17, lane 12: fraction 19, lane 13: fraction 21, lane 14: fraction 23, lane 15: fraction 25.

2.3.16) Purification of DsRed-LPETGG-His_6

After large scale expression, clear lysate containing DsRed-LPETGG-His_6 was loaded onto a Ni affinity column. The eluted protein fractions were analysed by SDS-PAGE (Fig. 2.15). Fractions containing the purest protein (13-22) were pooled and concentrated. The red protein solution was loaded onto a S200 gel filtration column for further purification and removing excess imidazole. The fractions were analysed by SDS-PAGE. The purest fractions were pooled together and concentrated to 418 μM, as termined by Bradford assay, and stored at -80 ℃.

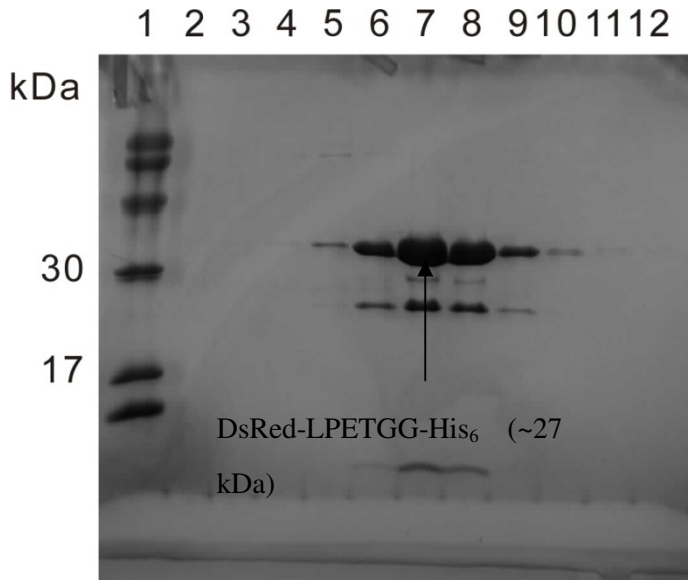


Figure 2.17 SDS-PAGE analysis of the eluted protein fractions from Ni-affinity column. 15% gel was stained with Coomassie blue for visualisation. Lane 1: molecular weight marker, lane 2: fraction 1, lane 3: fraction 4, lane 4: fraction 7, lane 5: fraction 10, lane 6: fraction 13, lane 7: fraction 16, lane 8: fraction 19, lane 9: fraction 22, lane 10: fraction 25, lane 11: fraction 28, lane 12: fraction 30.

2.3.17) Purification of Fpr-LPETGG-His_6

The clear lysate containing Fpr-LPETFF-His_6 extracted after large scale expression was loaded onto a Ni affinity column and the protein eluted with an increasing concentration of imidazole (50-500 mM). The eluted protein fractions had a pale yellow colour. The fractions were analysed by SDS-PAGE (Fig. 2.16), and the purest fractions (9 to 11) were combined and concentrated. The concentrated protein was loaded onto a PD10 desalting column to remove excess imidazole. The resulting pale yellow protein solution with a final concentration of 153 μM was stored at -80 ℃.

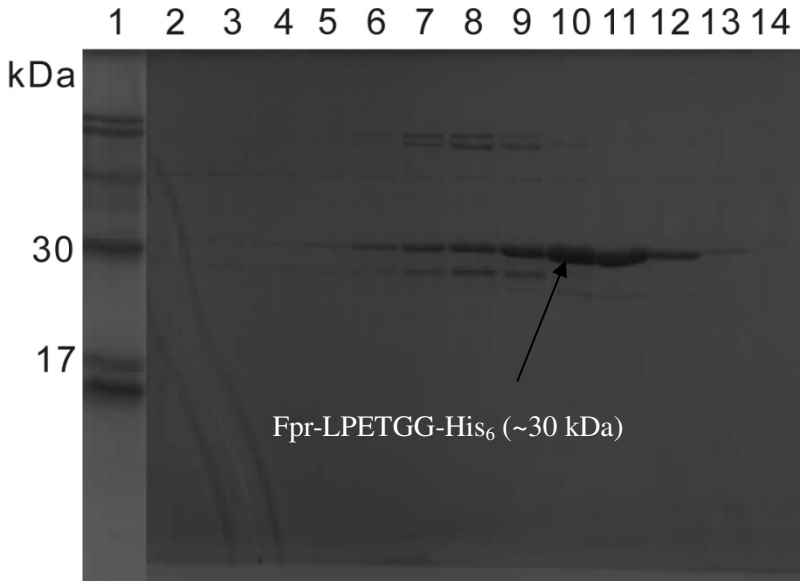


Figure 2.18 SDS-PAGE analysis of the eluted protein fractions from Ni-affinity column. 15% gel was stained with Coomassie blue for visualisation. Lane 1: molecular weight marker, lane 2: fraction 1, lane 3: fraction 2, lane 4: fraction 3, lane 5: fraction 4, lane 6: fraction 5, lane 7: fraction 6, lane 8: fraction 7, lane 9: fraction 8, lane 10: fraction 9, lane 11: fraction 10, lane 12: fraction 11, lane 13: fraction 12, lane 14: fraction 13.

2.4) Summary and conclusion

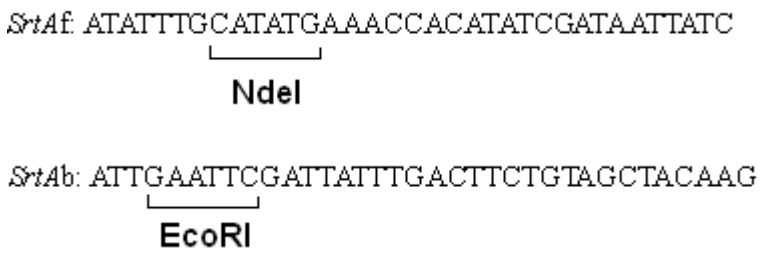
pLLC064 and pLLC066 were successfully constructed for the expression of His_6-SrtA and BFP-LPETGG-His_6 proteins under the control of isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter. Plasmids pLLC146, pLLC152 and pLLC155 were constructed from pLLC066 for the expression of EGFP-LPETGG-His_6, DsRed-LPETGG-His_6 and Fpr-LPETGG-His_6 proteins. As pLLC155 was subcloned into a pBAD vector, the expression was controlled by an arabinose inducible promoter.

A high expression level was observed in all proteins from the expression plasmid in *E. coli* strain BL21(DE3). All proteins were successfully purified by Ni-affinity chromatography and showed high purity. The expression of LPETGG-His_6 tagged proteins was successful as no undesired *in situ* cleavage between the target proteins and the tag was observed. All proteins were found to be stable at room temperature and were stored at and -80 ℃ in storage buffer (50 mM Tris, 150 mM NaCl, pH 8).

The purified proteins were used for solution phase labelling experiments (chapter 3) and protein immobilisation experiments (chapter 4).

2.5) Experimental methods

2.5.1) PCR primers



LC01: ATCGGAATTCAATGGTGATGGTGATGGTGGCCACCAGTTTCCCCTAAC

SOT015: CGCGAAATTAATACGACTCAC

Primers for *srtA* (*SrtA*f and *SrtA*b), were designed such that the PCR products contained 5' *Nde*I and 3' *Eco*RI restriction sites. Primers for *bfp-lpetgg-his*$_6$ (LC01 and SOT015) were designed to incorporate 5' *Nde*I and 3' *Eco*RI restriction sites and also introduce a *Xho*I restriction site before the LPETGG-His$_6$ motif.

2.5.2) Assembly of pLLC064: His$_6$-SrtA expression vectors

srtA from *Staphylococcus aureus* subsp. genomic DNA (ATCC 35556)was amplified by PCR using *SrtA*f and *SrtA*b primers. The stock genomic DNA (1 mg/mL) was diluted into 3 different concentrations (0.1 mg/mL, 0.01 mg/mL and 0.001 mg/mL) with deionised water. Two sets of PCR reactions were set (according to chapter 5) for each concentration of genomic DNA, one with $MgSO_4$ and one without. The PCR products were analysed on a 1.5% agarose gel stained with ethidium bromide. The PCR products were then purified using Wizard® PCR Prep DNA purification system and the purified DNA was digested with the restriction enzymes *Eco*R I and *Nde* I overnight at 37 ℃ and then analysed on a 1.5% agarose gel. The pETMCSIII plasmid was digested with *Eco*R I

and *Nde* I overnight at 37 ℃ and analysed on a 1.5% agarose gel. The purified *srtA* PCR product and pETMCSIII backbone were ligated using T4 DNA ligase to generate pLLC064. The ligation reaction mixture was transformed into XL1-Blue competent cells and then plated in LB agar plate supplemented with ampicilin. A single colony was isolated and used to inoculate 10 mL LB medium plasmid DNA prepared. Plasmid DNA was analysed by restriction digestion and DNA sequencing.

2.5.3) Assembly of pLLC066: BFP-LPETGG-His_6

bfp was amplified by PCR from pKLC065 using primers *LC01* and *SOT015*. The amplified gene was analysed by gel electrophoresis and the 750 bp product was isolated. The purified PCR product was digested with *Eco*RI and *Nde*I and gel purified. Plasmid pSOT048 (pETMCSI) was digested with *Nde*I and *Eco*RI to produce a pETMCSI plasmid backbone which was gel purified. The digested and purified PCR product was ligated with the pETMCSI plasmid backbone overnight to generate pLLC066. The ligation mix was transformed into XL1-Blue cells, a single colony was isolated and plasmid DNA was prepared. The plasmid DNA was analysed by sequencing and restriction digestion.

2.5.4) Assembly of pLLC146, pLLC152: EGFP-LPETGG-His_6, DsRed-LPETGG-His_6

pJS110 (*egfp-intein-His_6*) and pLLC066 were digested with *Nde*I and *Xho*I enzymes. *egfp* and the LPETGG-His_6 backbone were gel purified and ligated to form pLLC146. The plasmid was transformed into XL1-Blue competent cells. A colony was picked to prepare overnight cultures and the plasmid was extracted by miniprep and then analysed by analytical digestion.

pJKS120 (*dsred-arg9-intein*) and pLLC066 were digested with *Nde*I and *Xho*I enzymes to

obtain the *dsred* and the plasmid backbone. After gel purification the two gragments were ligated to form pLLC152 (*dsred-lpetgg-His$_6$*). The plasmid was transformed into XL1-Blue competent cells. A colony was picked to prepare overnight cultures and the plasmid was extracted by miniprep. The plasmid DNA was analysed by restriction digestion.

2.5.5) Assembly of pLLC155: Fpr-LPETGG-His$_6$

pRJW/2960/88 and pLLC066 plasmid were digested with *Pci*I *and Xho*I. *fpr* and the plasmid backbone were gel purified and then ligated to form pLLC155. The plasmid was transformed into XL1-Blue competent cells. A colony was picked to prepare overnight cultures and the plasmid extracted by miniprep. The plasmid DNA was analysed by restriction digestion.

2.5.6) Expression of target-LPETGG-His$_6$ proteins

pLLC064, pLLC066, pLLC146 and pLLC152 were transformed into *E. coli* strains BL21(DE3). Target proteins were purified by Ni-affinity chromatography and then analysed by SDS-PAGE. When identification of the expressed fluorescent protein was unclear colonies were picked and plated onto an agar plate contains IPTG, left in an incubator overnight at 37 ℃ and visualised under UV light.

2.5.7) Expression of Fpr-LPETGG-His$_6$ proteins

pLLC155 was transformed into competent *E. coli* BL21(DE3). Protein expression was induced by the addition of 0.1% v/v of 20% arabinose. Target protein was purified by Ni-affinity chromatography and then analysed by SDS-PAGE.

2.5.8) Large scale expression and purification of SrtA and target-LPETGG-His_6 proteins

Buffer A = 50 mM Tris/HCl pH 8.1 + 500 mM NaCl + 50 mM imidazole

Buffer B = 50 mM Tris/HCl pH 8.1 + 500 mM NaCl + 500 mM imidazole

Buffer C = 50 mM Tris/HCl pH 8.1 + 500 mM NaCl

a) Ni affinity chromatography

His_6-SrtA was expressed on a large scale (15 L culture) from pLLC064 in BL21 (DE3). Cell pellet was lysed and the cleared lysate applied to a pre-equilibrated Ni affinity column (50 mL column volume, flow rate = 5 mL / min, washed with 150 mL buffer A, 150 mL buffer B and 200 mL buffer A). Once loading was completed, the column was washed with 200 mL buffer A. Protein was eluted at 5 mL / min, over a gradient of buffer B from 0 – 100% over 600 mL, 12 mL fractions were collected. A sample was taken out from each fraction and analysed with Bradford assay to detect the protein concentration in each fraction. Fractions with proteins were assessed by SDS-PAGE (15% gel). Fractions were combined and concentrated by ultrafiltration (10 kDa MW cut off filter) to a volume of ~ 5 mL.

b) Gel filtration chromatography

To a pre-equilibrated S-200 gel filtration column (column volume = 600 mL, washed with 2 L buffer C) was applied concentrated supernatant from the Ni affinity purification. Protein was eluted at 3 mL / min, collecting fractions with 15 mL fraction size. Protein concentration was assessed by Bradford Assay, fractions with proteins were assessed by SDS-PAGE. Fractions with puriest proteins were combined and concentrated by ultrafiltration (50 kDa MW cut off filter) to a volume of ~15 mL with a final concentration of 123 μM.

2.5.8) Purification of BFP-LPETGG-His_6

a) Ni affinity chromatography

BFP-LPETGG-His_6 was expressed on a large scale (15 L culture) from pLLC066 in BL21 (DE3). Cell pellet was lysed and the cleared lysate applied to a pre-equilibrated Ni affinity column (50 mL column volume, flow rate = 5 mL / min, washed with 150 mL buffer A, 150 mL buffer B and 200 mL buffer A). Once loading was complete, the column was washed with 200 mL buffer A. Protein was eluted at 5 mL / min, over a gradient of buffer B from 0 – 100% over 600 mL. 12 mL fractions were collected, light fluorescent blue colouration was observed. A sample was taken out from each fraction and analysed with Bradford assay to detect the protein concentration in each fraction. Fractions with proteins were assessed by SDS-PAGE (15% gel). Fractions were combined and concentrated by ultrafiltration (10 kDa MW cut off filter) to a volume of ~ 5 mL.

b) Gel filtration chromatography

Protein was eluted at 3 mL / min, collecting fractions with 15 mL fraction size. Protein concentration was assessed by Bradford Assay, fractions with proteins were assessed by SDS-PAGE (15% gel). Fractions with puriest proteins were combined and concentrated by ultrafiltration (10 kDa MW cut off filter) to a volume of ~15 mL with a final concentration of 930 μM with light blue colouration.

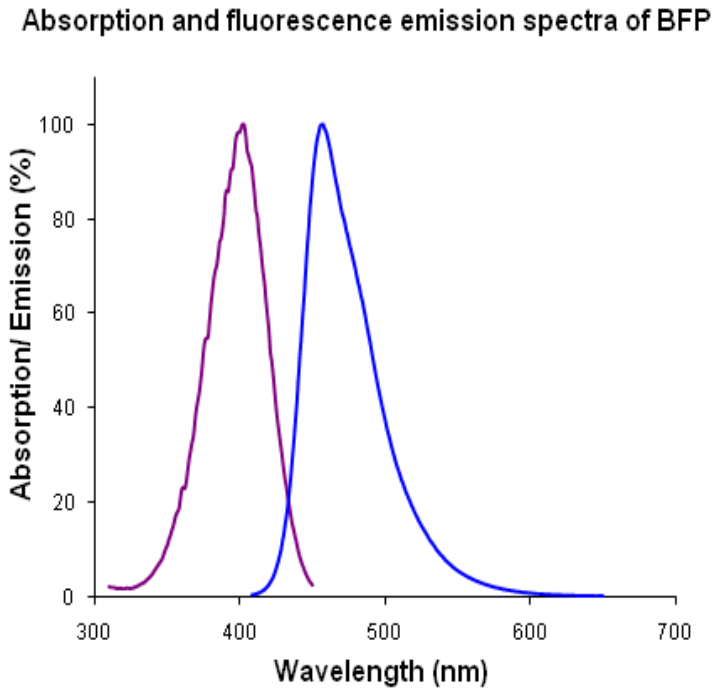


Figure 2.19 Absorption and fluorescence emission spectra of BFP.

2.5.9) Purification of EGFP-LPETGG-His_6

a) Ni affinity chromatography

EGFP-LPETGG-His_6 was expressed on a large scale (15 L culture) from pLLC146 in BL21 (DE3). Cell pellet was lysed and the cleared lysate applied to a pre-equilibrated Ni affinity column (50 mL column volume, flow rate = 5 mL / min, washed with 150 mL buffer A, 150 mL buffer B and 200 mL buffer A). Once loading was complete, the column was washed with 200 mL buffer A. Protein was eluted at 5 mL / min, over a gradient of buffer B from 0 – 100% over 600 mL, 12 mL fractions were collected. A sample was taken out from each fraction and analysed with Bradford assay to detect the protein concentration in each fraction. Fractions with proteins were assessed by SDS-PAGE (15% gel). Fractions were combined and concentrated by ultrafiltration (10 kDa MW cut off filter) to a volume of ~5 mL.

b) Gel filtration chromatography

Protein was eluted at 3 mL / min, collecting fractions with 15 mL fraction size. Protein concentration was assessed by Bradford Assay, fractions with proteins were assessed by SDS-PAGE (15% gel). Fractions with puriest proteins were combined and concentrated by ultrafiltration (10 kDa MW cut off filter) to a volume of ~15 mL with a final concentration of 940 μM with bright green colouration.

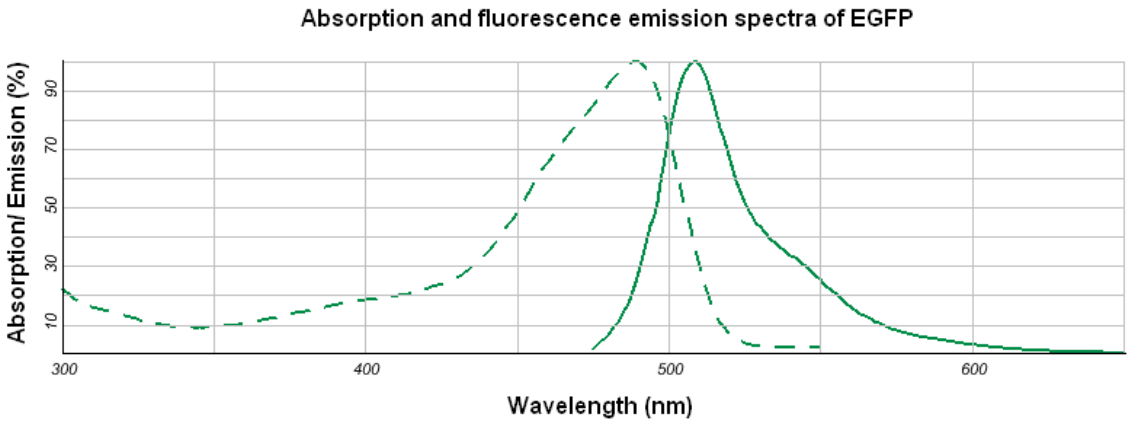


Figure 2.20 Absorption and fluorescence emission spectra of EGFP.

2.5.10) Purification of DsRed-LPETGG-His_6

a) Ni affinity chromatography

His_6-SrtA was overexpressed on a large scale (15 L culture) from pLLC152 in BL21 (DE3). Cell pellet was lysed and the cleared lysate applied to a pre-equilibrated Ni affinity column (50 mL column volume, flow rate = 5 mL / min, washed with 150 mL buffer A, 150 mL buffer B and 200 mL buffer A). Once loading was complete, the column was washed with 200 mL buffer A. Protein was eluted at 5 mL / min, over a gradient of buffer B from 0 – 100% over 600 mL, 12 mL fractions were collected. A sample was taken out from each fraction and analysed with Bradford assay to detect the protein concentration in each fraction. Fractions with proteins were assessed by SDS-PAGE (15% gel). Fractions

were combined and concentrated by ultrafiltration (10 kDa MW cut off filter) to a volume of ~5 mL.

b) Gel filtration chromatography

Protein was eluted at 3 mL / min, collecting fractions with 15 mL fraction size. Protein concentration was assessed by Bradford Assay, fractions with proteins were assessed by SDS-PAGE (15% gel). Fractions with puriest proteins were combined and concentrated by ultrafiltration (50 kDa MW cut off filter) to a volume of ~15 mL with a final concentration of 418 μM with bright red colouration.

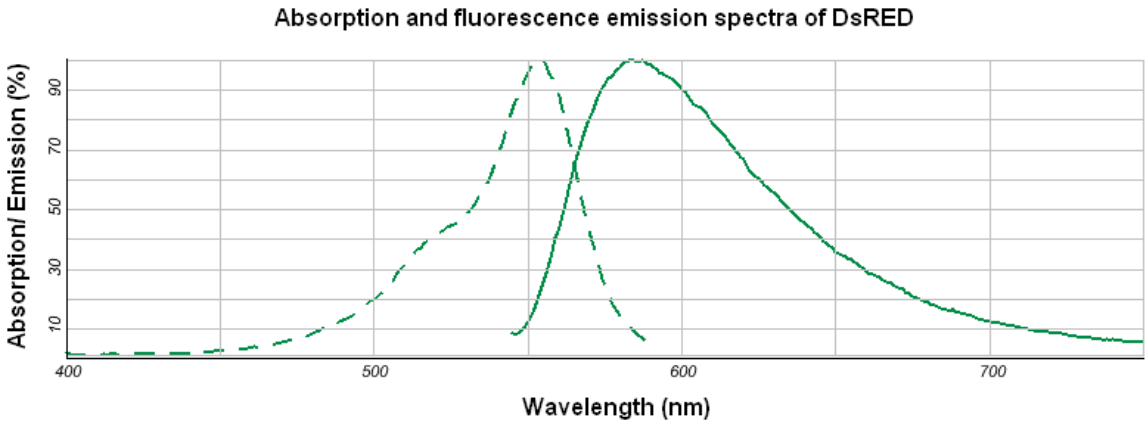


Figure 2.21 Absorption and fluorescence emission spectra of DsRED.

2.5.11) Purification of Fpr-LPETGG-His_6

a) Ni affinity chromatography

Fpr-LPETGG-His_6 was overexpressed on a large scale (15 L culture) from pLLC155 in BL21 (DE3). Cell pellet was lysed and the cleared lysate applied to a pre-equilibrated Ni affinity column (50 mL column volume, flow rate = 5 mL / min, washed with 150 mL buffer A, 150 mL buffer B and 200 mL buffer A). Once loading was complete, the column was washed with 200 mL buffer A. Protein was eluted at 5 mL / min, over a

gradient of buffer B from 0 – 100% over 600 mL, yellow colouration was observed, 12 mL fractions were collected. A sample was taken out from each fraction and analysed with Bradford assay to detect the protein concentration in each fraction. Fractions with proteins were assessed by SDS-PAGE (15% gel). Fractions were combined and concentrated by ultrafiltration (10 kDa MW cut off filter) to a volume of ~5 mL.

b) Gel filtration chromatography

Protein was eluted at 3 mL / min, collecting fractions with 15 mL fraction size. Protein concentration was assessed by Bradford Assay, fractions with proteins were assessed by SDS-PAGE (15% gel). Fractions with puriest proteins were combined and concentrated by ultrafiltration (50 kDa MW cut off filter) to a volume of ~15 mL with a final concentration of 153 μM with pale yellow colouration.

2.6) References

(1) Mao, H.; Hart, S. A.; Schink, A.; Pollok, B. A. *J Am Chem Soc* **2004**, *126*, 2670-1.

(2) Pritz, S.; Wolf, Y.; Kraetke, O.; Klose, J.; Bienert, M.; Beyermann, M. *J Org Chem* **2007**, *72*, 3909-12.

(3) Lesaicherre, M. L.; Lue, R. Y.; Chen, G. Y.; Zhu, Q.; Yao, S. Q. *J Am Chem Soc* **2002**, *124*, 8768-9.

(4) Tan, L. P.; Lue, R. Y.; Chen, G. Y.; Yao, S. Q. *Bioorg Med Chem Lett* **2004**, *14*, 6067-70.

(5) Wood, R. J.; Pascoe, D. D.; Brown, Z. K.; Medlicott, E. M.; Kriek, M.; Neylon, C.; Roach, P. L. *Bioconjug Chem* **2004**, *15*, 366-72.

(6) Chan, L.; Cross, H. F.; She, J. K.; Cavalli, G.; Martins, H. F.; Neylon, C. *PLoS ONE* **2007**, *2*, e1164.

(7) Prasher, D. C.; Eckenrode, V. K.; Ward, W. W.; Prendergast, F. G.; Cormier, M. J. *Gene* **1992**, *111*, 229-33.

(8) Heim, R.; Prasher, D. C.; Tsien, R. Y. *Proc Natl Acad Sci U S A* **1994**, *91*, 12501-4.

(9) Heim, R.; Tsien, R. Y. *Curr Biol* **1996**, *6*, 178-82.

(10) Yang, T. T.; Sinai, P.; Green, G.; Kitts, P. A.; Chen, Y. T.; Lybarger, L.; Chervenak, R.; Patterson, G. H.; Piston, D. W.; Kain, S. R. *J Biol Chem* **1998**, *273*, 8212-6.

(11) Ropp, J. D.; Donahue, C. J.; Wolfgang-Kimball, D.; Hooley, J. J.; Chin, J. Y.; Cuthbertson, R. A.; Bauer, K. D. *Cytometry* **1996**, *24*, 284-8.

(12) Inouye, S. *FEBS Lett* **2004**, *577*, 105-10.

(13) Inouye, S.; Sasaki, S. *FEBS Lett* **2006**, *580*, 1977-82.

(14) Mitra, R. D.; Silva, C. M.; Youvan, D. C. *Gene* **1996**, *173*, 13-7.

(15) Dietrich, C.; Maiss, E. *Biotechniques* **2002**, *32*, 286, 288-90, 292-3.

(16) Gross, L. A.; Baird, G. S.; Hoffman, R. C.; Baldridge, K. K.; Tsien, R. Y. *Proc Natl Acad Sci U S A* **2000**, *97*, 11990-5.

(17) Jakobs, S.; Subramaniam, V.; Schonle, A.; Jovin, T. M.; Hell, S. W. *FEBS Lett* **2000**, *479*, 131-5.

(18) Hill, T. M.; Tecklenburg, M. L.; Pelletier, A. J.; Kuempel, P. L. *Proc Natl Acad Sci U S A* **1989**, *86*, 1593-7.

(19) Pelletier, A. J.; Hill, T. M.; Kuempel, P. L. *J Bacteriol* **1989**, *171*, 1739-41.

(20) Kobayashi, T.; Hidaka, M.; Horiuchi, T. *EMBO J* **1989**, *8*, 2435-41.

(21) Sista, P. R.; Mukherjee, S.; Patel, P.; Khatri, G. S.; Bastia, D. *Proc Natl Acad Sci U S A* **1989**, *86*, 3026-30.

(22) Hidaka, M.; Kobayashi, T.; Takenaka, S.; Takeya, H.; Horiuchi, T. *J Biol Chem* **1989**, *264*, 21031-7.

(23) Natarajan, S.; Kelley, W. L.; Bastia, D. *Proc Natl Acad Sci U S A* **1991**, *88*, 3867-71.

(24) Gottlieb, P. A.; Wu, S.; Zhang, X.; Tecklenburg, M.; Kuempel, P.; Hill, T. M. *J Biol Chem* **1992**, *267*, 7434-43.

(25) Sista, P. R.; Hutchinson, C. A., 3rd; Bastia, D. *Genes Dev* **1991**, *5*, 74-82.

(26) Coskun-Ari, F. F.; Skokotas, A.; Moe, G. R.; Hill, T. M. *J Biol Chem* **1994**, *269*, 4027-34.

(27) Bianchi, V.; Reichard, P.; Eliasson, R.; Pontis, E.; Krook, M.; Jornvall, H.; Haggard-Ljungquist, E. *J Bacteriol* **1993**, *175*, 1590-5.

(28) Ingelman, M.; Bianchi, V.; Eklund, H. *J Mol Biol* **1997**, *268*, 147-57.

(29) Jenkins, C. M.; Waterman, M. R. *J Biol Chem* **1994**, *269*, 27401-8.

(30) Jenkins, C. M.; Waterman, M. R. *Biochemistry* **1998**, *37*, 6106-13.

(31) Krapp, A. R.; Rodriguez, R. E.; Poli, H. O.; Paladini, D. H.; Palatnik, J. F.; Carrillo, N. *J Bacteriol* **2002**, *184*, 1474-80.

(32) McIver, L.; Leadbeater, C.; Campopiano, D. J.; Baxter, R. L.; Daff, S. N.; Chapman,

S. K.; Munro, A. W. *Eur J Biochem* **1998**, *257*, 577-85.

(33) Wan, J. T.; Jarrett, J. T. *Arch Biochem Biophys* **2002**, *406*, 116-26.

(34) Fujii, K.; Huennekens, F. M. *J Biol Chem* **1974**, *249*, 6745-53.

(35) Hall, D. A.; Vander Kooi, C. W.; Stasik, C. N.; Stevens, S. Y.; Zuiderweg, E. R.; Matthews, R. G. *Proc Natl Acad Sci U S A* **2001**, *98*, 9521-6.

Chapter 3: Solution phase assays

3.1) Introduction

Studying biological interactions at the molecular level is essential in many disciplines. Since biological molecules are often very small in size, they normally cannot be seen easily using conventional optical instruments, such as an optical microscope, without being labelled with a tag. These can be a fluorescent label (fluorescent dyes or proteins) or even an enzyme that catalyse a visualable reactions. Other methods include measuring the absorbance of the protein of interest at a particular wavelength; measuring the size of biological molecules by mass spectrometry; solving the structure of the of the protein:substrate complex by X-ray crystallography or NMR spectroscopy; detecting the emitting radioactivity through the radioactive label, etc.[1]

Fluorescence polarisation is another powerful technique for detecting molecular interactions and conformational changes. This technique was first described by Perrin in 1926 and has since been used to study many types of molecular binding. The principle behind this is that when a fluorecenet molecule is excited with a polarised light, the resulting fluorescence is emitted in the same polarised plane if the molecule remains stationary. In solution, if molecules tumble and roate during the excited state, the fluorescent light is therefore emitteed in a different plane from the incident light. The rate of rotation and tumbling is inversely proportional to the size of the molecule, so that small molecules rotate faster than larger ones. By measuring the emitted fluorescence light in

vertical and horizontal planes, we can estimate the size of the molecules. Real-time fluorescence polarisation measurement can even detect the binding or dissociation of two or more molecules (**Figure 3.1**).[2-4]

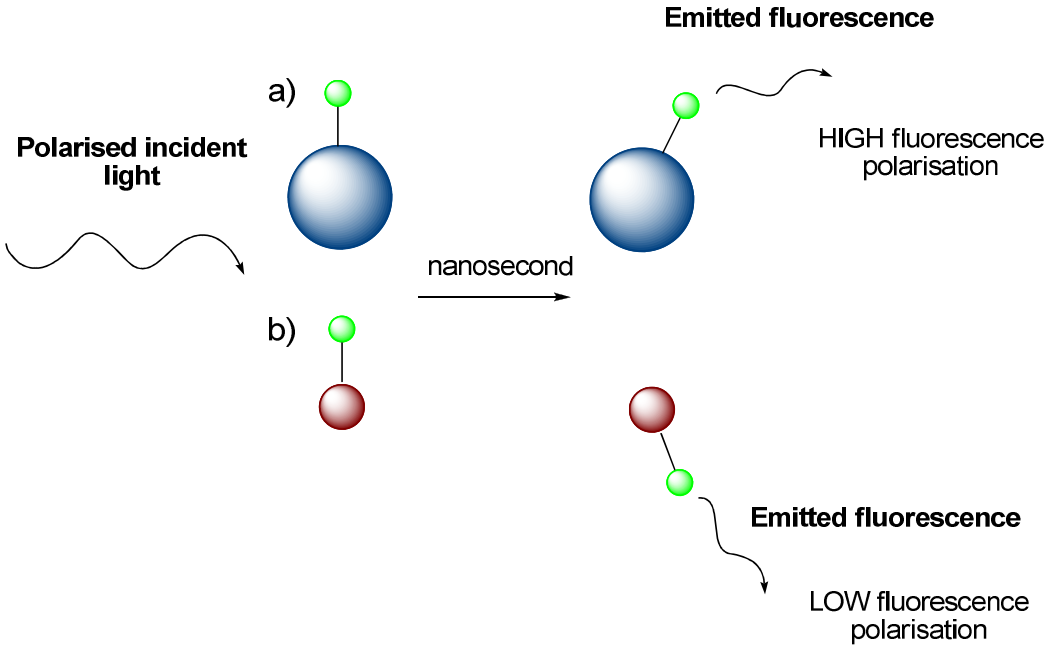


Figure 3.1 Schematic diagram of fluorescence polarisation. (a) When a large fluorescent molecule is excited with a plane polarised light, little movement occurs and as a result the emitted fluorescence light is highly polarised. (b) When a small fluorescent molecule is excited, however, the rotation is more rapid and therefore the emitted fluorescence is less polarised.

3.1.1) FRET

Another fluorescence-based technique for monitoring molecular interactions,[5,6] conformational changes within a molecule,[7-9] and protein complex formation[10] is Fluorescence resonance enery transfer (FRET). Although FRET was discovered by Forster as early as in 1948[11] it was not popular until 1970s.[12]

FRET is an energy transfer mechanism between two fluorophores where one act as a donor

and the other act as an acceptor (**Figure 3.2**). The efficiency of FRET depends on three main factors, the first is the distance between the acceptor and the donor, which normally should be between 10 – 100 Å.[12] Secondly, it depends on the spectral overlap between the donors emission spectrum and the acceptors excitation spectrum, and finally the orientation of the donor emission dipole moment and the acceptor absorption dipole moment.

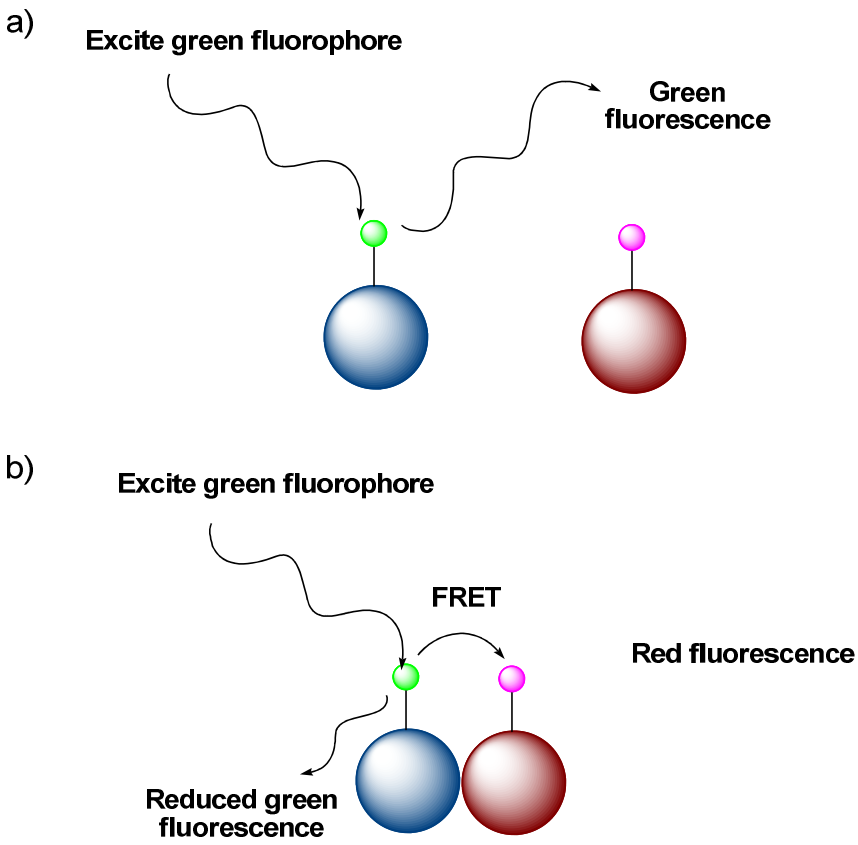


Figure 3.2 Schematic diagram of FRET. a) When the donor (blue molecule) and acceptor molecules (red molecule) are separated, the green fluorophore (donar) emits green fluorescence in a normal way. b) When the donor are in close approximity, the fluorescence light emitted from the green fluorophore is used to excite the red fluorophore, resulting in an increase of red fluorescence from the acceptor molecule.

A vast number of fluorophores have been used as a FRET pair. The notable pairs are

fluorescent proteins: cyan fluorescence protein (CFP)/ yellow fluorescence protein (YFP) and BFP/GFP.[13] Fluorescent proteins such as GFP can be expressed in different cells and is highly fluorescent.[13] It can easily be fused with another protein to produce a fusion and retain its functionality. The gene of GFP can be mutated to form different kinds of fluorescent proteins with different spectral properties which can be used as donors and acceptors of FRET.[13-19] This provides a better way to investigate biological mechanism and physiological functions of cells.[20-25] Different researchers used FRET to to study the interaction between different molecules, for example: between the CD38, contains an ADP ribosylcyclase domain that mediates intracellular Ca^{2+} signaling by the production of cyclic ADP-ribose (cADPR), and lipid raft component ganglioside M(1).[26-34]

3.1.2) Fluorescent labelling of proteins and DNA

Most proteins are not fluorescent in their nature. The need to understand the dynamic behavior of the non-fluorescent proteins, organelles and cell, has lead to the development of methods to fluorescently label biological molecules, including proteins, DNA and RNA.

One of the methods to label proteins is to express fluorescent protein fusion tags. It works by inserting the gene that encodes the fluorescent protein next to the target gene sequence. After protein expression, the desired protein is fused with the fluorescent protein tag. However, the product of this fusion is large. For example, ECFP-venus, has a overall molecular weight of 55 kDa, which is almost three times bigger than the original protein. This could decrease the mobility of the proteins.

Another common method to label proteins in solution is the use of synthetic fluorescent dyes, such as the amine-reactive label fluorescein isothiocyante (FITC). FITC has a maximum excitation and emission wavelengths at 495 nm and 525 nm respectively. It

forms a very stable thiourea bond with the free primary amines on the target biomolecule. However, FITC reacts with any free amine available and so the labelling reaction is non-specific and uncontrollable.[35] Nonetheless, this fluorescent dye is very easy to use and many labelled protein examples can be found in the literature.[36-41]

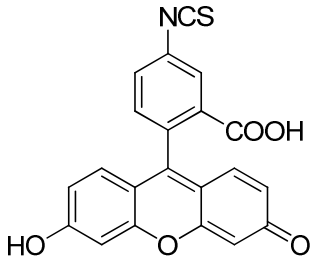


Figure 3.3 Molecular structure of fluorescein-5-isothiocyanate.

Intein mediated labelling is another method to label proteins,[42-44] peptides.[45] However, the expression of target-intein fusion proteins was proved to be difficult, as the intein domain of the fusion protein can be easily cleaved non-specifically.[46, 47]

By using the SrtA system, we aim to develop a new and specific method to fluorescently label proteins under a mild condition. Our next goal was to ligate a range of proteins with different substrates, including fluorecein derivaties and other biological molecules.

3.2) Results and Discussion

3.2.1) Assay for Sortase A

With all the proteins expressed and purified in Chapter 2, the activity of SrtA was analysed by ligating BFP-LPETGG-His_6 with mono-, di- and tetra-glycine.

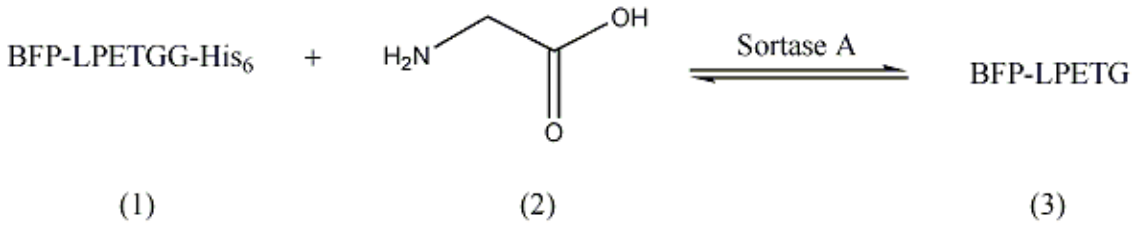


Figure 3.4 Ligation of BFP-LPETGG-His_6 to glycine using SrtA, (1) BFP-LPETGG-His_6 protein, (2) glycine, (3) ligation product BFP-LPETG.

BFP-LPETGG-His_6 was incubated with the glycine substrates in the present of SrtA in assay buffer. The reaction was left overnight in the dark at room temperature. Aliquotes were taken at different time points and then loaded onto a pre-eqilibrated Ni-affinity spin column. During the ligation reaction the His_6 tag on the C-terminus of BFP-LPETGG-His_6 was removed, therefore losing the ability to bind to the affinity column. The flow through from the spin Ni column was collected and transferred to a 96-well plate for fluorescence analysis using a microplate reader. **Figure 3.5** shows a kinetic diagram of the SrtA mediated ligation. With increasing time, the concentration of product increased, leading to an increase in the fluorescent intensity of the flow through from the Ni-column. Two different negative controls were used, one without SrtA and the other one without glycine substrate.

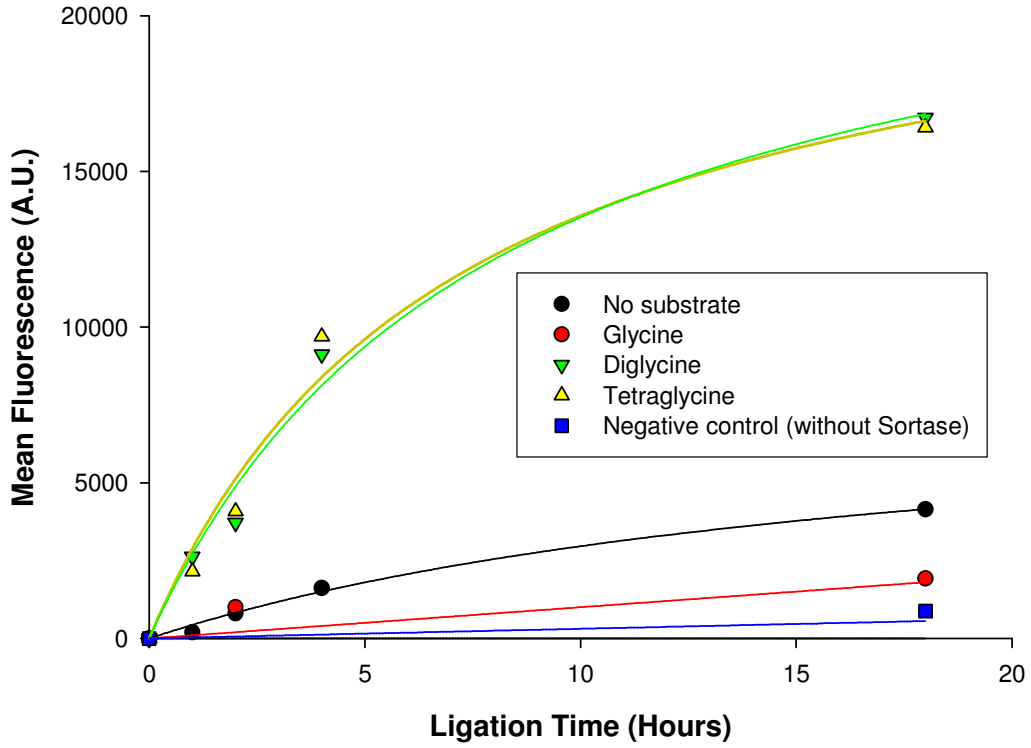


Figure 3.5 Fluorescence intensity of Ni column flow through of each assay and fitted to a ligand binding one site saturation curve.

From the graph above it shows that diglycine and tetraglycine were the best substrates, as they produced the highest increase of fluorescent intensity with a similar rate. With glycine as the substrate, the reaction was very slow and close to the two negative controls. This suggested that mono-glycine was a poor substrate for the SrtA systemin solution. In the negative control where no substrate was added, an increase in fluorescent intensity was also observed. A sample from this reaction was taken and analysed by HPLC (See appendix A). The results indicated that the BFP-LPET-SrtA intermediate was hydrolysed by water, forming BFP-LPET-OH. As this hydrolysed product lost the His_6 tag from the C-terminus, the protein failed to bind to the affinity column which results in an increase in fluorescent intensity in the flow-through.

3.2.2) Labelling of BFP-LPETGG-His_6 with fluorescein labelled oligoglycine

After successfully conjugating tagged BFP with different oligoglycines using our noval SrtA system, our next goal was to fluorescently label proteins. We investigated the labelling of BFP-LPETGG-His_6 with different fluorescein derivaties at different time points.

Three different fluorescein-labelled glycines were selected as substrates for the SrtA labelling system: diglycine-fluorescein (GGF), triglycine-fluorescein (GGGF) and tetraglycine-fluorescein (GGGGF). Tagged target protein (50 μM) was incubated with the substrate (500 μM) and SrtA (100 nM) in assay buffer (50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.5) at room temperature ($CaCl_2$ was added to the reaction was some research suggest that it could increase the reaction rate). Aliquots containing the protein sample were withdrawn at different time points, denatured at 95 °C for 10 min and then analysed by SDS-PAGE.

Figure 3.6 shows the fluorescence image (lower image, no staining) and brightfield image (upper panel, stained with Coomassie blue) of the 15% SDS-PAGE gel. Since the fluorescent protein was denatured at high temperature, the matured chromophore was no longer in the correct orientation and lost its fluorescence properties. This can be seen from lane 2-4 on the fluorescence and brightfield image. Over a course of 16 h, all three fluorescein-labelled substrated were conjugated to the target proteins.

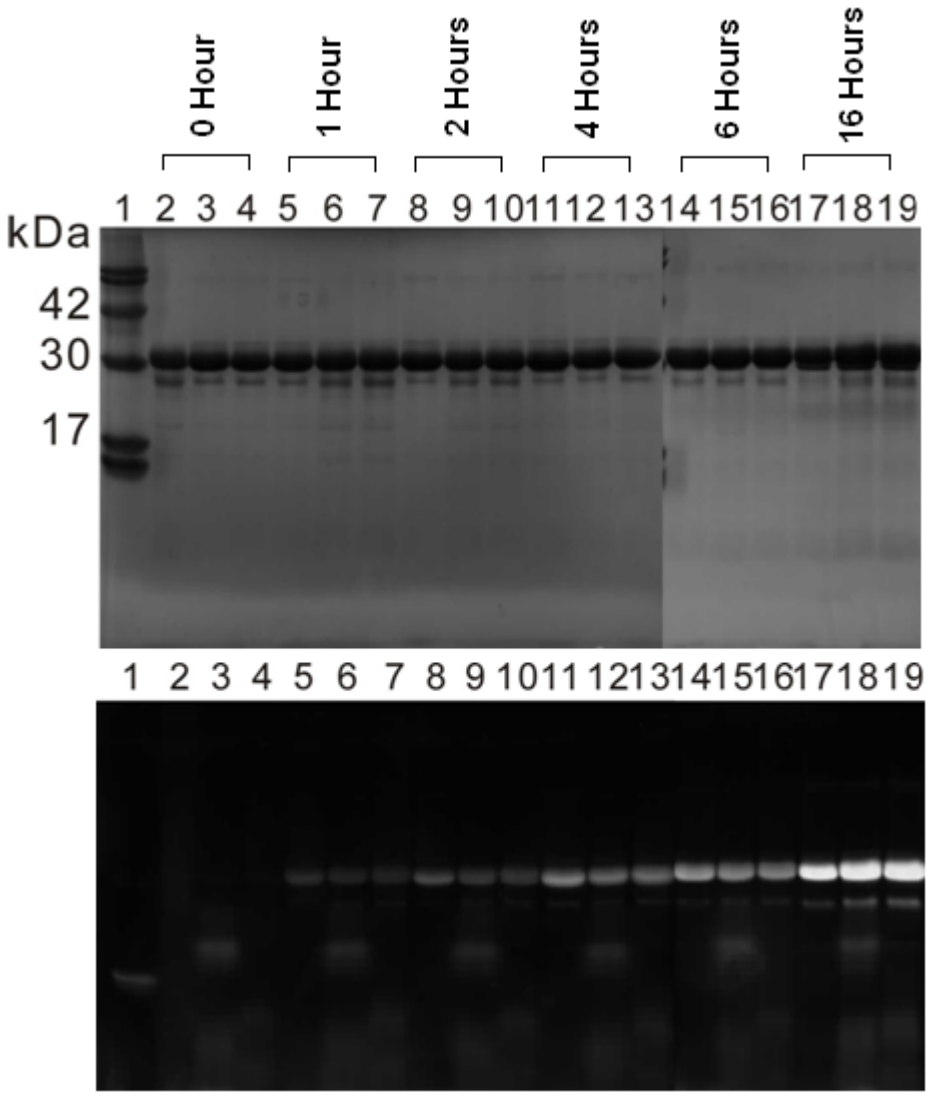


Figure 3.6 SDS-PAGE analysis of labelling BFP-LPETGG-His_6 with different fluorescein labelled glycines using the SrtA system. Top image: Coomassie blue stained brightfield image. Lower image: fluorescence images (illuminated by transilluminator, $\lambda = 302$ nm, no staining). In both cases: lane 1: protein marker, lane 2 -4: GGF, GGGF and GGGGF labelling sample at 0 hour, lane 5-7: GGF, GGGF and GGGGF labelling sample at 1 hour, lane 8-10: GGF, GGGF and GGGGF labelling sample at 2 hour, lane 11-13: GGF, GGGF and GGGGF labelling sample at 4 hour, lane 14-16: GGF, GGGF and GGGGF labelling sample at 6 hour, lane 17-19: GGF, GGGF and GGGGF labelling sample at 16 hour.

The fluorescent intensity from the gel shown above was measured by ImageJ (version 1.42) and the corresponding kinetic curves are represented in **Figure 3.7**.

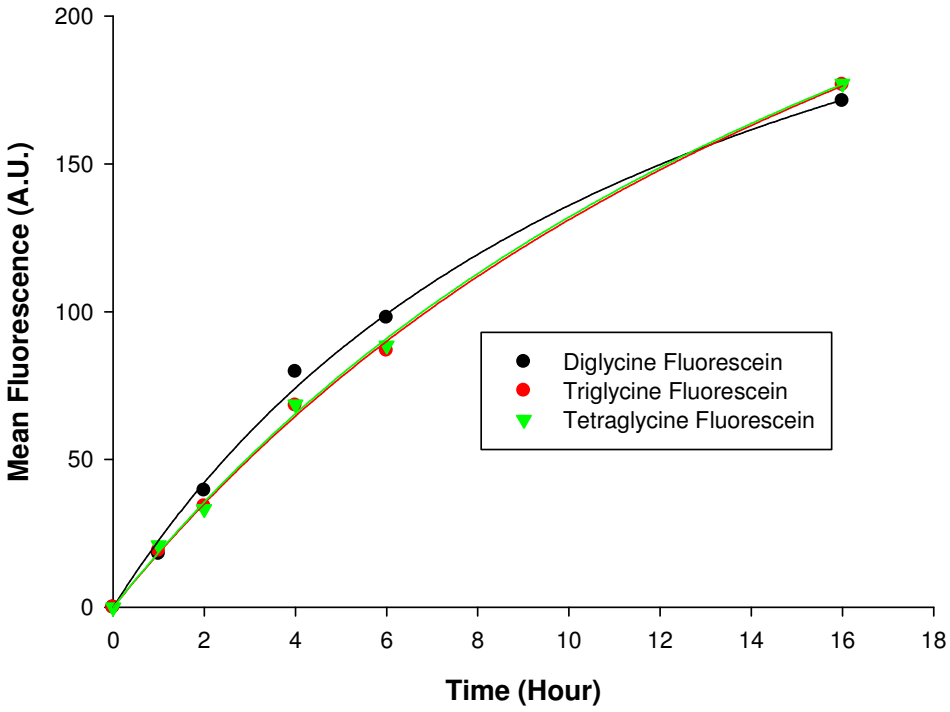


Figure 3.7 Labelling of BFP-LPETGG-His_6 with fluorescein labelled oligoglycine. The points were fitted to a ligand binding one site saturation curve. The mean fluorescent intensity of each band from figure 3.2 under UV light was measured by ImageJ.

From the graph shown above, all selected substrates showed good labelling efficiency, as they yielded high amount of labelled protein at the end of the reaction. Suprisingly, unlikely previous analysis where reaction kinetics were monitored by a plate reader, all the substrates showed similar reaction kinetics. This could be due to the fact that SDS-PAGE analysis is not as sensitive as the plate reader. Although there were some random bands below the labelled products, which might due to the degradation of proteins with a LPETGG motif and results in the labelling of degradated protein, the quantity of the bands were neglectabe when compare to the products.

3.2.3) Labelling of EGFP-LPETGG-His_6 with different concentrations of fluorescein labelled diglycine

Our next goal was to optimise the concentration of fluorescein-labelled oligoglycine substrate used in the reaction. EGFP-LPETGG-His_6 and GGF were used for this labelling experiment. Similar to the previous kinetic experiment, the tagged protein (50 μM) was incubated with increasing concentration of GGF (0 μM - 1 mM) in the present of SrtA (150 nM) in assay buffer. After 16 h, the samples were denatured at 95 ℃ for 10 min, and then analysed by SDS-PAGE. Similar to BFP, the fluorescent protein was unfolded after denaturation and therefore lost the fluorescence properties, as see from lane 2 in Figure 3.8.

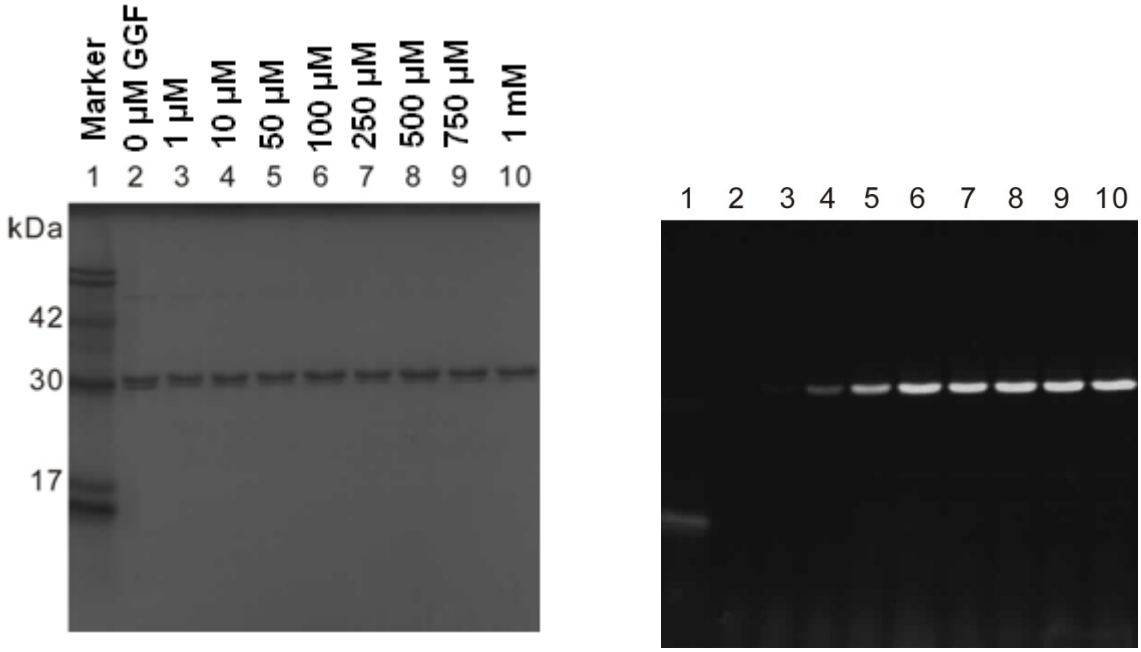


Figure 3.8 SDS-PAGE analysed of the fluorescent labelling reaction of EGFP-LPETGG-His_6 with increasing concentration of GGF using the SrtA system. On the left: 15% gel brightfield image stained with Coomassie blue; on the right: fluorescence image of the same gel before staining (illuminated by transilluminator, λ =

302 nm). In each case: lane 1: protein marker, lane 2: 0 μM GGF, lane 3: 1 μM GGF, lane 4: 10 μM GGF, lane 5: 50 μM GGF, lane 6: 100 μM GGF, lane 7: 250 μM GGF, lane 8: 500 μM GGF, lane 9: 750 μM GGF, lane 10: 1 mM GGF.

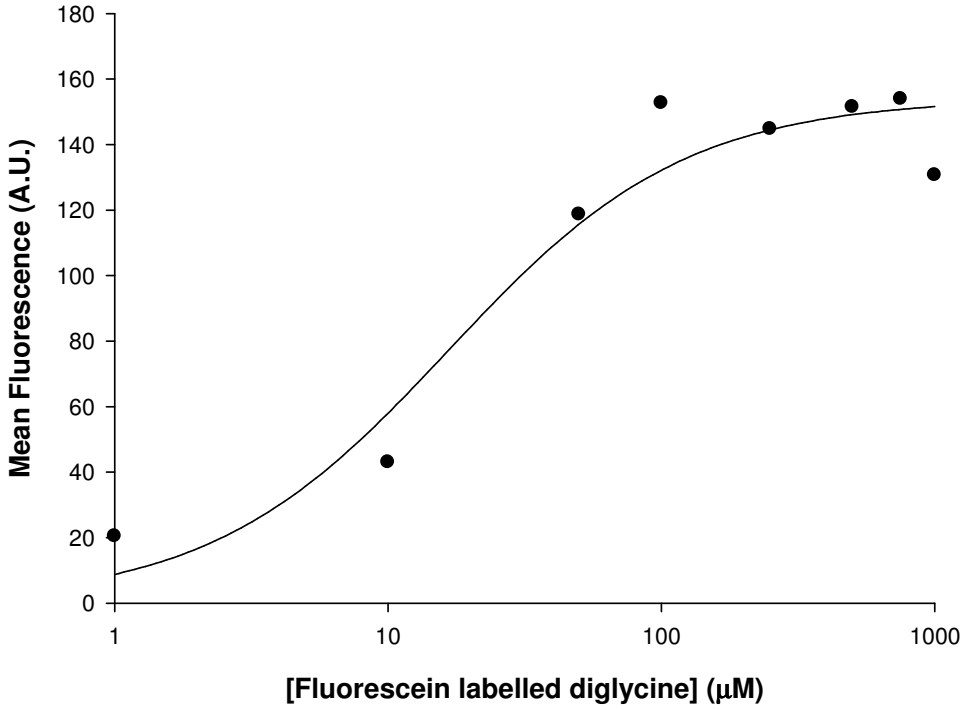


Figure 3.9 Labelling of EGFP-LPETGG-His_6 with different concentrations of fluorescein labelled diglycine. The mean fluorescent intensity of each band from figure 3.8 under UV light was measured by ImageJ and fit curve to ligand binding one site saturation.

The graph above shows that with increasing concentration of GGF substrate, the fluorescent intensity of the product band increased. The minimum concentration of fluorescein labelled diglycine needed for a reasonable amount of product was about 50 μM. The optimized concentration of substrate was determined to be 150 μM

In order to study the labelling efficiency of target protein with GGF at lower

concentrations, BFP-LPETGG-His_6 was labelled with the substrate at 0 μM – 25 μM. The reaction was monitored by FRET at 37 ℃ over 16 h.

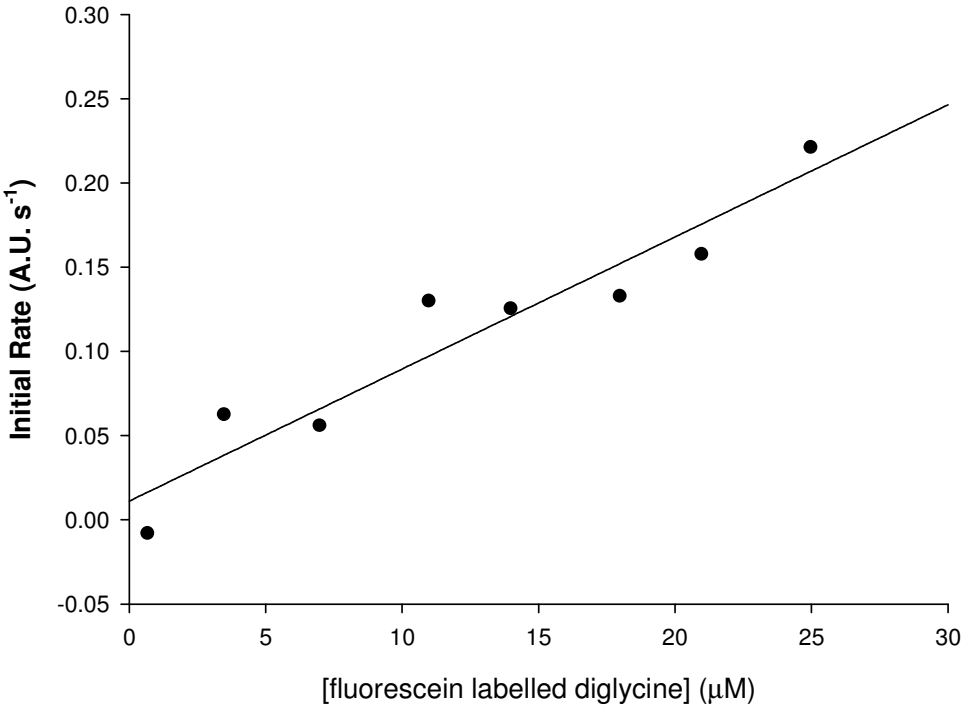


Figure 3.10 Initial rate of fluorescent intensity measurement at 518 nm of BFP FRET assay with different GGF concentrations and fitted to a linear line.

From the graph above shown the initial rate of the labelling reaction increased as a function of substrate concentration. This finding confirmed previous observation where the labelling reaction roughly followed a one-site saturation binding profile.

3.2.4) Labelling of BFP-LPETGG-His_6 with fluorescein labelled diglycine using different concentrations of SrtA

We were also interested in studying the minium amount of SrtA needed for the labelling reaction. Similar to the previous experiments, target protein (50 μM) and substrate (GGF, 10 μM) were incubated with increasing concentration of SrtA (0 nM – 1000 nM) in assay buffer overnight. Samples were denatured at 95 ℃ for 10 min and then analysed by SDS-PAGE.

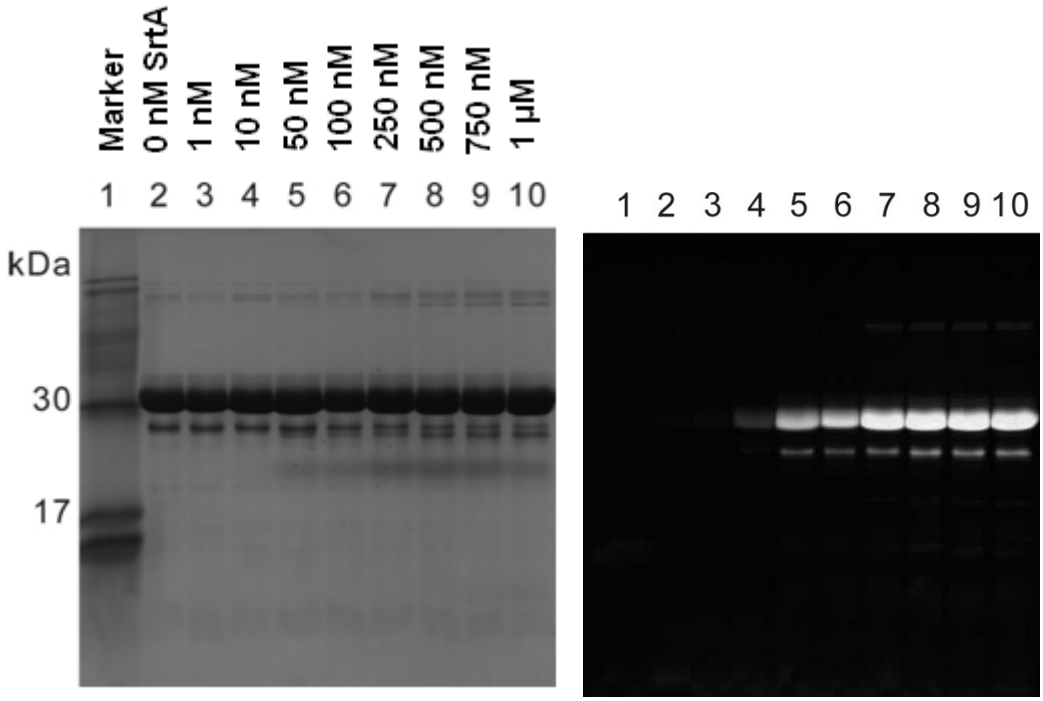


Figure 3.11 SDS-PAGE analysis of the SrtA-mediated protein labelling of BFP-LPETGG-His_6 with GGF in the present of increasing concentration of SrtA for 16 hours. On the left is Coomassie blue stained brightfield image; on the right is fluorescence image before staining (illuminated by transilluminator, λ = 302 nm). In each case: lane 1: protein marker, lane 2: 0 nM SrtA, lane 3: 1 nM SrtA, lane 4: 10 nM SrtA, lane 5: 50 nM SrtA, lane 6: 100 nM SrtA, lane 7: 250 nM SrtA, lane 8: 500 nM SrtA, lane 9: 750 nM SrtA, lane 10: 1000 nM SrtA.

The images above shown the brightfield image (left, stained with Coomassie blue) and fluorescence image (right, no staining) of the 15% SDS-PAGE gel. With increasing concentration of SrtA in the reaction the amount of labelled target protein increased. Apart from the target proteins there were a small amount of other proteins labelled with the fluorescent substrate. These could be the truncated proteins and target protein dimers. The quantity of these impurities, when compare with the major project, were neglectable. The fluorescent intensity of the gel was analysed by ImageJ (version 1.42) and a saturation-binding curve was plotted.

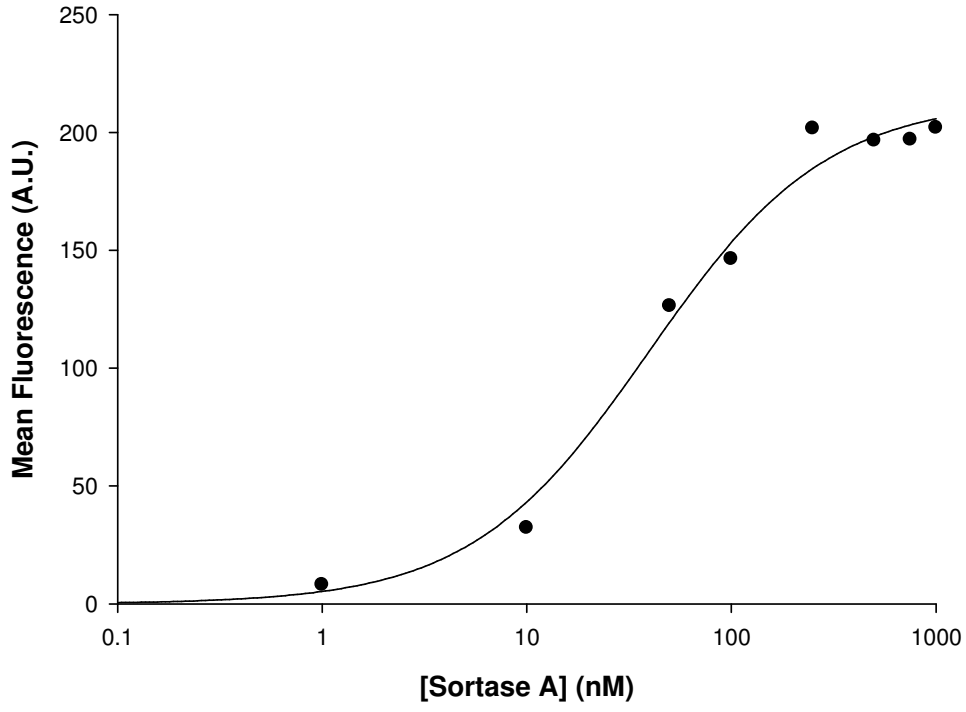


Figure 3.12 Labelling of BFP-LPETGG-His_6 with fluorescein labelled diglycine using different concentrations of SrtA. The mean fluorescent intensity of each band from Figure 3.11 under UV light was measured by ImageJ and the graph was fitted to a ligand binding one site saturation curve.

From the saturation-binding curve shown above, it was clear that 10 nM of SrtA was enough to start to catalyse the labeling reaction. The amount of labelled products increased as a function of [SrtA] until the reaction reached saturation at ~200 nM.

3.2.5) Labelling of different proteins with fluorescein labelled diglycine

By using the optimised ligation conditions, we attempted to label different proteins using the SrtA system. BFP-, EGFP-, DsRed-, Tus- (prepared by H. Cross) and Fpr-LPETGG-His_6 were selected to be labelled with GGF. In the panel of target proteins, BFP, EGFP and DsRed had similar protein structures. They are known to be robust under a wide range of conditions and have been used for a number of labelling technologies. Fpr and Tus, on the other hand, have more delicate protein structures. Previously, Wood *et al.* attempted to label Fpr with fluorecein derivatives using the intein labelling method. In the first step, a fusion protein of Fpr-intein had to be expressed. This was proved to be difficult since the thioester linkage between Fpr and intein was easily hydrolysed in aqueous environment. Tus is a DNA-binding protein which recognises the *ter* sequence. It was our interest to study the labelling effiency of these potenitaly challenging proteins using our novel system.

Target proteins (50 μM), substrate (10 μM) and SrtA (100 nM) were incubated in assay buffer at r.t. for overnight. SrtA was omitted from the reaction mixtures to serve as negative controls. After which, the reaction mixtures were denatured at 95 ℃ for 10 min and then analysed by SDS-PAGE (Figure 3.13)

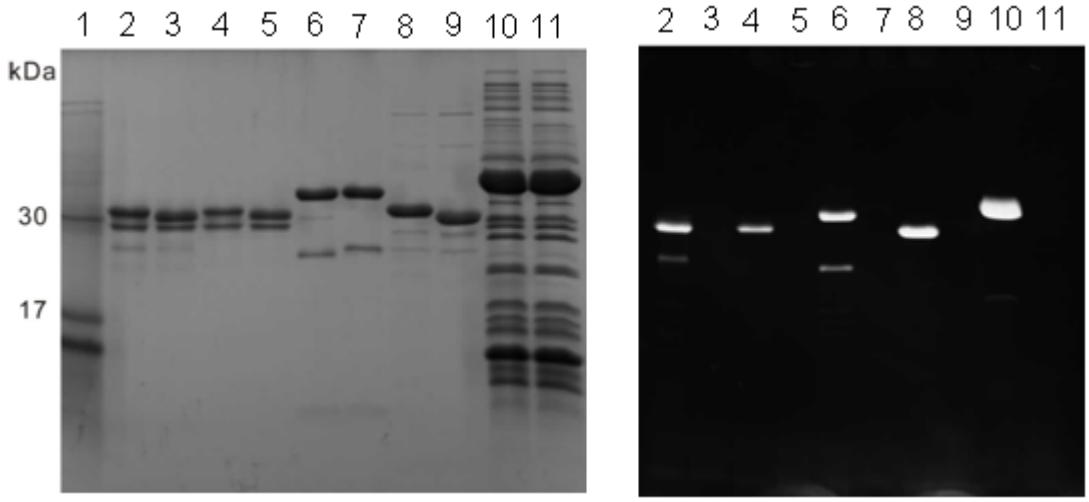


Figure 3.13 SDS-PAGE analysis of the Srt-A mediated labelling of BFP-, EGFP-, DsRed-, Fpr-, and Tus-LPETGG-His_6 with GGF. Left imgae: 15% gel brightfield image (stained with Coomassie blue). Right image: fluorescence image of the same gel before staining (illuminated by transilluminator, λ = 302 nm). On the left, lane 1: protein marker, lane 2: BFP ligation reaction, lane3: BFP negative control, lane 4: EGFP ligation reaction, lane 5: EGFP negative control, lane 6: DsRed ligation reaction, lane 7: DsRed negative control, lane 8: Fpr ligation reaction, lane 9: Fpr negative control, lane 10: Tus ligation reaction, lane 11: Tus negative control. On the right, the same as on the left but without the protein marker.

As expected, all fluorescent proteins were labelled with GGF mediated by SrtA successfully (left image lane 2-7). Fpr and Tus were also labelled with the fluorescent substrate in high yield. In all cases, in the absence of SrtA no target protein was labelled. Interestingly, SrtA-mediated labelling was found to be highly specific. This was demonstrated in the Tus labelling experiment (left image lane 10 and 11) where in the present of a relatively high amount of other proteins only Tus was labelled with the

fluorescent substrate. The functionality of the labelled Tus after SrtA reaction will be discussed in Chapter 4.

3.2.6) DNA labeling of EGFP-LPETGG-His_6 with different concentrations of DNA and pH

We have demonstrated that SrtA could be used for labelling proteins site-specifally with relatively small substrates under mild conditions. We have now turned our interest in labelling proteins with other biological molecules, such as DNA, using our system. This potentially could open up opportunity for a number of applications, such as drug delivery. In this experiment, we aimed to label EGFP-LPETGG-His_6 with *ter* conjugated with diglycin at either 5'- or 3'- end (*ter*-GG, a strand of DNA that bind specifically to Tus protein).

The pH of the assay buffer (50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.5) had to be optimised for DNA labelling. At this condition, the protonated amine group of diglycine could bind to the *ter* DNA phosphate backbone by electrostatic interactions, resulting in a self-aggregated cluster which might not be recongised by SrtA. With this in mind, we attempted to label our EGFP-LPETGG-His_6 (50 μM) with increasing concentrations of *ter*-GG (0 μM, 1 μM, 10 μM, 20 μM, 40 μM and 60 μM) in the present of SrtA (100 nM) at either pH 7.5 or 9. EGFP-His_6 was used as negative control. Without the LPETGG motif, *ter*-GG should not conjugate with the protein in the present of SrtA.

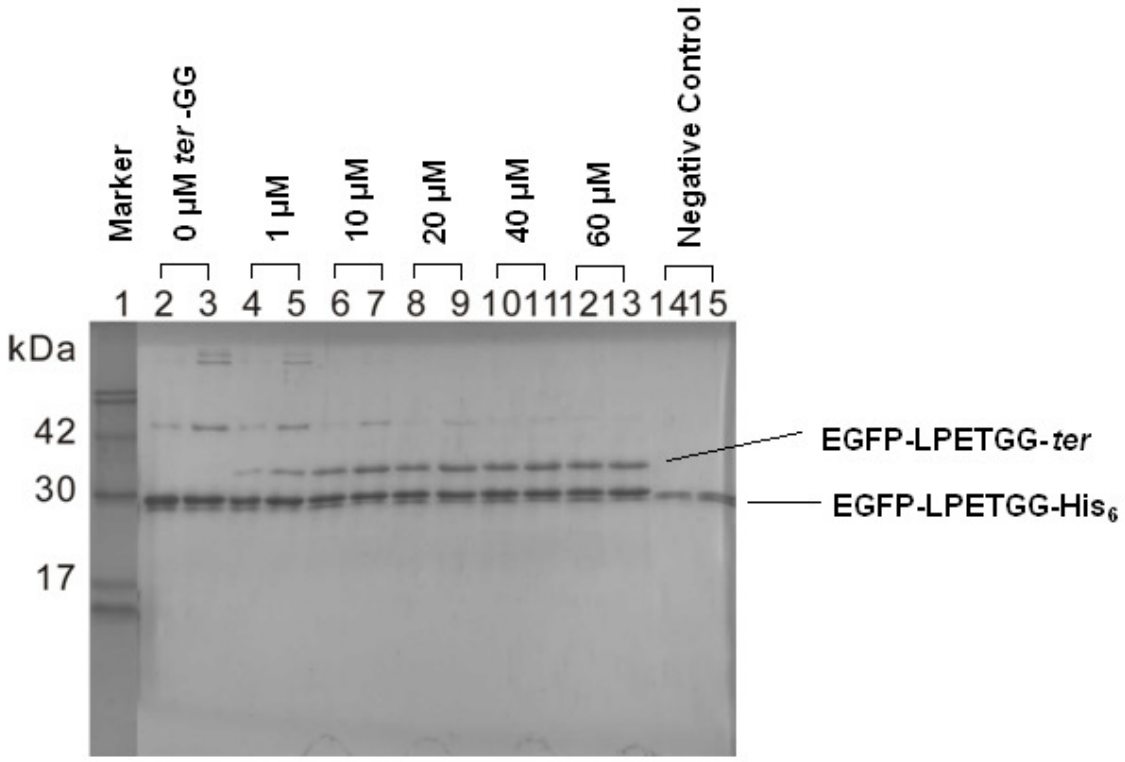


Figure 3.14 SDS-PAGE analysis of the conjugation of *ter*-GG with EGFP-LPETGG-His_6 at pH 7.5 or 9. Brightfield image of the 15% gel was acquired after staining with Commassie blue. Lane 1: protein marker, lane 2: 0 μM at pH 7.5, lane 3: 0 μM at pH 9, lane 4: 1 μM at pH 7.5, lane 5: 1 μM at pH 9, lane 6: 10 μM at pH 7.5, lane 7: 10 μM at pH 9, lane 8: 20 μM at pH 7.5, lane 9: 20 μM at pH 9, lane 10: 40 μM at pH 7.5, lane 11: 40 μM at pH 9, lane 12: 60 μM at pH 7.5, lane 13: 60 μM at pH 9, lane 14: EGFP-His_6 at pH 7.5 and lane 15: EGFP-His_6 at pH 9.

Figure 3.14 shows the SDS-PAGE analysis of the conjugation reaction. The unconjugated protein (EGFP-LPETGG-His_6) had a MW of 29 kDa, whereas the DNA-conjugated protein (EGFP-LPETGG-*ter*) appeared at ~35 kDa. As seen from the gel, the conjugation reaction performed equally well at either pH 7.5 or 9. The conjugating reaction reached satuation when *ter*-GG was ~10 μM. The reaction was highly specific as demonstrated in the negative control and about 30% of EGFP was modified to the DNA-conjugated protein.

This experiement proved that SrtA labelling system could be use to label proteins with relatively large substrate, such as DNA, under very mild conditions.

3.2.7) DNA labeling of EGFP-LPETGG-His_6 with different concentrations of EGFP-LPETGG-His_6

After optimising the pH of the assay buffer and substrate concentration, our next goal was to optimise the target protein concentration. The substrate *ter*-GG (10 μM) was incubated with increasing concentration of EGFP-LPETGG-His_6 (0 μM, 1 μM, 5 μM, 10 μM, 20 μM, 50 μM, 75 μM, 100 μM, 150 μM, 250 μM) in the present of SrtA (100 nM) in assay buffer (pH 9) at r.t. overnight. Afterwhich, the reaction mixture was analysed by SDS-PAGE.

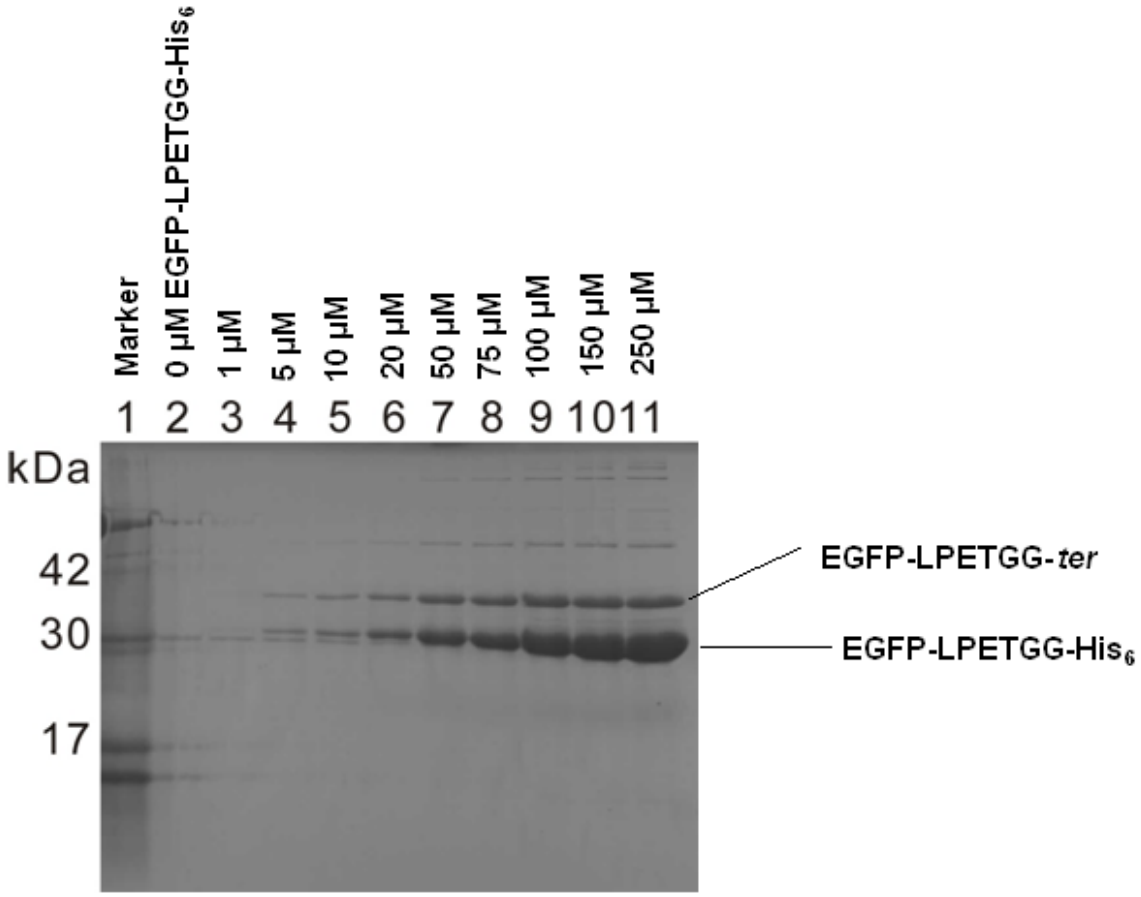


Figure 3.15 SDS-PAGE analysis of the conjugation of *ter*-GG to increasing concentration of EGFP-LPETGG-His_6 at r.t. over 16 h. 15% gel was stained with Commassie blue for visualisation. Lane 1: protein marker, lane 2: 0 μM; lane 3: 1 μM; lane 4: 5 μM; lane 5: 10 μM; lane 6: 20 μM; lane 7: 50 μM; lane 8: 75 μM; lane 9: 100 μM;, lane 10: 150 μM; lane 11: 250 μM.

Unconjugated EGFP-LPETGG-His_6 had a MW of 29 kDa, whereas the DNA-conjugated product appeared at~35 kDa. Despite of the increase in target protein concentration the conjuation reaction reached saturation at 50-75 μM of EGFP-LPETGG-His_6. This indicated that the yield of the reaction was less than 20%.

3.2.8) DNA labeling of EGFP-LPETGG-His_6 with different concentrations of SrtA

To improve the yield of the conjugation reaction, we attempted to optimise the SrtA concentration in the reaction. Target protein (50 μM) and *ter*-GG (10 μM) were incubated with increasing concentration of Srt A (0 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM, 100 nM, 150 nM, 200 nM, 250 nM, 500 nM and 1 μM) in assay buffer (pH 9) at r.t. overnight. The samples were analysed by SDS-PAGE.

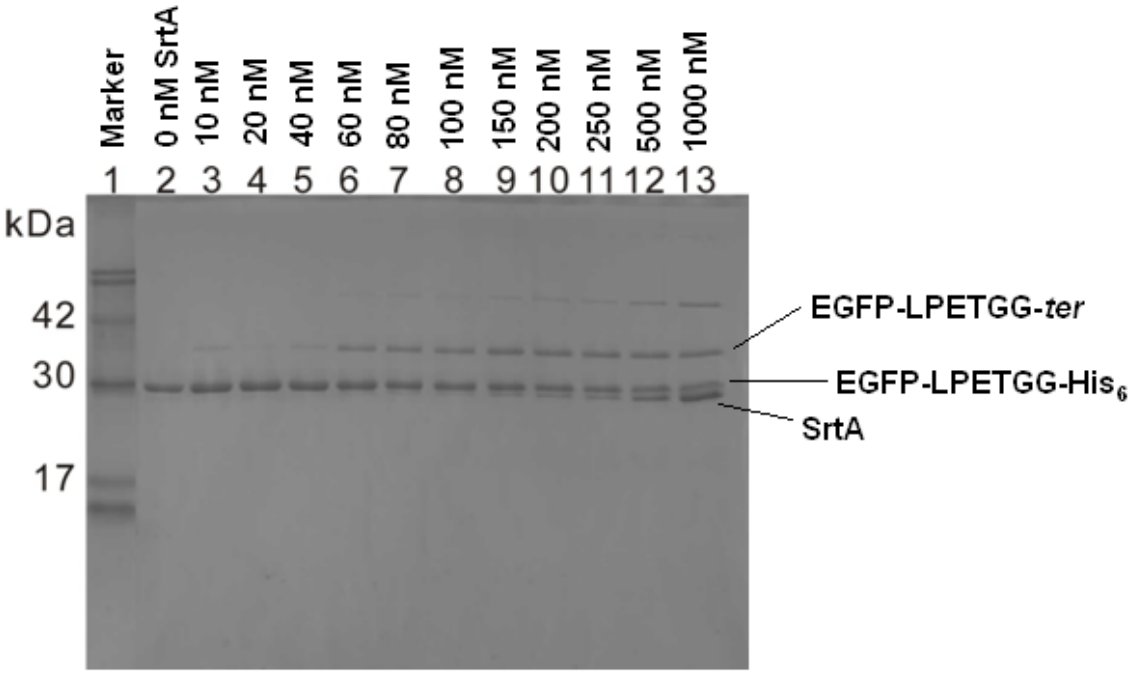


Figure 3.16 SDS-PAGE analysis of the conjugation of *ter*-GG to EGFP-LPETGG-His_6 with increasing concentration of SrtA at r.t. over 16 h. 15% gel was stained with Coomassie blue for visualisation. Lane 1: protein marker, lane 2: 0 nM; lane 3: 10 nM; lane 4: 20 nM; lane 5: 40 nM; lane 6: 60 nM; lane 7: 80 nM; lane 8: 100 nM; lane 9: 150 nM; lane 10: 200 nM; lane 11: 250 nM; lane 12: 500 nM; and lane 13: 1000 nM.

The reaction mixtures were analysed by SDS-PAGE and the conjugated product was seen at ~35 kDa. Although the concentration of SrtA was increasing it had little effect in the product concentration. The amount of product produced was fairly uniform when 60-1000 nM of SrtA was used. The band appeared at ~55 kDa might be due to the EGFP-LPET-SrtA intermediate. This suggested the *ter*-GG substrate did not ligate to the target protein efficiently. From lane 9, a band just below 29 kDa (EGFP-LPETGG-His_6) could be seen on the gel. This could be due to the hydrolysed product of the intermediate.

3.3) Summary and conclusion

In the past few years, the potential use of SrtA in biotechnology was greatly underestimated.[48-53] It was not until recently that researchers started to have increasing interest in using the SrtA system for monitoring protein-protein interactions,[54] cell labelling[55,56] or even anchoring cell surface proteins to cell walls.[57]

We have demonstrated the use of SrtA-meidated ligations for labelling target proteins (BFP, EGFP, DsRed, Tus and Fpr) site-specifcally with fluorescein-labelled oligoglycine substrates.[51,58] The reaction conditions for labelling target protein with GGF were optimised and showed good labelling yield. We also attempted the labelling of EGFP with a 21 bp diglycine labelled double-stranded *ter* DNA sequence. From the labelling experiments and SDS-PAGE analysis, we discoverded that *ter*-GG was a poor substrate for SrtA- labelling system in solution. The substrate was unable to bind to the EGFP-LPET-SrtA complex, probably due to steric hinderance. The target protein-SrtA complex was eventually hydrolysed in assay environment over time.

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3.4) Experimental Methods

3.4.1) Assay for Sortase

The reaction mixture contained 10 μM of BFP, 10 nM of SrtA, 200 mM diglycine (10 μL was added to each 1 mL reaction mixture) in assay buffer (150 mM NaCl, 50 mM Tris and 5 mM $CaCl_2$, pH 7.5). Another set without SrtA was prepared as negative control. For each reaction mixture, 50 μL of sample was taken at 0 minute, after which 10 nM of SrtA was added to the assay, and the same amount of deionised water was added to the negative control. Both reaction mixtures were incubated at 37 ℃ and samples were taken at the following time points, 5, 10, 20, 30, 60, 90, 120 and 240 minutes. These aliquots were immediately applied to a small Ni-NTA column to stop the reaction by separating SrtA and other by-products from the ligated proteins. Both reaction mixtures were left at 37 ℃ overnight and a final sample was taken the following day. The flow through from each Ni-NTA column was collected. As the product is fluorescent, the fluorescence intensity was measured by using the florescencene microplate reader (Safire^2, Tecan, Switzerland)..

3.4.2) Labelling of EGFP-LPETGG-His_6 with fluorescein labelled oligoglycine

Three set of reactions of EGFP-LPETGG-His_6 ligation with fluorescein labelled oligoglycine (GGF, GGGF, GGGGF) were set up.

Reagent	Quantity	Final concentration
EGFP-LPETGG-His_6	13.5 μL	50 μM
Fluorescein labelled oligoglycine	31.2 μL	500 μM
SrtA	2 μL	100 nM
Assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5)	Adjust volume to 250 μL	

Table 3.1 EGFP-LPETGG-His_6 with fluorescein labelled oligoglycine reaction mixture.

An aliquote of 20 μL from the reaction mixture was taken out at different time points (0 h, 1 h, 2 h, 4 h, 6 h, and 16 h). The aliquots were immediately heated up to 96 ℃ for 10 minutes for denaturation and stored at – 80 ℃ for storage. Once the last sample was taken and heated, the samples were then analysed with SDS-PAGE gel.

3.4.3) Labelling of EGFP-LPETGG-His_6 with different concentrations of fluorescein labelled diglycine

Different concentrations of fluorescein labelled diglycine (GGF) (0 μM, 1 μM, 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 750 μM and 1 mM) was used to ligate to EGFP-LPETGG-His_6. The reaction mixtures were incubated at room temperature overnight in the dark.

Reagent	Final concentration
EGFP-LPETGG-His_6	10 μM
GGF	Indicated above
SrtA	100 nM
Assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5)	Adjust volume to 50 μL

Table 3.2 Reaction mixture of EGFP-LPETGG-His_6 with GGF with different concentrations of GGF.

After which an aliquote of 20 μL was withdrawn from the samples and then analysed by SDS-PAGE.

3.4.4) Labelling of BFP-LPETGG-His_6 with fluorescein labelled diglycine using different concentrations of SrtA

Different concentrations of SrtA (0 nM, 1 nM, 10 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM and 1 μM) were used to catalyse the ligation of BFP-LPETGG-His_6 with GGF. The reaction mixtures were incubated at room temperature overnight in the dark.

Reagent	Final concentration
BFP-LPETGG-His_6	50 μM
GGF	100 μM
SrtA	Indicated above
Assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5)	Adjust volume to 200 μL

Table 3.3 Reaction mixture of BFP-LPETGG-His_6 with GGF with different concentrations of SrtA.

After which an aliquote of 20 μL was withdrawn from the samples and then analysed by SDS-PAGE.

3.4.5) Labelling of different proteins with fluorescein labelled diglycine using SrtA

To demonstrate that the SrtA system for fluorescently labelling proteins were mild and robust, several proteins (BFP-, EGFP-, DsRed-, Tus-, Fpr-LPETGG-His_6) were chosen to ligate to GGF. The reaction mixtures were incubated at room temperature overnight in the dark.

Reagent	Final concentration
Protein	Indicated above
GGF	20 μM
SrtA	100 nM
Assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5)	Adjust volume to 200 μL

Table 3.4 Reaction mixture of different proteins with GGF.

After which an aliquote of 20 μL was withdrawn from the samples and then analysed by SDS-PAGE.

3.4.6) DNA labeling of EGFP-LPETGG-His_6 with different concentrations of DNA and pH

ter-GG:

```
GG-ATAAGTATGTTGTAACTAAAG
   TATTCATACAACATTGATTTC
```

The double-stranded DNA *ter*-GG was used as substrate to label EGFP-LPETGG-His_6 using the SrtA system. Three sets of reaction were set up: 1) Reaction mixture with different concentrations of *ter*-GG. 2) Reaction mixture with different pH of the buffer. 3) Reaction mixture with EGFP-His_6 as negative control.

Set 1): Reaction mixture with different concentrations of *ter*-GG:

Different concentrations of *ter*-GG (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM and TBB (without any DNA)) were chosen to optimise the DNA concentration for ligation to EGFP-LPETGG-His_6.

Reagent	Final concentration
ter-GG	Indicated above
EGFP-LPETGG-His_6 (940 mM)	50 μM
SrtA (123 μM)	100 nM
Buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 9)	Adjust volume to 200 μL

Table 3.5 Reaction mixture of DNA labelling of EGFP-LPETGG-His_6.

Set 2): Reaction mixture with different pH of the buffer:

Similar to set 1 but using pH 9.0 buffer solution.

Set 3): Reaction mixture with EGFP-His_6:

Similar to set 1 (pH 7.5) and set 2 (pH 9.0) but replacing EGFP-LPETGG-His_6 with EGFP-His_6.

Reagent	Quantity	Final Concentration
ter-GG (250 μM)	16 μL	10 μM
EGFP- His_6 (178 mM)	22.5 μL	50 μM
SrtA (123 μM)	1.7 μL	100 nM
Buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$)	160 μL	

Table 3.6 Reaction mixture of DNA labelling of EGFP -His_6 with different pH of buffer.

3.4.7) DNA labeling of EGFP-LPETGG-His_6 with different Protein concentrations

Different concentrations of EGFP-LPETGG-His_6 (0 μM, 1 μM, 5 μM, 10 μM, 20 μM, 50 μM, 75 μM, 100 μM, 150 μM, 250 μM) were chosen to optimise the labelling reaction.

Reagent	Fincal Concentration
ter-GG (250 μM)	10 μM
EGFP-LPETGG-His_6 (940 mM)	Indicated above
SrtA (123 μM)	100 nM
Buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 9)	Adjust volume to 200 μL

Table 3.7 Reaction mixture of DNA labelling of EGFP-LPETGG-His_6 with different concentrations of EGFP-LPETGG-His_6.

3.4.8) DNA labeling of EGFP-LPETGG-His_6 with different concentrations of SrtA

To optimized the condition for ligation of DNA to EGFP-LPETGG-His_6, different SrtA concentrations were used (0 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM, 100 nM, 150 nM, 200 nM, 250 nM, 500 nM and 1 μM).

Reagent	Final Concentration
ter-GG (250 μM)	10 μM
EGFP-LPETGG-His_6 (940 mM)	50 μM
SrtA (123 μM)	Indicated above
Assay Buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 9)	Adjust volume to 200 μL

Table 3.8 Reaction mixture of DNA labelling of EGFP-LPETGG-His_6 with different concentrations of SrtA.

3.4.9) Ligation of BFP-LPETGG-His_6 to GGF with different concentrations of fluorescein labelled diglycine

Different concentrations of GGF (0.7 μM, 3.5 μM, 7.0 μM, 11 μM, 14 μM, 18 μM, 21 μM and 25 μM) were used for optimising the labelleding reaction.

Reagent	Final concentration
BFP-LPETGG-His_6	50 μM
GGF	Indicated above
SrtA	100 nM
Assay Buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 9)	Adjust volume to 200 μL

Table 3.9 Reaction mixture of BFP-LPETGG-His_6 with different concentrations of fluorescein labelled diglycine.

The reaction mixture was mixed in a black 96 well plate with three drops of mineral oil on top, and the fluorescence was monitored by plate reader at λ_{ex} = 395 nm, λ_{em} = 518 nm 37 ℃ overnight.

3.4.10) Ligation of BFP-LPETGG-His_6 to GGF with different concentrations of SrtA

Different SrtA concentrations (0 nM, 1 nM, 5 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM and 100 nM) were used for optimising the labelling reaction.

Reagent	Final concentration
BFP-LPETGG-His_6	50 μM
GGF	25 μM
SrtA	Indicated above
Assay Buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 9)	Adjust volume to 200 μL

Table 3.10 Reaction mixture of BFP-LPETGG-His_6 with different concentrations of SrtA.

The reaction was mixed in black 96-well plate with three drops of mineral oil on top, and the fluorescence monitored by plate reader at 37 ℃ overnight (at λ_{ex} = 395 nm, λ_{em} = 518 nm).

3.5) References

(1) Giot, L.; Bader, J. S.; Brouwer, C.; Chaudhuri, A.; Kuang, B.; Li, Y.; Hao, Y. L.; Ooi, C. E.; Godwin, B.; Vitols, E.; Vijayadamodar, G.; Pochart, P.; Machineni, H.; Welsh, M.; Kong, Y.; Zerhusen, B.; Malcolm, R.; Varrone, Z.; Collis, A.; Minto, M.; Burgess, S.; McDaniel, L.; Stimpson, E.; Spriggs, F.; Williams, J.; Neurath, K.; Ioime, N.; Agee, M.; Voss, E.; Furtak, K.; Renzulli, R.; Aanensen, N.; Carrolla, S.; Bickelhaupt, E.; Lazovatsky, Y.; DaSilva, A.; Zhong, J.; Stanyon, C. A.; Finley, R. L., Jr.; White, K. P.; Braverman, M.; Jarvie, T.; Gold, S.; Leach, M.; Knight, J.; Shimkets, R. A.; McKenna, M. P.; Chant, J.; Rothberg, J. M. *Science* **2003**, *302*, 1727-36.

(2) Dagher, R.; Pigault, C.; Bonnet, D.; Boeglin, D.; Pourbaix, C.; Kilhoffer, M. C.; Villa, P.; Wermuth, C. G.; Hibert, M.; Haiech, J. *Biochim Biophys Acta* **2006**, *1763*, 1250-5.

(3) Khoschsorur, G.; Fruehwirth, F.; Zelzer, S.; Stettin, M.; Halwachs-Baumann, G. *Clin Chim Acta* **2007**, *380*, 217-21.

(4) Rizzo, M. A.; Piston, D. W. *Biophys J* **2005**, *88*, L14-6.

(5) Hebert, T. E.; Gales, C.; Rebois, R. V. *Cell Biochem Biophys* **2006**, *45*, 85-109.

(6) Wang, C.; Bian, W.; Xia, C.; Zhang, T.; Guillemot, F.; Jing, N. *Cell Res* **2006**, *16*, 585-98.

(7) Mani, R. S.; Usova, E. V.; Cass, C. E.; Eriksson, S. *Biochemistry* **2006**, *45*, 3534-41.

(8) Nakanishi, J.; Takarada, T.; Yunoki, S.; Kikuchi, Y.; Maeda, M. *Biochem Biophys Res Commun* **2006**, *343*, 1191-6.

(9) Suzuki, Y.; Yasunaga, T.; Ohkura, R.; Wakabayashi, T.; Sutoh, K. *Nature* **1998**, *396*, 380-3.

(10) Kim, K.; Dimitrova, D. D.; Carta, K. M.; Saxena, A.; Daras, M.; Borowiec, J. A. *Mol*

Cell Biol **2005**, *25*, 2463-74.

(11) Forster, T. *Ann. Physik (Leipzig)* **1984**, *2*, 55-75.

(12) Stryer, L. *Annu Rev Biochem* **1978**, *47*, 819-46.

(13) Tsien, R. Y. *Annu Rev Biochem* **1998**, *67*, 509-44.

(14) Cormack, B. P.; Valdivia, R. H.; Falkow, S. *Gene* **1996**, *173*, 33-8.

(15) Delagrave, S.; Hawtin, R. E.; Silva, C. M.; Yang, M. M.; Youvan, D. C. *Biotechnology (N Y)* **1995**, *13*, 151-4.

(16) Heim, R.; Cubitt, A. B.; Tsien, R. Y. *Nature* **1995**, *373*, 663-4.

(17) Heim, R.; Prasher, D. C.; Tsien, R. Y. *Proc Natl Acad Sci U S A* **1994**, *91*, 12501-4.

(18) Miyawaki, A.; Llopis, J.; Heim, R.; McCaffery, J. M.; Adams, J. A.; Ikura, M.; Tsien, R. Y. *Nature* **1997**, *388*, 882-7.

(19) Rizzuto, R.; Brini, M.; De Giorgi, F.; Rossi, R.; Heim, R.; Tsien, R. Y.; Pozzan, T. *Curr Biol* **1996**, *6*, 183-8.

(20) Bastiaens, P. I.; Squire, A. *Trends Cell Biol* **1999**, *9*, 48-52.

(21) Gadella, T. W., Jr.; van der Krogt, G. N.; Bisseling, T. *Trends Plant Sci* **1999**, *4*, 287-291.

(22) Herman, B. *Methods Cell Biol* **1989**, *30*, 219-43.

(23) Jovin, T. M.; Arndt-Jovin, D. J. *Annu Rev Biophys Biophys Chem* **1989**, *18*, 271-308.

(24) Pepperkok, R.; Squire, A.; Geley, S.; Bastiaens, P. I. *Curr Biol* **1999**, *9*, 269-72.

(25) Wouters, F. S.; Bastiaens, P. I. *Curr Biol* **1999**, *9*, 1127-30.

(26) Yguerabide, J. *Biophys J* **1994**, *66*, 683-93.

(27) Wolber, P. K.; Hudson, B. S. *Biophys J* **1979**, *28*, 197-210.

(28) Veatch, W.; Stryer, L. *J Mol Biol* **1977**, *113*, 89-102.

(29) Shaklai, N.; Yguerabide, J.; Ranney, H. M. *Biochemistry* **1977**, *16*, 5585-92.

(30) Kenworthy, A. K.; Edidin, M. *J Cell Biol* **1998**, *142*, 69-84.

(31) Fung, B. K.; Stryer, L. *Biochemistry* **1978**, *17*, 5241-8.

(32) Dewey, T. G.; Hammes, G. G. *Biophys J* **1980**, *32*, 1023-35.

(33) Adair, B. D.; Engelman, D. M. *Biochemistry* **1994**, *33*, 5539-44.

(34) Woolhead, C. A.; McCormick, P. J.; Johnson, A. E. *Cell* **2004**, *116*, 725-36.

(35) Sweet, G. H.; Schindler, C. A. *J Bacteriol* **1967**, *93*, 1943-8.

(36) Hungerford, G.; Benesch, J.; Mano, J. F.; Reis, R. L. *Photochem Photobiol Sci* **2007**, *6*, 152-8.

(37) Weingart, C. L.; Broitman-Maduro, G.; Dean, G.; Newman, S.; Peppler, M.; Weiss, A. A. *Infect Immun* **1999**, *67*, 4264-7.

(38) Hed, J. *Methods Enzymol* **1986**, *132*, 198-204.

(39) Hazenbos, W. L.; van den Berg, B. M.; van't Wout, J. W.; Mooi, F. R.; van Furth, R. *Infect Immun* **1994**, *62*, 4818-24.

(40) Drevets, D. A.; Campbell, P. A. *J Immunol Methods* **1991**, *142*, 31-8.

(41) Sender, S.; Decker, B.; Fenske, C. D.; Sly, W. S.; Carter, N. D.; Gros, G. *J Histochem Cytochem* **1998**, *46*, 855-61.

(42) Evans, T. C., Jr.; Benner, J.; Xu, M. Q. *J Biol Chem* **1999**, *274*, 3923-6.

(43) Ando, T.; Tsukiji, S.; Tanaka, T.; Nagamune, T. *Chem Commun (Camb)* **2007**, 4995-7.

(44) Zuberek, J.; Wyslouch-Cieszynska, A.; Niedzwiecka, A.; Dadlez, M.; Stepinski, J.; Augustyniak, W.; Gingras, A. C.; Zhang, Z.; Burley, S. K.; Sonenberg, N.; Stolarski, R.; Darzynkiewicz, E. *Rna* **2003**, *9*, 52-61.

(45) Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc Natl Acad Sci U S A* **1998**, *95*, 6705-10.

(46) Wood, R. J.; Pascoe, D. D.; Brown, Z. K.; Medlicott, E. M.; Kriek, M.; Neylon, C.; Roach, P. L. *Bioconjug Chem* **2004**, *15*, 366-72.

(47) Mukhopadhyay, J.; Kapanidis, A. N.; Mekler, V.; Kortkhonjia, E.; Ebright, Y. W.; Ebright, R. H. *Cell* **2001**, *106*, 453-63.

(48) Chan, L.; Cross, H. F.; She, J. K.; Cavalli, G.; Martins, H. F.; Neylon, C. *PLoS One* **2007**, *2*, e1164.

(49) Kobashigawa, Y.; Kumeta, H.; Ogura, K.; Inagaki, F. *J Biomol NMR* **2009**, *43*, 145-50.

(50) Mao, H.; Hart, S. A.; Schink, A.; Pollok, B. A. *J Am Chem Soc* **2004**, *126*, 2670-1.

(51) Parthasarathy, R.; Subramanian, S.; Boder, E. T. *Bioconjug Chem* **2007**, *18*, 469-76.

(52) Pritz, S.; Wolf, Y.; Kraetke, O.; Klose, J.; Bienert, M.; Beyermann, M. *J Org Chem* **2007**, *72*, 3909-12.

(53) Pritz, S.; Wolf, Y.; Kraetke, O.; Klose, J.; Bienert, M.; Beyermann, M. *Adv Exp Med Biol* **2009**, *611*, 107-8.

(54) Popp, M. W.; Antos, J. M.; Ploegh, H. L. *Curr Protoc Protein Sci* **2009**, *Chapter 15*, Unit 15 3.

(55) Yamamoto, T.; Nagamune, T. *Chem Commun (Camb)* **2009**, 1022-4.

(56) Tanaka, T.; Yamamoto, T.; Tsukiji, S.; Nagamune, T. *Chembiochem* **2008**, *9*, 802-7.

(57) Perry, A. M.; Ton-That, H.; Mazmanian, S. K.; Schneewind, O. *J Biol Chem* **2002**, *277*, 16241-8.

(58) Tsukiji S., Nagamune T. *Chembiochem* **2009**, 10, 787-98.

Chapter 4: Protein immobilisation on solid supports and surfaces

4.1) Introduction

The immobilisation of proteins onto surfaces is crucial to biological applications such as *in vitro* diagnostic and drug discovery,[1-5] allowing researchers to monitor molecular interactions, protein conformational dynamics, protein expression, and protein translocation in living cells etc. In such techniques, the immobilised protein[5,6] must retain their functionality or configuration on the surface in order to function properly. This is an active and yet challenging area in research. Many efforts have been made to develop different immobilisation techniques. For example in protein microarrays, proteins are routinely immobilised onto glass substrates by a number of methods: adsorption; affinity binding; and covalent couplings etc.[7-11]

4.1.1) Adsorption

In adsorption, protein molecules are adsorped to the surface by a combination of hydrophobic, electrostatic and other intramolecular interactions. Although this method is very easy to implement, the protein molecules are not bound to the surface permanently. In fact, the molecules are in equilibrium of adsorption and desroption. Over time the protein activity isreduced due to protein unfolding and denaturation.

4.1.2) Affinity binding

Another popular technique for attaching proteins onto surfaces is by affinity binding. Many systems have been developed, for example the His_6-tag/Ni-affinity system. In this example, protein molecules are expressed with a histidine tag (his-tag) at either the N- or C-termini. The His-tagged protein molecules can easily be captured by surfaces functionalised with Ni-NTA (similar methodology is routinely used in protein purification which was decribed in Chapter 3).

However, the binding between the his-tag and Ni-NTA is not very strong and can be affected by many chemicals,[12] for example, DTT, SDS and EDTA. Another very popular approach is the biotin/(strept)avidin system.[13-18] In this case, the surface of interest is coated with a layer of avidin (or streptavidin). Biological molecules functionalised with biotin are captured through the strongest non-covalent linkage. The binding between biotin and (strept)avidin is considered to be almost irreversible (K_d ~10^{-15} M)[19] and is stable under very harsh conditions.[20]

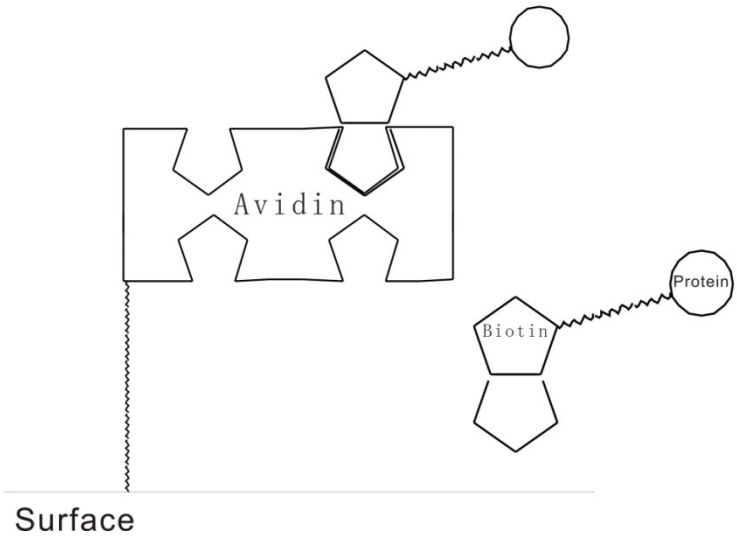

Figure 4.1 Schematic diagram of an immobilised avidin molecule with one of the four binding sites complexed with a biotinylated-protein.

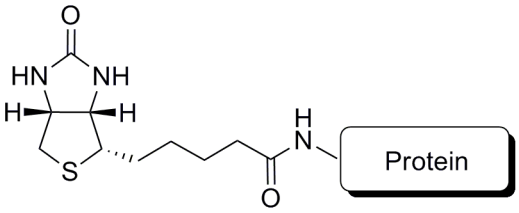

Figure 4.2 Structure of biotinylated protein.

This method is often use for DNA-based applications, such as DNA microarrays, where synthetic oligonucleotides can easily be modified with biotin. For proteins, this method provides limited solutions mainly due to the limitation of *in vitro* biotinylation of target proteins.[16]

4.1.3) Covalent attachment

Another popular approach is by covalent attachment.[22-32] For example, glass substrates can be modified with amino group functionality using (3-Aminopropyl)triethoxysilane (APTES).[26] Biological molecules can then be immobilised to the functionalised surface using a cross-linker (e.g. glutaraldehyde) or coupling reagens such as *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC).[23]

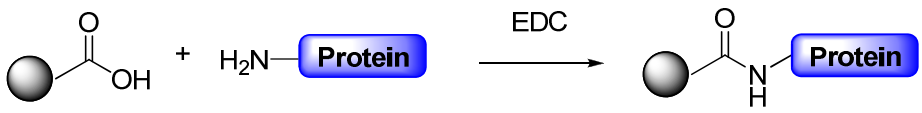

Figure 4.3 Protein ligation to GMA beads using EDC coupling.

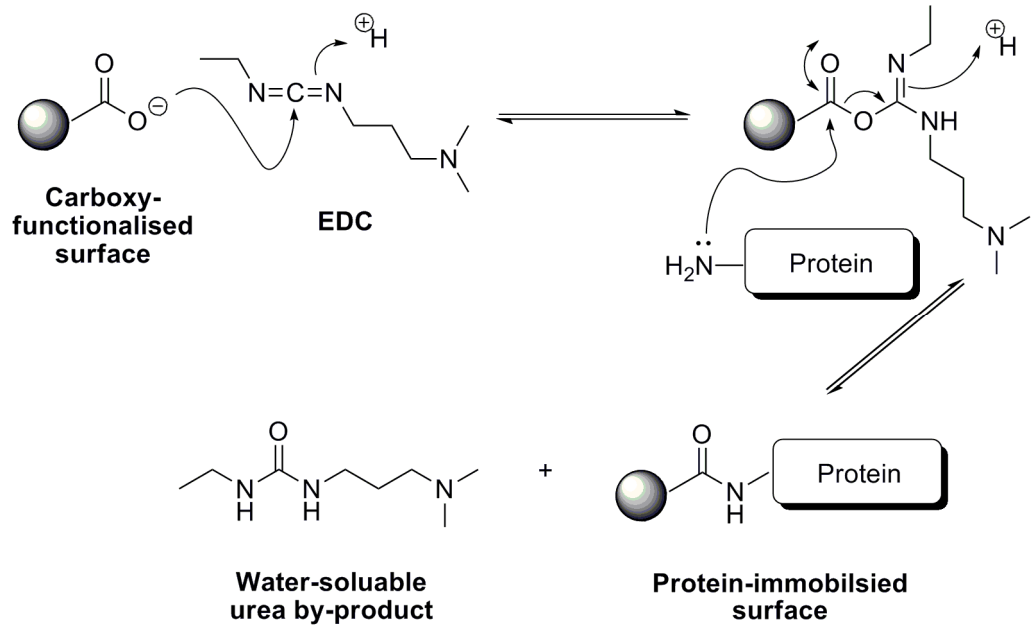

Figure 4.4 Mechanism of EDC-mediated protein coupling.

Although not as easy to implement as other methods, covalent attachment ensures the biological molecules are permenantly immobilised onto the surface substrate. The reaction conditions can be fine tuned for each application in order to reach the optimal loading level. There are still a number of disadvantages. For instance, employing EDC as a coupling reagent would result in covalent linkage between any carboxylic acids and free amines. This means the immobilisation is not site-specific and so the protein molecules are attached to the surface in random orientations. The use of toxic chemicals, such as EDC and glutaralhyde, also means a properly equipeed environment is needed for carrying out the chemical reactions.

4.1.4) Application of micron-sized beads

Other than planar surfaces, the immobilization of proteins has been extended to three-dimensional particles, for example, glycidyl methacrylate (GMA),[22-26] glass beads[27,28] and magnetic beads.[29-32] The immobilisation of biologically interesting

proteins onto micron-size beads like glass and magnetic beads could provide a lot of advantages. For instance, molecular interactions can be monitored on bead surfaces by flow cytometry,[23] and easy separation of immobilised proteins from reaction mixture by gravitation or magnetic sepearation. In biological assays, the use of micron-sized beads in suspension arrays increase the surface-to-volumn ratio and thereby reduce the volume needed for reactions.[23]

With the success of labelling target proteins with a number of substrates by SrtA-mediated ligation, we would like to develop novel methods for immobilising proteins to a range of surface substrates. These methods should improve the current immobilisation techniques which provide a mild and yet site-specific ligation of proteins onto solid supports.

4.2) Results and Discussion

With the success in fluorescently labelling proteins in solution with the SrtA system, our next goal was to explore the field in immobilising proteins onto solid supports. Three solid supports with distinctly different properties were chosen: 1) highly crossed linked glycidyl methacrylate polymer beads (GMA beads, 5 μm diameter, Bangs Laboratories, IN, USA); 2) porous gel matrix (Affi-Gel 102 resin, Bio-Rad, Hertfordshire, U.K.) and microscope glass coverslips (VWR, Leicestershire, U.K.).

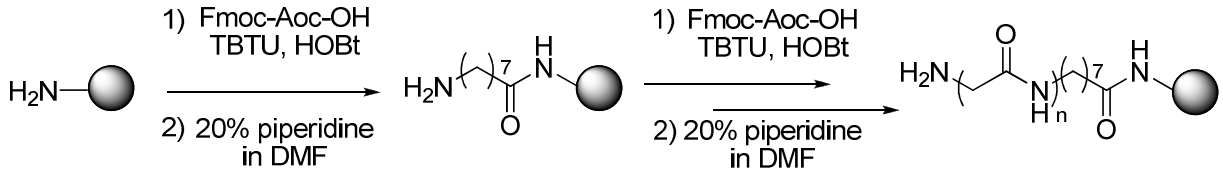

Figure 4.5 The modification of GMA beads with Aoc-OH followed by one, two or four glycine residues.

As the surface is not recognisable by SrtA, it had to be modified with glycines using the EDC coupling methods. For the GMA beads (modified by Joseph She), the amino-functionalised surface was modified with an Fmoc-protected 8-amino-octanoic acid spacer (Fmoc-Aoc-OH). After Fmoc deprotection, the surface was functionalised sequentially with one, two, or four glycine residues (Figure 4.5)

4.2.1) Ligation of EGFP-LPETGG-His_6 onto different oligoglycine labelled GMA beads

Our first target was the immobilization of proteins onto cross-linked polymer beads. Glycidyl methacrylate (GMA) beads were modified with one, two, or four glycine residues as described above. The mono-glycine, di-glycine, and tetra-glycine beads were incubated with EGFP-LPETGG-His_6 (85 μM) and SrtA (40 nM) in assay buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5). As negative controls, beads with no glycine coupled were incubated with SrtA and EGFP-LPETGG-His_6, and tetraglycine beads were incubated with EGFP-LPETGG-His_6 in the absence of SrtA.

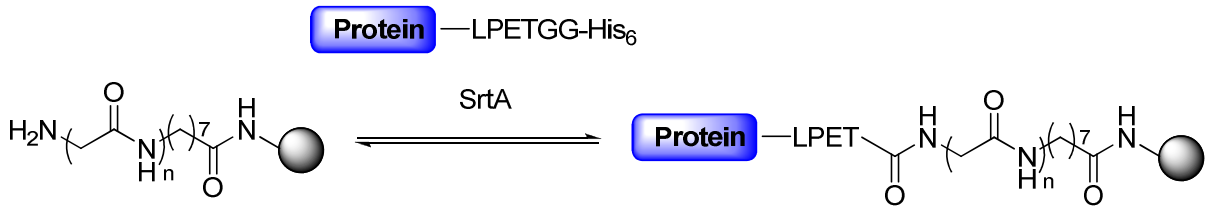

Figure 4.6 Attachment of a target protein to glycine-functionalised solid support using SrtA system.

Samples were taken at various time points and the beads were analysed using a fluorescence-activated cell sorter (FACS, FACSAria, BD, NJ, U.S.A.). Negative controls were amino-functionalised beads or diglycine beads without SrtA. Data were collected on 1000 events and the mean of the fluorescent population plotted. Error bars shows the standard errors in the mean fluorescence and were generally smaller than the data points.

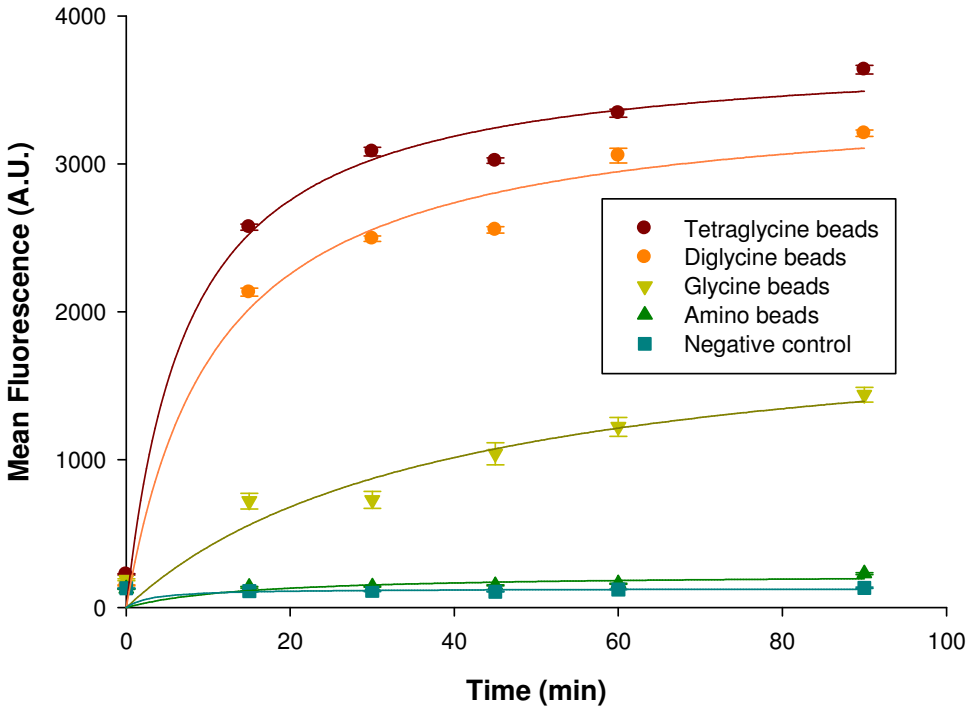

Figure 4.7 Kinetic analysis of EGFP-LPETGG-His_6 immobilisation on mono-, di-, and tetra-glycine functionalised GMA beadsusing the SrtA system.

Figure 4.7 shows the kinetic analysis of the SrtA-mediated protein immobilisation. GMA functionalised with tetra-glycine showed the most rapid fluorescence increase and the highest final fluorescence. Di-glycine beads were nearly as effective as tetra-glycine with mono-glycine beads showing slower increase and significantly reduced final fluorescence. In both negative controls, where the substrate beads were substituted with amino-functionalised solid support or SrtA was omitted, there was little background reaction, suggesting the non-specific binding of the fluorescent proteins to the bead was minimum. These observations were similar to our previous labelling experiments in solution.

4.2.2) Ligation of EGFP-LPETGG-His_6 and DsRed-LPETGG-His_6 on to GMA beads

After successfully immobilised EGFP-LPETGG-His_6 onto different functionalised GMA beads, our next goal was to attach different fluorescent proteins onto the di-glycine beads using the same SrtA system. As demonstrated from the result above, the di-glycine beads exibited similar initial rate as the tetraglycine beads and were easier to produce. EGFP and DsRed were separately immobilised onto di-glycine functionalised GMA beads and then mixed. As for negative control, SrtA was omitted from the reaction mixture (with EGFP-LPETGG-His_6). Brightfield and fluorescence micrographs were obtained on an Axiovert 200 fluorescence microscope with fluorescein and Cy3 filter sets, combined, and false colored.

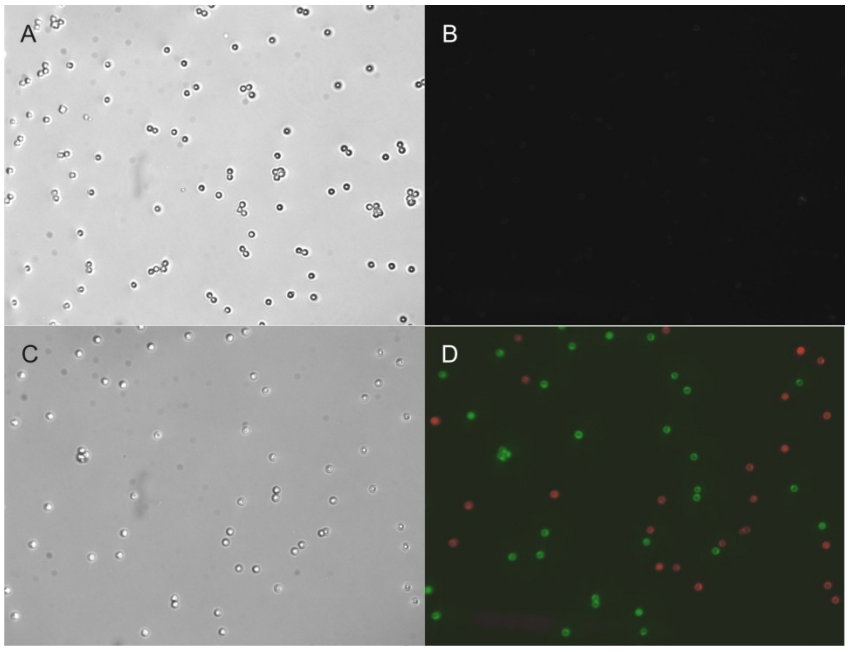

Figure 4.8 Immobilisation of fluorescent proteins to diglycine GMA beads. Diglycine GMA beads were incubated with fluorescent proteins in the presence (C and D) or absence of SrtA (A and B). Negative control: EGFP-LPETGG-His_6 proteins without SrtA (A:

brightfield; B: fluorescence image) Mixture of EGFP-LPETGG-His_6 and DsRed-LPETGG-His_6 ligated GMA beads (C: brightfield; DL fluorescence image)

Figure 4.8 shows the micrographs of the GMA beads after protein immobilisation reactions. EGFP and DsRed were separately immobilised onto di-glycine functionalised GMA beads and then mixed (C: brightfield image; D: fluorescence image). As seen on the fluorescence image, the beads were highly fluorescent. In the negative control where SrtA was omitted from the reaction mixture (with EGFP-LPETGG-His_6 only) it showed little background reactions (A: brightfield image; D: fluorescence image), suggesting a very low amount of target proteins were non-specifically bound to the surface.

4.2.3) DNA binding to Tus ligated GMA beads

The major limitation of exisiting immobilisation techniques is that the attached proteins are captured in a random orientation and sometimes lost the activity due to protein unfolding. In this experiment, we ought to demonstrate that immobilised protein molecules using the SrtA system was functional and accessible to other molecules. Tus-LPETGG-His_6 was selected to immobilised onto tetraglycine GMA beads using the standard attaching protocol and then tested the ability for binding to specific DNA sequence.

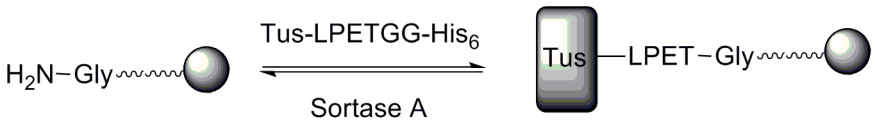

Figure 4.9 Attachment of Tus to functionalised solid support using SrtA system.

Tus is a sequence specific DNA-binding protein that recognises the 21 bp *ter* sites.[33] In order to demonstrate the functionality of the immobilised Tus protein of GMA beads, the solid supports were incubated with different proportions of fluorescein-labelled *terB* double-stranded sequence and a 21 bp Cy5-labelled dsDNA sequence unrelated to *terB* in binding buffer (50 mM Tris-HCl, 250 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, pH 9). The total DNA concentration (*terB* plus nonspecific DNA) was 100 nM for all samples.

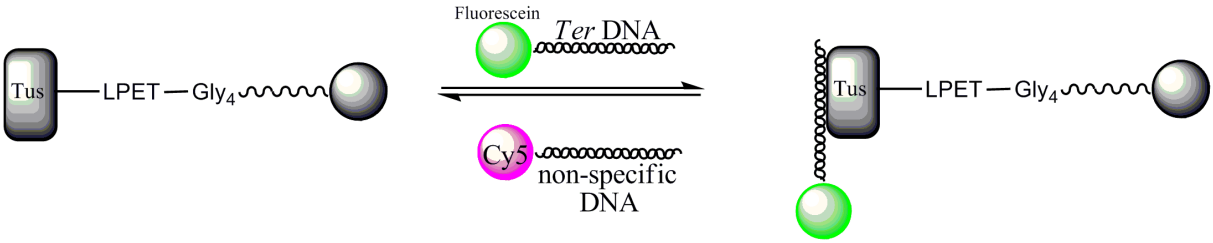

Figure 4.10 Attachment of a target protein to a solid support using SrtA system.

After the DNA binding reaction, the beads were analysed by FACS. A total of 1000 beads from each reaction were recorded and the fluorescein and Cy5 fluorescence of each bead was measured. If the Tus is still functionable, the fluorescein labelled *ter* should be able to bind to the Tus specifically while the Cy5 labelled non specific DNA will not bind

to the Tus. This will result in a high fluorescent intensity in fluorecein channel and low fluorescent intensity in Cy5 channel. The resulting fluorescence-saturation binding curve is shown in Figure 4.11. Non-specific DNA binding (Cy5) was very low in all cases. This was in agreement with previous observation in solution in 250 nM KCl.[34] The binding between *ter* and immobilised Tus showed a concentration-dependent single-binding process. The equilibrium dissociation constant (K_d) was determined to be 29±8 nM, which was comparable with the values measured by fluorescence anisotropy (~15 nM at 37 °C) or Biacore (~1 nM at 25 °C).[34]

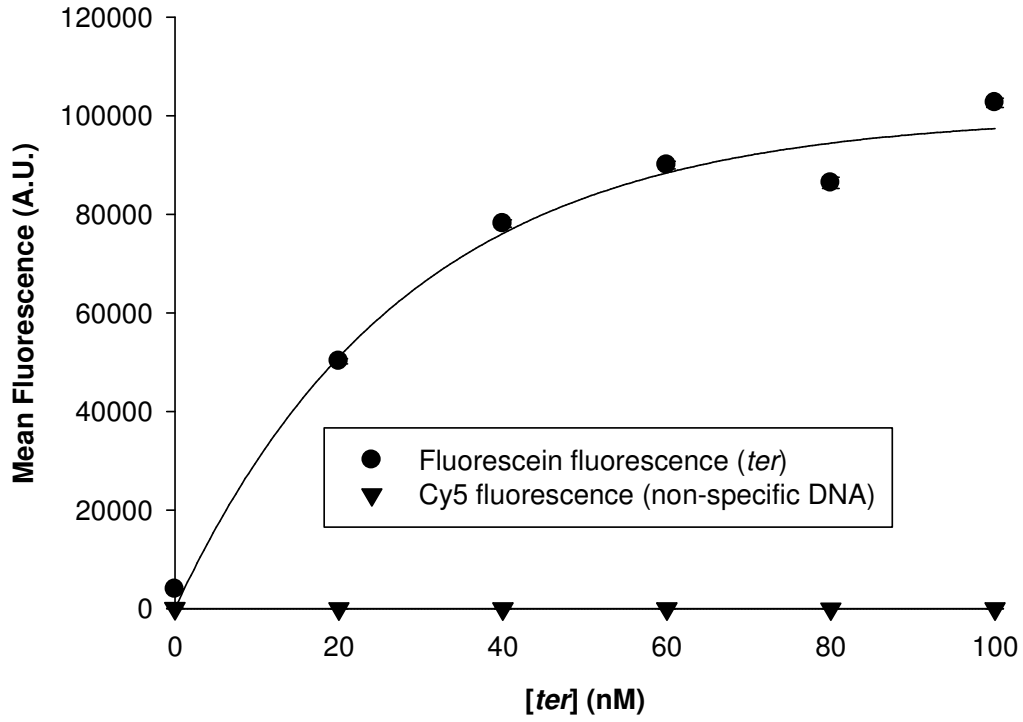

Figure 4.11 Tus protein ligated to GMA beads is accessible to its cognate DNA ligand (*ter*). The sequence specific DNA-binding protein Tus was ligated to diglycine GMA beads. The Tus-labelled beads were incubated with varying proportions of fluorescein labelled *ter* and Cy5 labelled non-specific DNA and the bead fluorescence analysed by FACS. The curve is a model fit for a single binding process with a K_d of 29 nM.

This experiment demonstrated that proteins with delicate 3-D structures, like Tus, could be immobilised onto hard polymeric supports using the SrtA system while retaining the protein functionality. This important finding potentially provides an improved, clean and straightforward technique for the exisiting biotechnologies.

4.2.4) Assay of Tus-LPETGG-His_6 ligated tetraglycine GMA beads with different concentrations of DNA

In addition to the above demonstration, we were eagered to provide futher evidence to show the *ter* DNA sequence was specifically bound to the immobilised Tus on the solid support. Seven different concentrations of flurocein-labelled *ter* (0 nM (TBB), 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM) were prepared. The Tus-coated GMA beads were washed by resuspending in DNA ligation buffer, centrifuged at 13500 rpm for 1 minute, and the supernatant discarded. The above washing steps were repeated twice. As a negative control, tetraglycine-functionalised GMA beads were used. The GMA beads were incubated with different concentrations of labelled *ter* at r.t. for 3 h with shaking. After which the beads were analysed by FACS and the fluorescent intensity was recored.

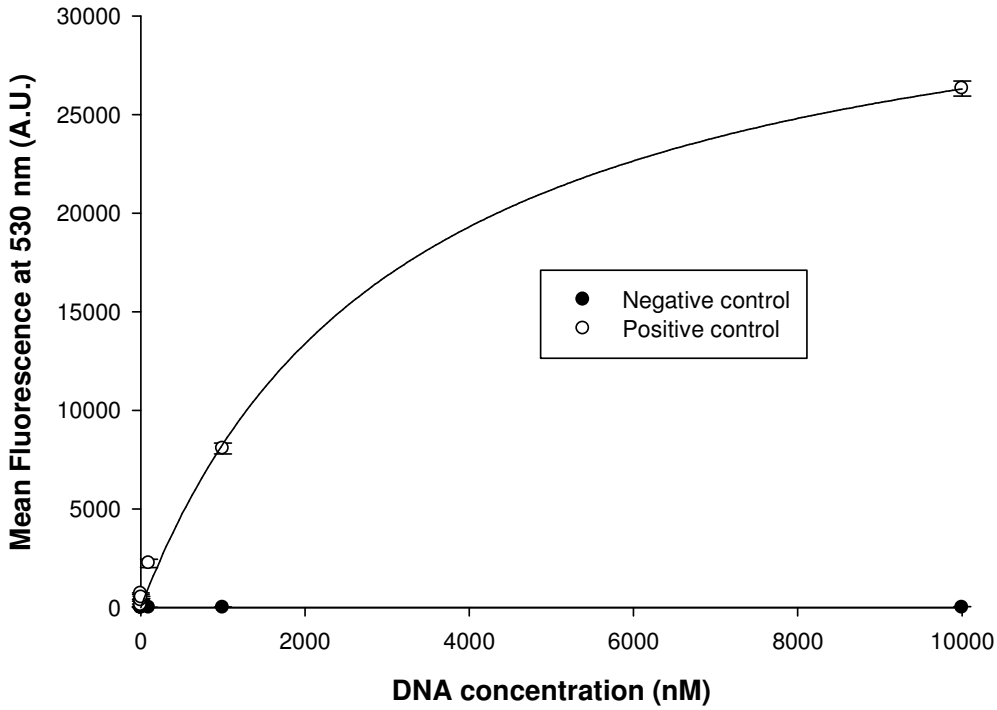

Figure 4.12 The fluorescent intensity measured at 530 nm as a function of *ter* concentration with the deduction of back ground fluorescent intensity.

The fluorescence-binding curve shown in Figure 4.12 provided further evidence that the *ter* sequence was binding to the immobilised Tus on GMA in a specific manner. When tetra-glycine GMA beads were used, the amount of non-speicifcally bound *ter* was neglectable. In the case of Tus-GMA, the binding increased as a function of *ter* concentration.

4.2.5) Removal of *ter* from immobilised Tus-GMA.

Our next attempt was to remove the bound *ter* sequence from the immobilised Tus on GMA. The samples were washed by resuspended in DNA removal buffer (50 mM Tris, 0.1 mM EDTA, 250 mM KCl, 0.1 DTT, 1 M $MgCl_2$, pH 7.5) at room temperature for 5

minutes, centrifuged at 13500 rpm for 1 minute, and the supernatant was discarded. The above washing steps were repeated for 3 times. The fluorescent intensity of the washed beads were analysed by FACS at 530 nm.

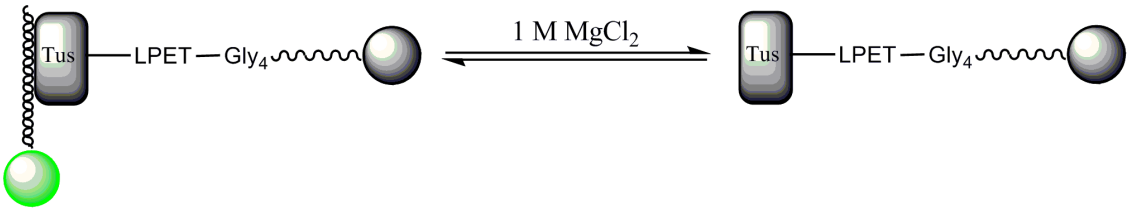

Figure 4.13 The removal of fluorescein labelled *ter* from Tus ligated GMA beads.

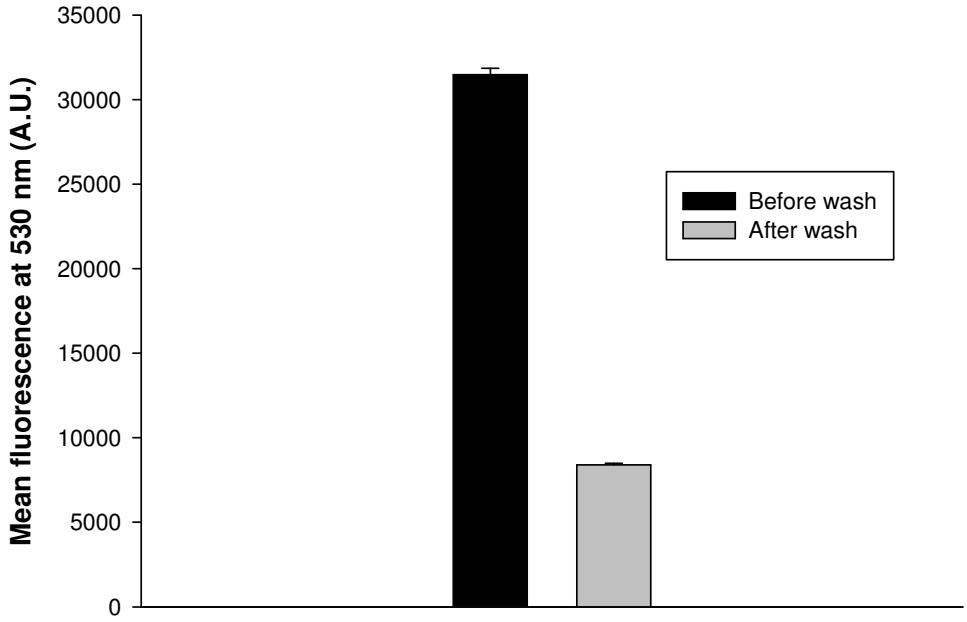

Figure 4.14 The mean fluorescent intensity at 530 nm of *ter*-Tus-GMA before and after 1 M $MgCl_2$ wash.

As shown in Figure 4.14, most of the bound *ter* DNA sequence was washed away from Tus-GMA, as indicated by the dramatic drop of fluorescence to near the background level (background: ~7000 A.U.). This indicated not only the DNA could bind the immobilised

Tus specifically, but also be released from the surface by treating with 1 M $MgCl_2$ solution. However, there was still a significant amount of *ter* remained on the surface. Probably the washing steps needed optimisation, or the DNA sequence was trapped on the surface.

4.2.6) Labelling of modified affi gel resin with different proteins

We have successfully demonstrated the immobilisation of different proteins onto functionalised GMA beads using the SrtA system. In addition, we have also proved that this attaching method retained the functionality of the immobilised proteins. Our next goal was to demonstrate protein attachment to other solid supports, such as soft-gel matrix like beaded agarose affinity support (Affi-Gel 102 resin, Bio-Rad). Similar to GMA beads, Affi-Gel was modified with oligoglycine by incubating the amino-resin with diglycine (0.5 M) and EDC (2.5 mM) for three hours at 50 °C. The resin was packed into a small spin column. Afterwhich BFP-, EGFP-, and DsRed-LPETGG-His_6 were ligated to the resin overnight at room temperature using the same SrtA system as decribed above, except that the spin rate was reduced to 3500 rpm to prevent damaging the resin.

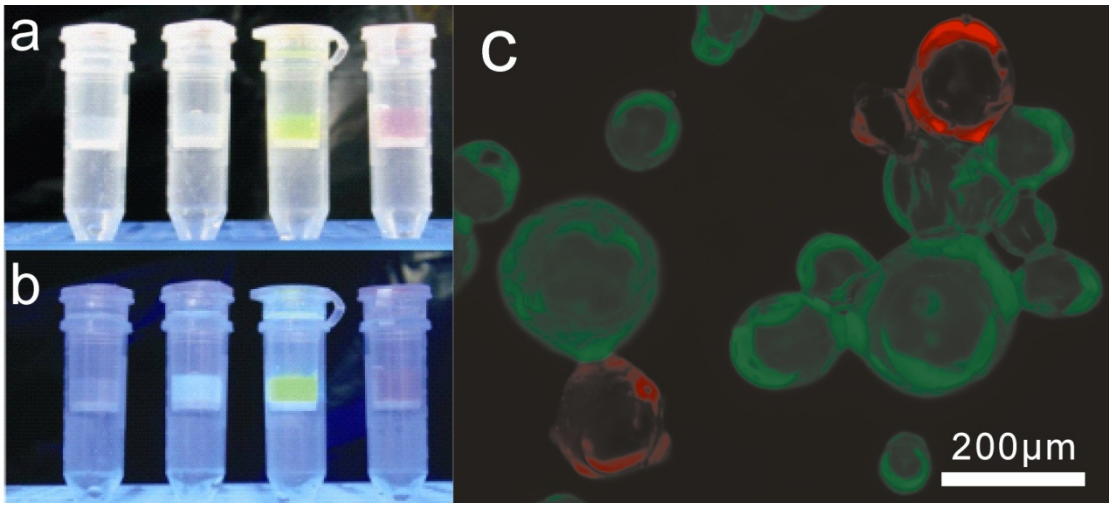

Figure 4.15 Ligation of fluorescent proteins onto affi-gel resin. Diglycine affi-gel resin was incubated with fluorescent proteins with the presence and absence of SrtA. Images were taken under white light (a) and UV light (b), the protein labelled in the tubes were (from left to right): Negative control (EGFP-LPETGG-His_6 proteins without SrtA), BFP-LPETGG-His_6, EGFP-LPETGG-His_6 and DsRed-LPETGG-His_6, (c) oligoglycine modified Affi-gel resin were separately labelled with EGFP- and DsRed-LPETGG-His_6 and mixed. Fluorescence images were obtained on an Axiovert 200 Fluorescence microscope with fluorescein and Cy3 filter sets, combined, and false colored.

After the washing steps, the resin could be clearly seen to be labelled with BFP and EGFP (Figure 4.15b, second and third tube from the left), whereas the negative control (EGFP-LPETGG-His_6 without SrtA) showed no fluorescence (Figure 4.15b, first tube from the left). Resin labelled with DsRed showed mimimum fluorescence (Figure 4.15b, right tube). This was due to the fact that the fluorecent protein was excited with UV illumination which was much lower than the λ_{ex}. However, under the fluorescence microscope with the correct filter sets the fluorescence was clearly visable (Figure 4.15c).

4.2.7) Protein immobilization onto glass surfaces

Finally, with the success on ligation of proteins to hard- and soft-polymeric beads, we extend our interest onto planer surfaces, such as a glass surface, to demonstrate the SrtA ligation method is applicable to the development of planar protein arrays or microdevices.

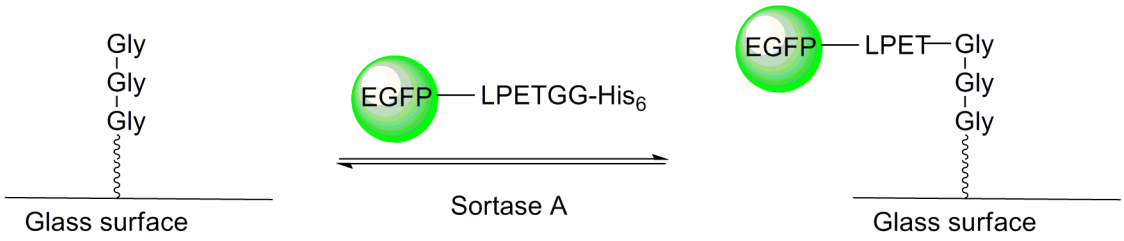

Figure 4.16 Fluorescent labelling of glass surface using SrtA system.

Clean glass coverslips were modified with 2% triethoxy(aminopropyl) silane and oligoglycine was then coupled to the amino-modified surface as decrobed above. EGFP-LPETGG-His_6 and SrtA mixture was dripped onto the glass coverslide and incubated overnight at room temperature. Afterwhich, the glass surface was cleaned with assay buffer (50 mM Tris 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5). To remove excess non-specific binding proteins the glass surface was incubated in wash buffer (50 mM Tris 150 mM NaCl, 5 mM $CaCl_2$, 1% SDS, pH 7.5) at room temperature for 1 hour. Subsequentlly the surface was washed again with assay buffer before being imaged using a fluorescence microscope equipped with fluorescein filter sets.

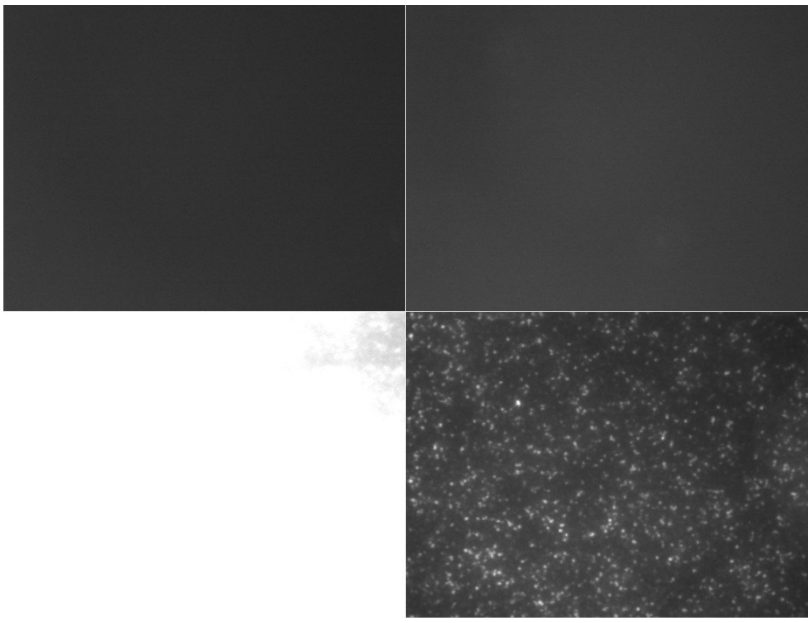

Figure 4.17 Glass surfaces were modified with triethoxy(aminopropyl) silane and oligoglycine before incubation with EGFP-LPETGG-His_6 and Sortase. The surface was washed with assay buffer with 1% SDS and imaged using the fluorescein filter sets on an Axiovert 200 microscope. (Top left) no SrtA; (Top right) no glycine modification, (Bottom left) with SrtA and glycine modification and imaged using the same exposure settings as negative controls, (Bottom right) same as (Bottom left) but with five-fold reduced exposure time.

In the absence of SrtA or oligoglycine, little fluorescence was observed on the glass slides (Figure 4.17, top panel). A high fluorescence was observed for the positive sample where SrtA was included on the oligoglycine-modified glass surface. The labeling is continuous across the modified surface with spots of higher fluorescence.

To prove that the EGFP-LPETGG-His_6 protein ligation on the glass surface was specific, a glass slide was modified with a layer of photosensitive layer moulded in a dolphin shape (prepared by Dr. M. E. Sanderson). As the mould was soluble in acetone, a modified method for functioning the glass surface with triethoxyl(aminopropyl) silane (APTES) in water was used. The photosensitive layer was then wet etched with methanol after

coupling of oligoglycine by EDC coupling method. The glass slide was treated with EGFP-LPETGG-His_6 and SrtA. After washing, the glass slide was imaged using a fluorescence microscope equipped with a FITC filter set.

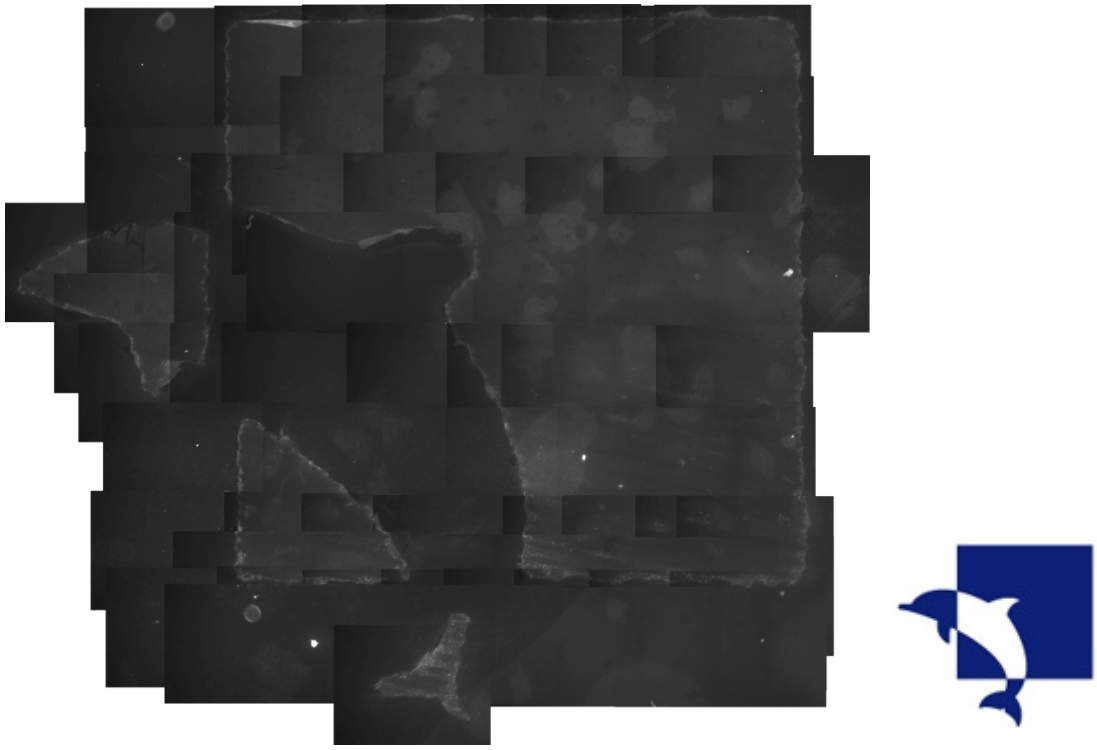

Figure 4.18 The dolphin-pattened fluorescent image was reassembled from a number of fluorescence images taken from the Axiovert 200 microscope equipeed with fluorescein filter set. Part of the glass surface was protected by a layer of photosensitive layer which was shaped as the University of Southampton dolphin logo (shown on the right). Glass surface was modified with APTES in water and oligoglycine before incubating with EGFP-LPETGG-His_6 and SrtA. The surface was washed with methanol and assay buffer containing 1% SDS.

It was clear from the image above that the SrtA-mediated protein immobilisation was highly specific. Areas on the glass slide previously protected by a layer of photosentive layer showed little non-specific binding, whereas other areas were visually distinquishable.

4.3) Summary and conclusion

There has been a growing interest on immobilising proteins onto solid supports. We extended our interest from fluorescently labeling proteins to attach different proteins (fluorescent proteins, Fpr and Tus) onto a range of surfaces (highly cross-linked GMA, soft polymeric matrix and glass surface). The coupling of proteins onto surfaces proved to be successful. Non-specific binding of target protein to these surfaces was undetectable. During the initial stage of immobilising proteins onto glass surfaces, we expereiced difficulty when using a short glycine linker. This was overcome by elongating the glycine chain (oligoglycine) to prevent steric hinderence. The activity of the immobilised protein was also tested. Tus was coated onto GMA beads and then used for capturing the double strainded *ter* DNA sequence. From our experiments, the immobilised Tus was highly functional. After extensive washing, the bound double stranded DNA was released from the Tus-coated solid support, potentially open up possibility of reusing protein-coated surfaces for biological assays.

4.4) Experimental Methods

4.4.1) Ligation of EGFP-LPETGG-His_6 onto different oligoglycine labelled GMA beads

GMA beads (~25 mg) were re-suspended with 100 μL of assay buffer, and calcium chloride (5mM) was added to the EGFP-LPETGG-His_6. The reaction (20 μL) was set up as following, unless otherwise stated:

Negative control:

Reagent	Quantity
Beads (~25 mg)	2.24μL
EGFP-LPETGG-His_6 (940 mM)	16.76 μL
Deionised water	1 μL

Table 4.1 Reaction mixture of GMA beads with EGFP-LPETGG-His_6 without sortase.

Reactions:

Reagent	Quantity
Beads (~25 mg)	2.24μL
EGFP-LPETGG (940 mM)	16.76 μL
Sortase (123 μM)	1 μL
Assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH7.5)	1 μL

Table 4.2 Reaction mixture of GMA beads with EGFP-LPETGG-His_6 with sortase.

A 2 μL sample was taken out as a control for time point 0 minutes. The sample was re-suspended with 50 μL of assay buffer, centrifuged at top speed for 1 minute and the supernatant was discarded. The wash step was repeated three times and after the final wash, the pellet was re-suspended with 20 μL of assay buffer. After the washes, Sortase or deionised water was added to the reactions and a 2 μL sample was taken out at the following time points: 15 minutes, 30 minutes, 45 minutes, 60 minutes and 90 minutes. Each sample taken out was washed and re-suspended following the same washing steps as the 0 minute sample. The sample was re-suspended with FACS solvent in a tube and loaded onto the FACS machine to measure the fluorescence intensity at 530/30 nm for 5000 events.

4.4.2) Tus-LPETGG-His_6 protein ligation to tetraglycine coupled GMA beads

An overnight ligation of Tus-LPETGG-His_6 protein to tetraglycine coupled GMA beads in assay buffer (50 mM Tris, 150 mM NaCl, 5mM $CaCl_2$, pH 7.5) for the DNA binding assay. The reaction was set up as the following, otherwise stated:

Assay reaction:

Reagent	Quantity
Tetraglycine GMA beads	11 μL
Tus-LPETGG-His_6 (3.59 μM)	103 μL
Sortase	6 μL
Assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH7.5)	1 μL

Table 4.3 Reaction mixture of tetraglycine GMA beads with Tus-LPETGG-His_6 with sortase.

The reaction was centrifuged at top speed for 1 minute and washed with DNA ligation buffer (50 mM Tris, 0.1 mM EDTA, 250 mM KCl, 0.1 DTT, pH 7.5), repeated 3 times. The resin was re-suspended with 20 μL assay buffer for storage at 4 ℃.

4.4.3) Preparation of double stranded fluorescein labelled *ter*

Fluorescein labelled *ter*: Fluorescein -ATAAGTATGTTGTAACTAAAG
TATTCATACAACATTGATTTC

Both the fluorescent oligo and the opposite strand of *ter* (ATDBio, Southampton, U.K.) were heated up to 96 ℃ and allowed to cool down to room temperature naturally and yield 10 μM fluorescein labelled double stranded DNA.

Reagent	Quantity
Fluorescent oligo (39.07 μM)	211.16 μL
Opposite strand	75 μL
Tus DNA binding buffer (50 mM Tris, 0.1 mM EDTA, 250 mM KCl, 0.1 mM DTT, pH 9)	463.84 μL

Table 4.4 Preparation of fluorescein labelled double stranded DNA

The double stranded fluorescein labelled *ter* (*ter*-F) was used to dilute into different dilutions using Tus DNA binding buffer (50 mM Tris, 0.1 mM EDTA, 250 mM KCl, 0.1 mM DTT, pH 9)

4.4.4) DNA binding to Tus ligated GMA beads

The DNA solution in DNA ligation buffer (50 mM Tris, 0.1 mM EDTA, 250 mM KCl, 0.1 DTT, pH 7.5) was loaded onto the Tus-LPETGG-His_6 protein ligated tetraglycine GMA

beads to test the function of Tus protein. The reaction was set up as the following, otherwise stated:

Negative control:

Reagent	Quantity
Tus-LPETGG-His_6 ligated diglycine affi-gel resin	500 μL
DNA ligation buffer (50 mM Tris, 0.1 mM EDTA, 250 mM KCl, 0.1 DTT, pH 7.5)	100 μL

Table 4.5 Reaction mixture of Tus-LPETGG-His_6 ligated GMA beads with fluorescein labelled *ter* without fluorescein labelled *ter*.

Assay reaction:

Reagent	Quantity
Tus-LPETGG-His_6 ligated diglycine affi-gel resin	500 μL
Fluorescein labelled *ter*	100 μL

Table 4.6 Reaction mixture of Tus-LPETGG-His_6 ligated GMA beads with fluorescein labelled *ter*.

The reaction was incubated at room temperature for 3 hours, observe it under the UV light to see the fluorescent light from the DNA.

A mixture of fluorescein labelled DNA (100 nM) with the specific Tus binding sequence site and Cy5 labelled non-specific DNA (100 nM) were prepared, the total DNA concentration in the reaction was 100 nM. The GMA beads was resuspended with DNA ligation buffer, spun at top speed with bench centrifuge for 1 minute, repeat twice, the GMA beads was resuspended in 100 μL DNA binding buffer. Seven sets of diglycine coupled GMA beads were prepared, one for the negative control and one for each mixture of DNA. The DNA was loaded into the beads and incubated at room temperature for 3 hours with shaking, the beads was centrifuged and resuspended with DNA binding buffer, repeat the wash step twice. The GMA beads samples were then loaded into the FACS machine to measure the fluorescent intensity of the beads at 530 nm and 660 nm.

4.4.5) Binding of *ter*-F to Tus ligated GMA beads with different concentrations of fluorescein labelled *ter*

The DNA was diluted into different concentrations by mixing with DNA ligation buffer (50 mM Tris, 0.1 mM EDTA, 250 mM KCl, 0.1 DTT, pH 7.5), and was loaded into the Tus-LPETGG-His_6 protein ligated tetraglycine GMA beads solution. The reaction was set up as the following for each DNA concentration, otherwise stated:

Negative control:

Reagent	Quantity
Tetraglycine GMA beads	1 mg
DNA binding buffer (50 mM Tris, 0.1 mM EDTA, 250 mM KCl, 0.1 DTT, pH 7.5)	100μL

Table 4.7 Reaction mixture of Tus-LPETGG-His_6 ligated GMA beads with fluorescein labelled *ter* without fluorescein labelled *ter*.

Assay reaction:

Reagent	Quantity
Tus-LPETGG-His_6 ligated tetraglycine GMA beads	1 mg
Fluorescein labelled *ter*	Adjust volume according to the concentration

Table 4.8 Reaction mixture of Tus-LPETGG-His_6 ligated GMA beads with different concentrations of fluorescein labelled *ter*.

The reaction was incubated at room temperature for 3 hours and loaded into the FACS machine to measure the fluorescent intensity at 530 nm.

4.4.6) Removal of fluorescein labelled *ter* from Tus immobilised on GMA beads

A sample was taken from each assay, the DNA binding buffer was removed and the beads was resuspended with DNA removal buffer (50 mM Tris, 0.1 mM EDTA, 250 mM KCl, 0.1 DTT, 1 M $MgCl_2$, pH 7.5) and incubated at room temperature for 45 minutes. The supernatant was discarded and the beads was resuspended with DNA binding buffer (100 μL), repeat this step twice. The resin was loaded into the FACS machine and fluorescent intensity was measured at 530 nm.

4.4.7) Functionalization of affi gel resin

EDC coupling reaction

Buffer A (23.4 mM immidazole in 50 mL of deionsed water, pH 7) and buffer B (2.5 mM EDC in 25 mL of buffer A) was prepared for the coupling reaction. The affi-gel resin (500 μL, 17 μM loading concentration) was added into 540 μL buffer B and 170 μM of diglycine was added into an eppendorf tube and left for gentle shaking at 50 ℃ for 3 hours. Transfer the resin into a spin column and centrifuged at 3500 rpm for 1 minute and washed with 700 μL assay buffer and spun at 3500 rpm for 1 minute for 5 times. The resin was then re-suspended with 500 μL of deionsed water and stored at 4 ℃.

4.4.8) Labelling of modified affi gel resin with different proteins

Diglycine coupled affi-gel resin (500 μL) in 500 μL of assay buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5), was added to the BFP-, EGFP- and DsRed-LPETGG-His_6 protein in a 1.5 mL eppendorf tube. The reaction was set up as following, unless otherwise stated:

Negative control:

Reagent	Quantity
Diglycine coupled affi-gel resin	500 μL
Assay buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5)	845 μL
BFP-, EGFP-, DsRed-LPETGG-His_6	76.6 μL
Deionised water	4.3 μL

Table 4.9 Reaction mixture of ligating different proteins onto diglycine affi-gel resins without SrtA.

Reactions:

Reagent	Quantity
Diglycine coupled affi-gel resin	500 μL
Assay buffer	845 μL
BFP-, EGFP-, DsRed-LPETGG-His_6	76.6 μL
SrtA	4.3 μL

Table 4.10 Reaction mixture of ligating different proteins onto diglycine affi-gel resin.

The reactions were incubated at room temperature for overnight with very gentle shaking. It was then washed with 700 μL assay buffer and centrifuged at 3500 rpm for 1.5 minutes in a spin column and repeated for three times. The flow through was discarded and the resin was re-suspended with 700 μL of assay buffer.

4.4.9) Activation and functionalization of glass surface

Activation of glass surface without photo sensitive layer

Before the immobilization of EGFP onto glass cover slide, it has to be activated and couple an amine onto the surface.

This method was used for the glass surface without the photo sensitive layer. The glass cover was first wash with dry acetone to remove grease and dirt on the surface and air dried. Then immerse the cleaned cover slide into the activation reagent (1 part 3-aminopropyltriethysilane and 49 parts of dry acetone) for 30 seconds. It is then rinsed with dry acetone for 3 times and air dried at room temperature. The glass slides were the

stored in 50 mL glass vials and stored in dark at room temperature before use.

b) Activation of glass surface with the photo sensitive layer

This method was used for the glass surface with the photo sensitive layer as the acetone from the previous method will dissolves the photo sensitive layer. Then place the cover slide into the Petri dish device with dolphin side up and slowly by drop wise drip the activation reagent (1% 3-aminopropyltriethysilane in deionised water) onto the glass surface just to cover up the dolphin and incubated at room temperature for 20 minutes. It is then rinsed with water for 3 times to remove excess activation reagent and air dried at room temperature.

4.4.10) Functionalization of glass surface

Once the glass surface was activated, both kinds can be functionalized with oigoglycine using the same method. For the SrtA be able to recognize the glycine on the glass surface, an overnight EDC coupling of glycine was done on the prepared glass cover slides in a 50 mL glass vial. So a long chain of glycine is coupled onto the glass surface, which will increase the distance between the glycine for ligation and the glass surface and act as a linker. After the overnight EDC coupling reaction, the glass surface was immerse in ammonium solution at 60 ℃ for 3 hours to remove excess glycine. The glass surface without the dolphin molding was then washed carefully with deionised water for three times to make sure that all the ammonium solution was removed. The glass surface with dolphin molding was first wash with deionised water for three times, followed by wash with methanol to remove the entire photo sensitive layer. The glass surface finally

washed with deionised water for another three times to remove the methanol. They were then stored in assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5) before use.

4.4.11) EGFP-LPETGG-His_6 immobilization onto glass surface

Four different sets of reaction were prepared, three different sets of negative control (set 1: without SrtA, set 2: glass cover slide was not prepared by EDC coupling and with SrtA in the reaction mixture and set 3: glass cover slide was not prepared by EDC coupling and without SrtA in the reaction mixture) were designed, and one positive reaction. A 20 mL reaction mixture was prepared as below and poured into a 50 mL glass vial with the prepared glass cover slide. The glass vials were stored at room temperature in dark overnight.

Reagent	Quantity	Final concentration
EGFP-LPETGG-His_6	800 μL	50 mM
SrtA	17 μL	100 nM
Ligation buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5)	Adjust volume to 20 mL	

Table 4.11 Reaction mixture for immobilization EGFP-LPETGG-His_6 onto modified glass surface.

After the overnight ligation, the glass surface was first cleaned with assay buffer (50 mM Tris 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5). To remove excess non-specific binding proteins the glass surface was incubated in wash buffer (50 mM Tris 150 mM NaCl, 5 mM $CaCl_2$, 1% SDS, pH 7.5) at room temperature for 1 hour, and followed by the cleaning with

assay buffer and imageed using the fluorescein filter set on an Axiovert 200 microscope.

4.4.12) EGFP-LPETGG-His_6 immobilisation onto glass surface with a dolphin mold

The immobilisation of EGFP-LPETGG-His_6 onto glass surface was the same as method 54, except the glass surface was placed horizontally in a Petri dish with the dolphin mold side up. The reaction mixture was dripped onto the glass surface covering the dolphin mold and left at room temperature overnight in dark. After overnight incubation, the glass slide was washed with ligation buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5), followed by a wash with wash buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 1% SDS, pH 7.5) and with a final wash with ligation buffer before imageed using the fluorescein filter set on an Axiovert 200 microscope.

4.5) References

(1) Fields, S. *Science* **2001**, *291*, 1221-4.

(2) Zhu, H.; Snyder, M. *Curr Opin Chem Biol* **2001**, *5*, 40-5.

(3) MacBeath, G. *Nat Biotechnol* **2001**, *19*, 828-9.

(4) Hodneland, C. D.; Lee, Y. S.; Min, D. H.; Mrksich, M. *Proc Natl Acad Sci U S A* **2002**, *99*, 5048-52.

(5) Chen, G. Y.; Uttamchandani, M.; Lue, R. Y.; Lesaicherrea, M. L.; Yao, S. Q. *Curr Top Med Chem* **2003**, *3*, 705-24.

(6) Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293*, 2101-5.

(7) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760-3.

(8) Zhu, H.; Klemic, J. F.; Chang, S.; Bertone, P.; Casamayor, A.; Klemic, K. G.; Smith, D.; Gerstein, M.; Reed, M. A.; Snyder, M. *Nat Genet* **2000**, *26*, 283-9.

(9) Wang, D.; Liu, S.; Trummer, B. J.; Deng, C.; Wang, A. *Nat Biotechnol* **2002**, *20*, 275-81.

(10) Kindermann, M.; George, N.; Johnsson, N.; Johnsson, K. *J Am Chem Soc* **2003**, *125*, 7810-1.

(11) Sielaff, I.; Arnold, A.; Godin, G.; Tugulu, S.; Klok, H. A.; Johnsson, K. *Chembiochem* **2006**, *7*, 194-202.

(12) Paborsky, L. R.; Dunn, K. E.; Gibbs, C. S.; Dougherty, J. P. *Anal Biochem* **1996**, *234*, 60-5.

(13) Yin, J.; Liu, F.; Li, X.; Walsh, C. T. *J Am Chem Soc* **2004**, *126*, 7754-5.

(14) Cull, M. G.; Schatz, P. J. *Methods Enzymol* **2000**, *326*, 430-40.

(15) Cronan, J. E., Jr.; Reed, K. E. *Methods Enzymol* **2000**, *326*, 440-58.

(16) Taki, M.; Sawata, S. Y.; Taira, K. *J Biosci Bioeng* **2001**, *92*, 149-53.

(17) Smith, P. A.; Tripp, B. C.; DiBlasio-Smith, E. A.; Lu, Z.; LaVallie, E. R.; McCoy, J. M. *Nucleic Acids Res* **1998**, *26*, 1414-20.

(18) Lesaicherre, M. L.; Lue, R. Y.; Chen, G. Y.; Zhu, Q.; Yao, S. Q. *J Am Chem Soc* **2002**, *124*, 8768-9.

(19) Green, N. M.; Toms, E. J. *Biochem J* **1973**, *133*, 687-700.

(20) Reznik, G. O.; Vajda, S.; Cantor, C. R.; Sano, T. *Bioconjug Chem* **2001**, *12*, 1000-4.

(21) Sano, T.; Cantor, C. R. *Proc Natl Acad Sci U S A* **1990**, *87*, 142-6.

(22) Frengen, J.; Kierulf, B.; Schmid, R.; Lindmo, T.; Nustad, K. *Clin Chem* **1994**, *40*, 420-5.

(23) Holmes, D.; She, J. K.; Roach, P. L.; Morgan, H. *Lab Chip* **2007**, *7*, 1048-56.

(24) Saunders, G. C.; Jett, J. H.; Martin, J. C. *Clin Chem* **1985**, *31*, 2020-3.

(25) Lindmo, T.; Bormer, O.; Ugelstad, J.; Nustad, K. *J Immunol Methods* **1990**, *126*, 183-9.

(26) Frengen, J.; Schmid, R.; Kierulf, B.; Nustad, K.; Paus, E.; Berge, A.; Lindmo, T. *Clin Chem* **1993**, *39*, 2174-81.

(27) Berenson, J. A.; Benemann, J. R. *FEBS Lett* **1977**, *76*, 105-7.

(28) Lappi, D. A.; Stolzenbach, F. E.; Kaplan, N. O.; Kamen, M. D. *Biochem Biophys Res Commun* **1976**, *69*, 878-84.

(29) Kim, Y. G.; Shin, D. S.; Yang, Y. H.; Gil, G. C.; Park, C. G.; Mimura, Y.; Cooper, D. K.; Rudd, P. M.; Dwek, R. A.; Lee, Y. S.; Kim, B. G. *Chem Biol* **2008**, *15*, 215-23.

(30) Kuan, I.; Liao, R.; Hsieh, H.; Chen, K.; Yu, C. *J Biosci Bioeng* **2008**, *105*, 110-5.

(31) Centi, S.; Messina, G.; Tombelli, S.; Palchetti, I.; Mascini, M. *Biosens Bioelectron* **2008**, *23*, 1602-9.

(32) Scouten, W. H.; Konecny, P. *Anal Biochem* **1992**, *205*, 313-8.

(33) Neylon, C.; Kralicek, A. V.; Hill, T. M.; Dixon, N. E. *Microbiol Mol Biol Rev* **2005**, *69*, 501-26.

(34) Neylon, C.; Brown, S. E.; Kralicek, A. V.; Miles, C. S.; Love, C. A.; Dixon, N. E. *Biochemistry* **2000**, *39*, 11989 -11999.

Chapter 5: General Experimental Methods

5.1) Materials

Restriction enzymes were purchased from either New England Biolab or Promega. T4 DNA Ligase, Wizard® *Plus* Minipreps DNA Purification System, Wizard® PCR Preps DNA Purification System and dNTPs were purchased from Promega. Primers were obtained from Sigma Genosys or synthesised at the University of Southampton. Agarose was purchased from Melford and low melting point agarose from Sigma. *Staphylococcus aureus* subsp. genomic DNA was obtained from ATCC, LB Medium was made using LB Broth mix from Sigma or chemicals from Oxoid. Ampicillin, IPTG and DTT were obtained from Melford Laboratories Ltd. Bacteriological Agar was purchased from Sigma. Polyacrylamide-bis polyacrylamide (30% w/v, 37:5:1) was purchased from Amresco. Competent cells were obtained from frozen stocks at the University of Southampton. pH measurements were performed using a Mettler Delta 340 pH meter connected to a Mettler Toledo Inlab 413 Combination Electrode.

PCR was carried out in an Eppendorf Mastercycler gradient machine. Centrifugation was carried out in a Heraeus Contifuge Stratos D-37520 Osterode with either rotor Heraeus #3057 for 50 mL centrifugation or Heraeus #3048 for plate centrifugation. Microcentrifugation was carried out in an Eppendorf centrifuge 5415D. Gels and buffers for SDS-PAGE were prepared as described (Sambrook and Russell 2001). All polyacrylamide gels were 15% and run on a Biorad Protein-3 electrophoresis unit, at a

constant voltage (200 V). Agarose Gel electrophoresis was carried out as described (Sambrook and Russell 2001). Incubation of cell cultures was carried out in Infors Minitron, Innova™ 4400 or Innova™ 4230. DNA concentration was determined using NanoDrop® ND-1000 spectrophotometer at a wavelength of 260 nm. Sonication was carried out using Soniprep150. Sequencing was carried out in Beckman Coulter CEQ 200 XL machine. Vacuo was carried out in Büchi rotavapor R-114 and RE 111, with Büchi water bath 461 and B-480. Fermentation was carried out in an Incubator Shaker innova™4400 (New Brunswick Scientific). Bacterial plate cultures were grown in a Economy Incubator Size 2 (Gallenkamp). Samples were centrifuged at 4 °C in a Avanti™ J-25 centrifuge (Beckman) centrifuge. For small volumes (sample less than 1.5 mL), a microCentrifugette 4214 (ALC) was used.

Superdex 75 (S-75), Superdex 200 (S-200) and Chelating Fast Flow resins were purchased from Pharmacia. All other chemicals used were of the highest quality available and purchased from Aldrich, Sigma or Fluka.

Standard sterile techniques were applied during microbiological experiments. Growth media and heat stable solutions were autoclaved, whilst heat labile solutions (antibiotics, IPTG and arabinose) were filter sterilised through 0.22 μm filters.

Growth media were supplemented with the appropriate antibiotic at the following concentrations; ampicillin, 100 μg / mL, kanamycin, 30 μg / mL, streptomycin, 10 μg / mL. Protein expression was induced by the addition of arabinose (0.2% w/v) for pBAD derived plasmids and IPTG (1 mM) for pET derived plasmids.

5.2) General Methods

5.2.1) PCR

PCR reactions (20 µL) were set up on ice in sterile 200 µL PCR tubes and processed in the thermocycler using the conditions below, unless otherwise reported.

	Volume	Concentration	Total Quantity
Sterile Water	12 µL		
Thermopol buffer (10x)	2 µL		
dNTPs	2 µL	2 mM	10 nmoles
DNA template	1 µL	1mg/mL , 0.1 mg/mL, 0.001 mg/mL	1 mg, 100 µg, 1 µg
Forward / Reverse primers	1 µL Each	4 µM	0.05 nmoles
Vent polymerase (1 in 20 dilution)	1 µL	2.5 U / mL	2.5 U

Table 5.1 PCR general reaction mixture.

Reactions were carried out in the thermocycler as follows:

1 cycle	30 cycles	1 cycle
95 ℃, 10 minutes	95 ℃, 30 seconds	72 ℃, 10 minutes
60 ℃, 12 minutes	50 ℃, 30 seconds	4 ℃, for storage
	72 ℃, 1 minute	

Table 5.2 PCR cycle programme.

PCR products were analysed by agarose gel electrophoresis and purified using Wizard® PCR Preps DNA purification system, following the manufacturer's guidelines.

5.2.2) Transformation

Competent cells were purchased or prepared as described in 5.2.6.

Competent cell aliquots (50-100 μL) were thawed on ice for 10 minutes before addition of a ligation reaction mixtures (10 μL) or purified plasmid DNA (1 μL) with gentle mixing. Reaction mixture were maintained on ice for 30 minutes before being heat shocked in a temperature controlled waterbath (42 ℃, 45 seconds). The cultures were then placed on ice and SOC medium added (250 μL). Cultures were then incubated in a shaker for 1 hour (37 ℃, 180 rpm), and then spread onto agar plates containing the appropriate antibiotic and incubated overnight (37 ℃). Colonies were processed by minipreps and then characterised by analytical digestion.

5.2.3) Minipreps

Plasmid DNA was isolated using Wizard® *Plus* Minipreps DNA Purification System, used as stated in manufacturers instructions. Sterile water was used to elute the isolated plasmid DNA.

5.2.4) Restriction enzyme digestion (analytical and preparative)

Analytical digestion of plasmid DNA (50 μL, 50-75 ng / μL) isolated from bacterial culture (5 mL) was carried out using the following conditions.

Restriction enzyme digestion reactions of plasmid were set up on ice in sterile 200 μL tubes. Reactions were carried out at 37 ℃ overnight using the conditions below, unless otherwise reported.

	Volume	Concentration	Total Quantity
Plasmid	50 μL	50 - 75 ng / μL	2.5 – 3.75 μg
Buffer	10 μL	10X	1X
BSA	10 μL	1 mg / mL	10 μg
Restriction Enzyme (each)	5 μL	10 U / μL	50 U
Sterile Water	25 μL		

Table 5.3 Analytical digestion reaction mixture

Preparative digestion of plasmid DNA (15 μL, 100 - 300 ng / μL):

	Volume	Concentration	Total Quantity
Plasmid	15 μL	100 - 300 ng/μL	1.5 – 4.5 μg
Buffer	2 μL	10X	1X
BSA	2 μL	1 mg/mL	2 μg
Restriction Enzyme (each)	0.5 μL	10 U/μL	5 U

Table 5.4 Preparative digestion reaction mixture.

Reactions were incubated at 37 ℃ for 1.5 hours with a total volume of 20 μL.

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5.2.5) Purification of digested fragments

Completed preparative digestion reactions (30 μL) were loaded onto a 1% low melting point agarose gel. The desired product was excised from the gel and purified using the QIAquick® Gel Extraction Kit or Wizard® PCR Preps DNA purification system following the manufacturer's instructions.

5.2.6) Competent cell preparations

TFB I buffer:

Reagent	Final concentration
Potassium Acetate ($KCOOCH_3$)	30 mM
Rubidium Chloride ($RuCl_2$)	100 mM
Calcium Chloride ($CaCl_2$)	10 mM
Manganese Chloride ($MnCl_2$)	50 mM
Glycerol	30 mL
Deionised water	Adjust volume to 200 mL

Adjust the pH to 5.8 by using CH_3COOH, filtered the buffer through 0.22 μm membrane before storage.

Table 5.5 TFB I buffer mixture.

TFB II buffer:

Reagent	Final concentration
MOPS	10 mM
$CaCl_2$	75 mM
$RuCl_2$	10 mM
Glycerol	15 mL
Deionised water	Adjust volume to 100 mL

Adjust the pH of the beffer to pH 6.5, filtered the buffer through 0.22 μm membrane before storage.

Table 5.6 TFB II buffer mixture.

An overnight culture of competent cells with 10 mL of LB medium was prepared and incubated at 37 ℃ overnight with shaking. Every utility for preparing the competent cells, eg, pipette tips, eppendorf tubes, pipette and buffers were chilled overnight. 1 mL of the overnight culture was added into 100 mL of fresh LB medium and then incubated at 37 ℃ with shaking. When the OD_{600} of the cell culture reached 0.6, the flask was removed from the incubator and placed on ice immediately. The cell culture was then centrifuged at 4000 rpm for 10 minutes at 4 ℃. The supernatant was discarded and the cell pellet was placed on ice immediately. The pellet was resuspended with 10 mL of chilled TFB I buffer and kept on ice for 10 minutes at 4 ℃. The solution was then centrifuged at 4000 rpm for 10 minutes at 4 ℃. The supernatant was discardedand the cell pellet was chilled on ice and kept at 4 ℃. The pellet was resuspended in 1.5 mL of chilled TFB II buffer, aliquoted and stored at -80 ℃.

5.2.7) Fluorescent intensity measurement

The fluorescent intensity of the solution phase assays were analysed by plate reader (SaFire^2, Tecan, Switzerland). For GMA beads, the fluorescent intensity was measured by FACS machine (FACSAria, BD) and fluorescence microscope (Zeiss Axiovert 200) and for affi-gel resins, glass surface were observed by fluorescence microscope.

5.2.8) Ligation into an expression vector

Purified fragments from preparative digestions (vector and fragment) were ligated in three different ratios between the insert DNA and the plasmid backbone to a final volume of a 10 μL reaction mixture.

	Set		
	1	2	3
Insert DNA	6 μL	4 μL	2 μL
T4 Ligase buffer	1 μL	1 μL	1 μL
Plasmid backbone	2 μL	4 μL	6 μL
T4 DNA ligase	1 μL	1 μL	1 μL

Table 5.7 Ligation reaction mixture

Ligation reactions were incubated overnight at 4 ℃ and transformed into XL1Blue competent cells. Cultures were then incubated in a shaker for 1 hour (37 ℃, 180 rpm),

and then spread onto agar plates containing the appropriate antibiotic and incubated overnight (37 ℃). Colonies were analysed by digestions of minipreps.

5.2.9) Preparation of plasmid for sequencing

The concentration of the plasmid was determined by measuring the absorbance at 260 nm, where 50 μg/mL of dsDNA = 1 absorbance unit.

Plasmid DNA (400 ng) was diluted with sterile deionsed water to a total volume of 11 μL. The plasmid was incubated at 96 ℃ for 1 minute, after which 1 μL of primer and 8 μL of DTCS Quick start Mix was added. The reactions were carried out in the thermocycler as follows:

30 cycles	1 cycle
96 ℃, 20 seconds	hold at 4 ℃
50 ℃, 20 seconds	
60 ℃, 4 minutes	

Table 5.8 Thermocycler cycle for plasmid sequencing

After which 5 μL of stop solution was added to each reaction.

Reagent	Quantity
3 M sodium acetate, pH 5.2	2 μL
100 mM EDTA	2 μL
glycogen	1 μL

Table 5.9 Stop solution mixture for plasmid sequencing.

The reaction mixtures were transferred to a 96 well sample plate, and 60 μL of 95% ethanol was added to each sample. The sample plate was sealed with adhesive foil, mixed thoroughly and centrifuged at 1280 x g for 30 minutes at 4 ℃.

The supernatant was discarded, after which 200 μL of 70% ethanol was added to each well. The plate was sealed with adhesive foil and then centrifuged at 2900 x g for 15 minutes at 4 ℃. The supernatant was discarded carefully from the plate and the washing step was repeated wih 200 μL of 70% ethanol described above.

The sample plate was placed upside down on a piece of tissue, centrifuged at 124 x g for 10 seconds. The supernatant was removed and the sample plate air dried until no ethanol remained. Separation buffer was added to the buffer plate until 2/3 filled. Then 40 μL of loading buffer was added to each reaction in the sample plate. The sample plate was sealed with foil and mixed well. One drop of mineral oil was added to each reaction to prevent evaporation. Both plates were placed into the sequencing machine (CEQ system, Beckman Coulter) and analysed according to the manufacturer's instructions.

5.2.10) Glycerol freeze preperation

Permanent stocks of plasmid bearing *E. Coli* strains were prepared by adding sterilised glycerol (75% v/v, 125 μL) to bacterial culture (500 μL). These were stored at -80 °C.

5.2.11) Protein concentration determination

Protein concentration was assayed using the method of Bradford [1].

5.2.12) 15% SDS-PAGE gel

For 10 mL of resolving gel solution (5 mL per plate) the following components were mixed in the order as shown in Table 7.7:

Reagent	Quantity
H_2O	2.3 mL
30% Acrylamide / bis acrylamide	5 mL
1.5 M Tris/HCl (pH 8.8)	2.5 mL
10% SDS	0.1 mL
10% Ammonium Persulphate	0.1 mL
TEMED	0.004 mL

Table 5.10 Resolving gel mixture

5 mL of this solution was then added to each plate mould and allowed to set for 45 minutes. The top of the gel mixture was covered with a thin layer of water, which was then removed carefully. Once the gel solidified stacking gel was then prepared as in Table 5.11.

Reagent	Quantity
H_2O	2.7 mL
30% Acrylamide / bis acrylamide	0.67 mL
1.5 M Tris/HCl (pH 6.8)	0.5 mL
10% SDS	0.04 mL
10% Ammonium Persulphate	0.04 mL
TEMED	0.004 mL

Table 5.11 Stacking gel mixture

This mixture was then directly added on the resolving gel and a Teflon comb was inserted into the gel solution. Teflon combs were removed once the gel was set and gels immediately used or stored at 4 ℃.

Samples were prepared by mixing 20 µL protein sample with 20 µL sample loading buffer (Table 7.9), denaturing at 95 ℃ for 5 minutes. Samples were then loaded to the gel.

Reagent	Quantity
0.2 M Tris/HCl (pH 6.8)	2.5 mL
DTT	154 mg
SDS	200 mg
Bromophenol Blue	10 mg
Glycerol	1 mL
Deionised water	Adjust volume to 10 mL

Table 5.12 Sample loading buffer stock solution

Reagent	Quantity
Tris Base	15.1 g
Glycine	94 g
10% SDS solution	50 mL
Deionised water	Adjust volume to 1000 mL

Table 5.13 SDS-PAGE running buffer (×5 stock solution)

The proteins were separated according to their size by applying voltage at 180 V (~15V/cm) in SDS-PAGE running buffer for 45 minutes. After which gel was stained by Coomassie brilliant blue (Table 5.14) and then destained in destain solution (Table 5.15) for overnight. The stained gel was visualised under white light.

Reagent	Quantity
Coomassie brilliant blue	2.5 g
Methanol : water (1 : 1)	90 mL
Glacial acetic acid	10 mL

Table 5.14 Coomassie brilliant blue protein stain

Reagent	Quantity
Deionised water	4375 mL
Methanol : water (1 : 1)	375 mL
Glacial acetic acid	250 mL

Table 5.15 Destain solution

5.2.13) Small scale expression experiments

An overnight starter culture (10 mL) (inoculated from glycerol freeze stock) was used to inoculate LB medium (100 mL) containing 100 μg/mL of Ampicillin. Culture was incubated in a shaker (37 ℃, 180 rpm), and growth monitored by measuring the OD_{600}. Cells were induced at OD_{600} = 0.6 by the addition of arabinose for pBAD expression vectors (final concentration 0.2%) or IPTG (final concentration 1 mM) for the pET expression vector. The cells were grown for a further 4 h before harvested by centrifugation (8 min, 10,000 rpm). Cell pellets were resuspended in lysis buffer (750 μL) (5.2.15) and lysed by sonication (6 × 3 s on / off). Cell debris was separated by

centrifugation (13,000 rpm, 10 min) and the supernatant isolated. Cell debris was resuspended in lysis buffer (100 × pellet volume). Protein concentration was then estimated by Bradford assay. The protein content of both supernatant and pellet were analysed by SDS-PAGE.

5.2.14) Large Scale Expression Experiments

An overnight starter culture was used as a 1% innoculum for 4 × 1.25 L LB medium (containing appropriate antibiotic). Cultures were incubated in an orbital shaker (37 ℃, 180 rpm) and cell growth monitored by OD_{600}. At OD_{600} 0.6 cells were induced by IPTG and incubated at 37 ℃. Cells were then harvested four hours post induction by centrifugation (JA14 rotor, 10,000 rpm, 10 min, 4 ℃) and stored at -80 ℃.

5.2.15) Cell Lysis

The frozen cell pellet was resuspended in 5 × w/v lysis buffer (50 mM Tris/HCl pH 8.1, 0.5 M NaCl, 10% glycerol). Lysosyme (final concentration of 0.1 mg/mL) were added, and the mixture stirred for 30 minutes. The mixture was then sonicated (10 × 30 s bursts) whilst cooled on ice. Cell debris was separated by centrifugation (JA14 rotor, 10,000 rpm, 30 min, 4 ℃). The supernatant was then carefully isolated.

5.2.16) Protein purification: Fast Protein Liquid Chromatography (FPLC)

All enzyme purifications were performed using a Pharmacia FPLC System. Buffers were prepared in Milli-Q water. Resin specifications are given in Table 5.14.

Resin	Particle Size / μm	Matrix	Use
Superdex 75	13	Spherical composite of cross-linked agarose and dextran.	Size exclusion seperation of globular proteins mw 3 - 70 kDa
Superdex 200	13	Spherical composite of cross-linked agarose and dextran.	Size exclusion seperation of globular proteins mw 10 - 600 kDa
Chelating Sepharose Fast Flow	45 - 165	Highly cross linked 6% agarose, derivatised with chelating iminodiacetic acid groups.	Medium for immobilised metal ion affinity chromatography

Table 5.16 Resin types used for protein purification

5.2.17) Small scale expression study / nickel spin column purification

Cells from a cell culture (25 mL of a 100 mL growth) were harvested by centrifugation, resuspended in 1 mL lysis buffer and lysed by sonication (5 × 5s bursts). Cell debris was separated by centrifugation (13,000 rpm, 15 mins).

Nickel spin columns were prepared by adding chelating sepherose (150 µL) (Pharmacia chelating sepherose^{TM} fast flow) to a Quiagen QIAquick spin column, excess buffer was removed by centrifugation (3,000 rpm, 1 min). N.B all subsequent elutions were by centrifugation (3,000 rpm, 1 min, 4 ℃). The following protocol was then followed:-

Resin was charged with 0.2 M nickel sulphate (300 µL) and eluted by centrifugation.

The resin was washed with 50 mM Tris/HCl pH 8.1 (700 µL).

To bind the His tagged protein, cleared lysate (700 µL) was applied to the column, mixed, left to stand for 1 minute followed by centrifugation. The flow through was then reapplied, left to stand for one minute and eluted (repeated once).

The column was washed twice with 700 µL of 50 mM Tris/HCl buffer pH8.1 containing 50 mM imidazole and 0.5 M NaCl. After the final wash, the column was centrifuged once to remove excess buffer.

His tagged protein was eluted with Tris/HCl buffer pH 8.1 (50 µL, 50 mM) containing imidazole (0.5 M) and NaCl (0.5 M). This buffer was well mixed with the resin and left

to stand for five minutes before elution.

The eluted proteins were analysed by 10% SDS-PAGE (method 12).

5.2.18) Gel filtration column

Buffer A: 50 mM Tris, 150 mM NaCl, pH 7.5

Pre-equilibrated the S-200 column with 2 L buffer A overnight at 1 mL/min overnight. Concentrated protein from Ni affinity column purification was loaded onto the pre-equilibrated S-200 column to remove immidazole. Protein was eluted at 3 mL / min, collecting fractions with 15 mL fraction size.

5.2.19) Conditions for SDS-PAGE analysis of SrtA mediated fluorescent labelling reactions

Protein samples (10 μL) were denatured in Gel Loading Buffer (10 μL) for five minutes at 80 ℃ and analysed by SDS-PAGE on 15% gels. Gels were visualised under UV illumination and after Coomassie staining using a Syngene GeneGenius imager. The images were analysed using GeneTools software (Syngene, Cambridge, UK).

5.2.20) Desalting of protein solutions by NAP-10 column

The column was washed with 25 mL of buffer (50mM Tris/HCl, pH 8.1, 150 mM NaCl), then 1 mL of the protein solution was loaded onto the column. The column was then eluted with 1.5 mL of buffer and the elution was collected.

5.3) References

1) Bradford, M. M. *Anal. Biochem*. **1976**, 72, 248-254

Appendix A

Mass spectrometry result for the SrtA assay

Once we had a working assay, we would like to ensure that correct product was formed. We have performed another four assays, 1) first negative control (BFP-LPETGG-His_6), 2) second negative control (BFP-LPETGG-His_6 with water and SrtA), 3) assay with glycine (BFP-LPETGG-His_6 with glycine and SrtA), 4) assay with diglycine (BFP-LPETGG-His_6 with diglycine and sortase) and 5) assay with tetraglycine (BFP-LPETGG-His_6 with tetraglycine and SrtA). A sample was taken out from each reaction after overnight incubation at room temperature and analysed by LC/MS. Here we show the raw data of the HPLC traces of each sample. From the traces, we could conclude that the product from each reaction was formed and it was proved by the LC-MS.

a) BFP-LPETGG-His_6 sample

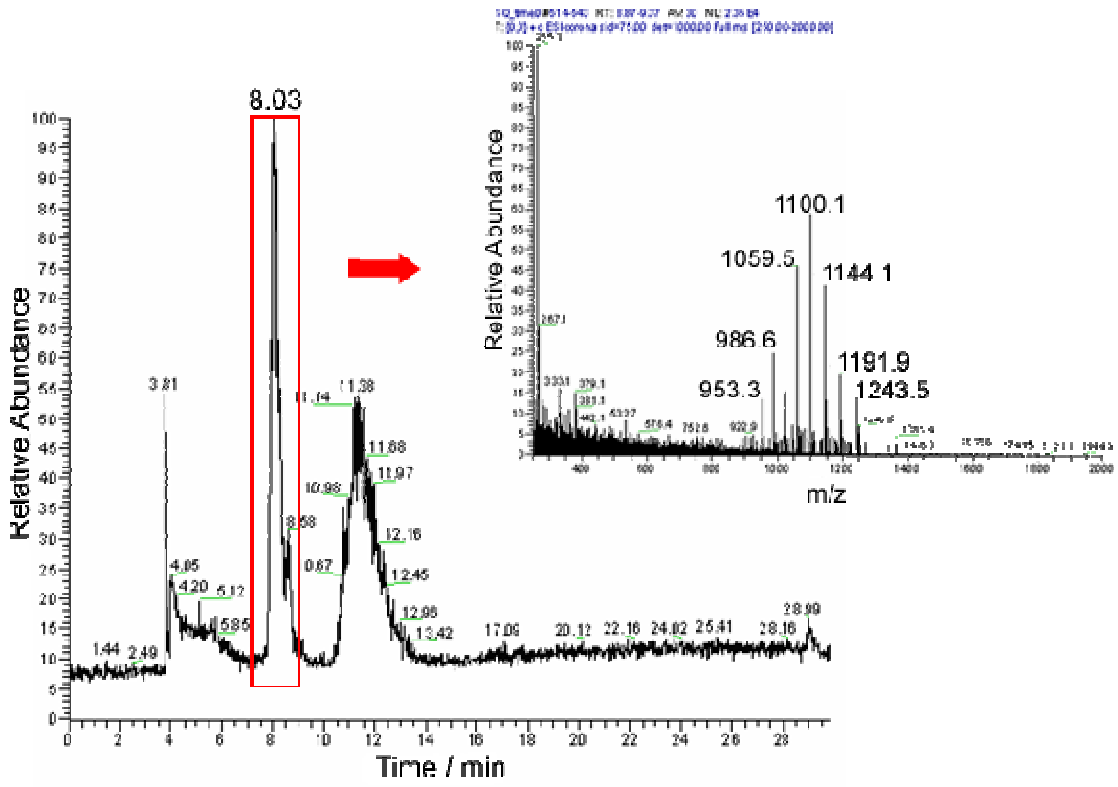

Figure A 1 Total ion current trace from LC-MS of BFP-LPETGG-His_6.

The expected m/z values of the BFP-LPETGG-His_6 were: 953.3, 986.3, 1021.6, 1059.4, 1100.1, 1144.0, 1191.6 and 1243.4. From the ion current trace, the main peaks observed were 953.3, 986.6, 1059.5, 1100.1, 1144.1, 1191.9 and 1243.5 which were very similar to the expected values. This proved that the correct BFP product has formed.

b) Deionised Water sample

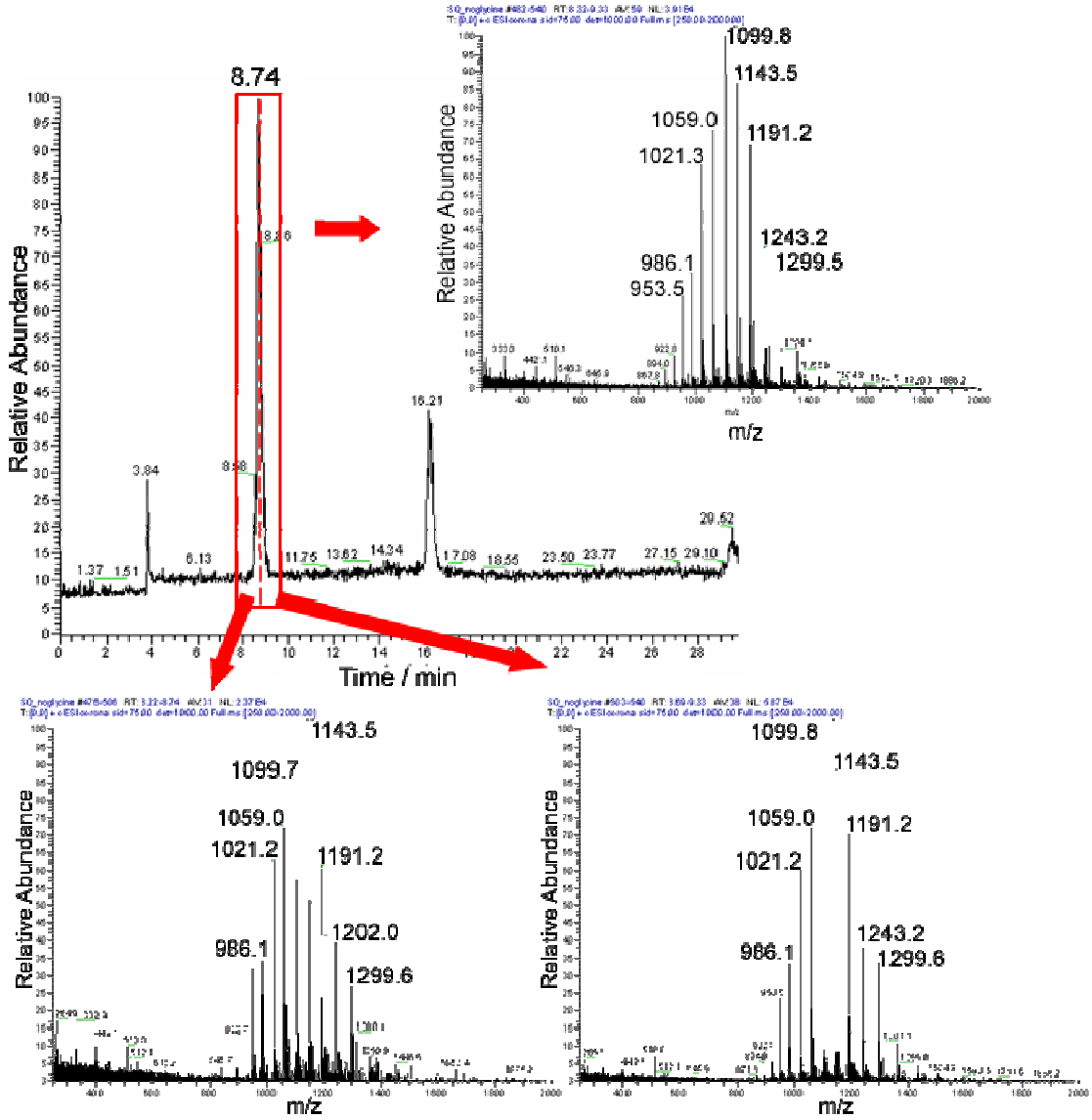

Figure A 2 Total ion current trace from LC-MS of assay with no substrate and the first and second half of the peak at 8.74 minutes.

The expected m/z values of the product BFP-LPET-OH were: 954.0, 988.1, 1024.6, 1064.0, 1106.5, 1152.6, 1202.6, 1257.3 and 1317.1. From the LC/MS result, the main peaks were different from the expected peaks, but similar to BFP-LPETGG-His_6. This showed most of the BFP were not cleaved. Some small peaks under the main peaks which has a mass

envelope corresponding to the hydrolysis product, which dedicated that only hydrolysis has happened in the deionised water assay.

c) Glycine sample

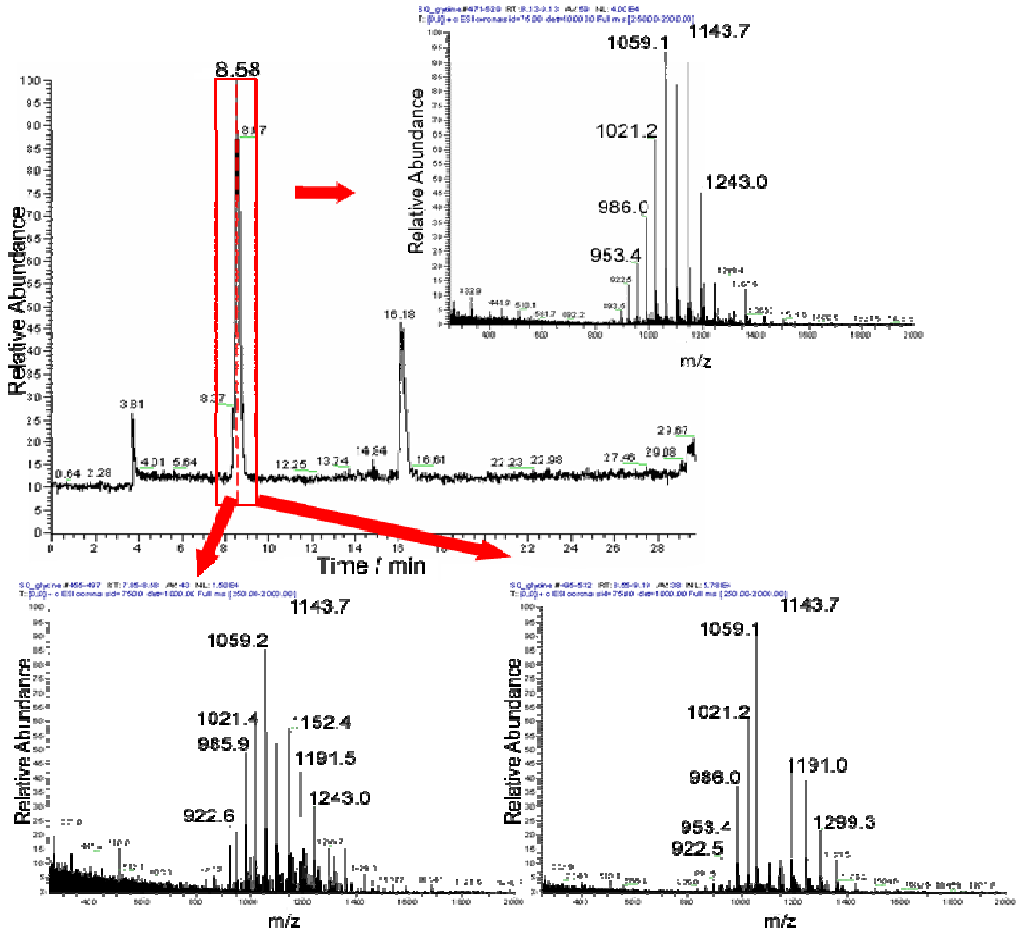

Figure A 3 Total ion current trace from LC-MS of assay with glycine as the substrate, and the first and second half of the peak at 8.58 minutes.

The expected m/z values of the glycine product BFP-LPETG were: 956.0, 990.1, 1026.7, 1066.2, 1108.8, 1154.9, 1205.1 and 1259.9. From the LC/MS result, the main peaks were different from the expected peaks, but similar to deionised water, where the peaks were BFP-LPETGG-His_6. This showed that most of the BFP were not cleaved. Some small peaks under the main peaks which has a mass envelope corresponding to the hydrolysis

product so it is the same with the assay with water and showed that only hydrolysis has happened in the glycine assay.

d) Diglycine sample

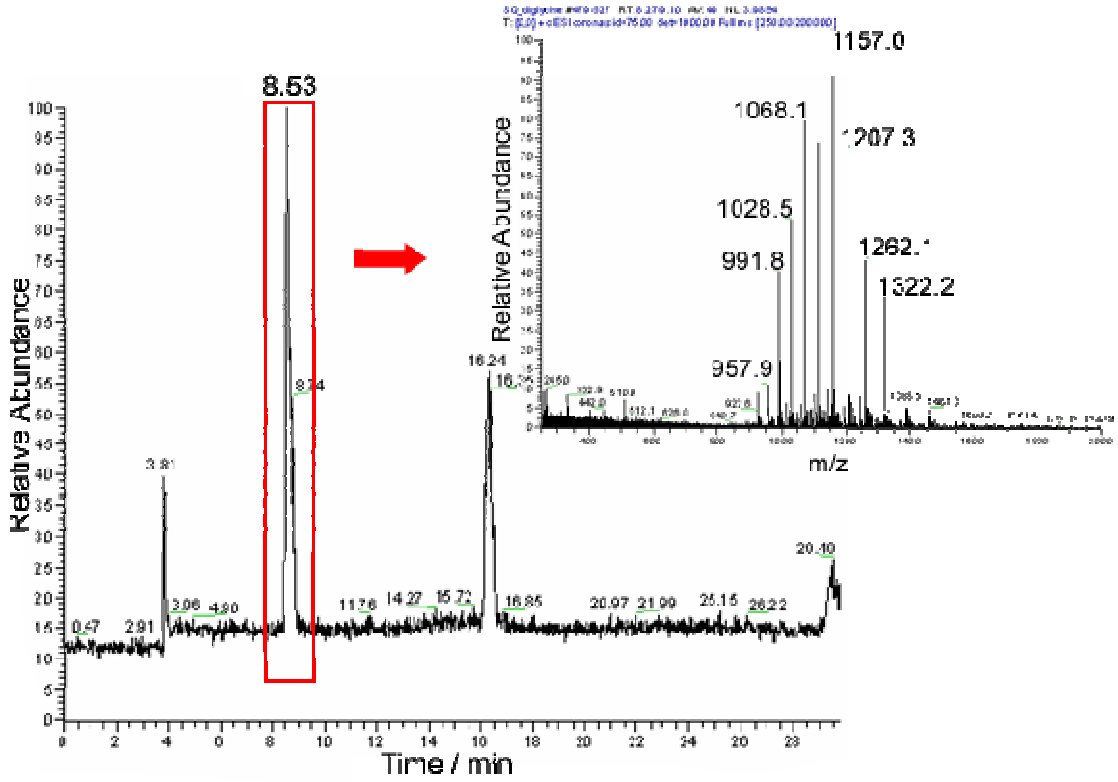

Figure A 4 Total ion current trace from LC-MS of assay with diglycine as substrate.

The expected m/z values of the diglycine assay's product BFP-LPETGG were: 958.0, 992.1, 1026.7, 1068.4, 1111.1, 1157.3, 1207.6, 1262.4 and 1322.5. The main peaks shown in the LC/MS result were: 957.9, 911.8, 1028.5, 1068.1, 1157.0, 1207.3, 1262.1 and 1322.2. So this shows that the diglycine assay worked as the expected peaks were found form the trace.

e) Tetraglycine sample

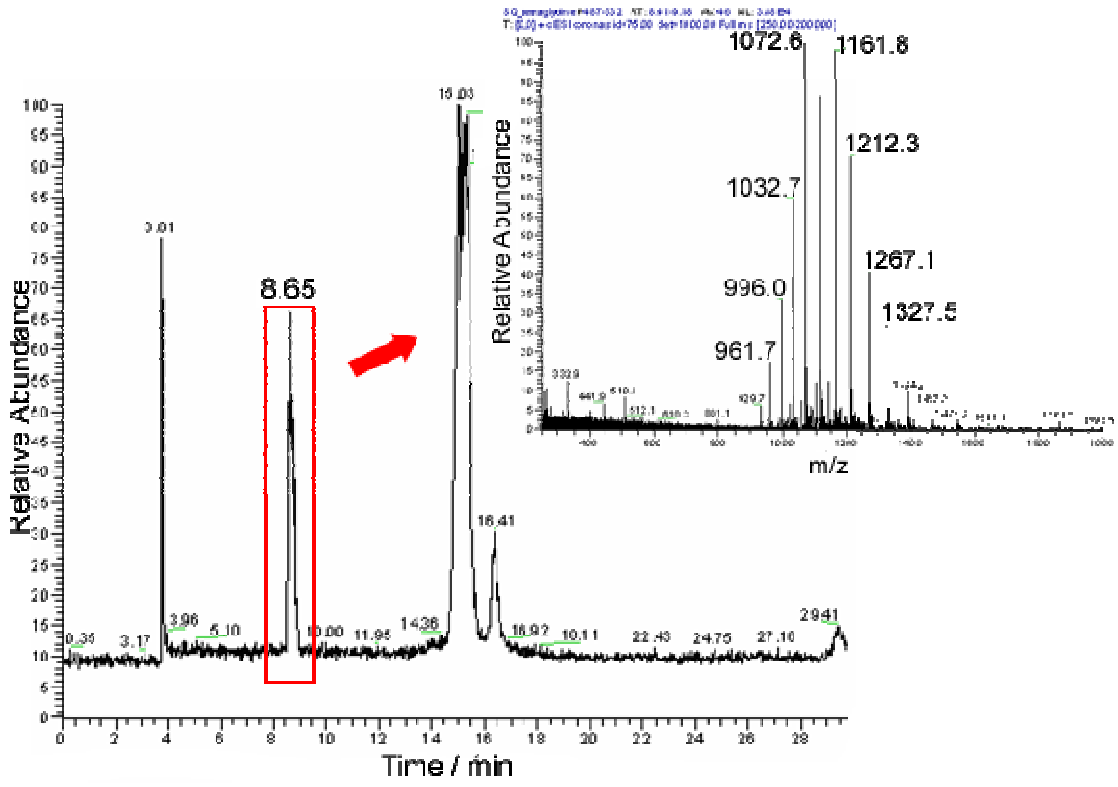

Figure A 5 Total ion current trace from LC-MS of assay with tetraglycine as substrate.

The expected m/z values of the tetraglycine assay product BFP-LPETGGGG were: 961.9, 996.2, 1033.1, 1072.8, 1115.6, 1162.1, 1212.6, 1267.6 and 1327.9. The expected peaks were shown in the LC/MS result. So this shows that the diglycine assay worked as the expected peaks were found form the trace.

With all the products being confirmed by the LC/MS as the correct product, further experiments were considered. The ligation using SrtA system is a very mild and rapid way to ligate different molecules to the protein, so the next goal would be to label the proteins using SrtA system and produce labelled proteins and fluorescence resonance energy transfer (FRET).

Appendix B

B1) Sequence of His_6 tagged Sortase A from *S. aureus*

```
  1       MGSSHHHHHH SSGLVPRGSH MKPHIDNYLH DKDKDEKIEQ YDKNVKEQAS
 51       KDKKQQAKPQ IPKDKSKVAG YIEIPDADIK EPVYPGPATP EQLNRGVSFA
101       EENESLDDQN ISIAGHTFID RPNYQFTNLK AAKKGSMVYF KVGNETRKYK
151       MTSIRDVKPT DVGVLDEQKG KDKQLTLITC DDYNEKTGVW EKRKIFVATE
201       VK
```

Figure B 1 Sequence of His_6 tagged sortase A from *S. aureus*.

B2) Plasmid Maps

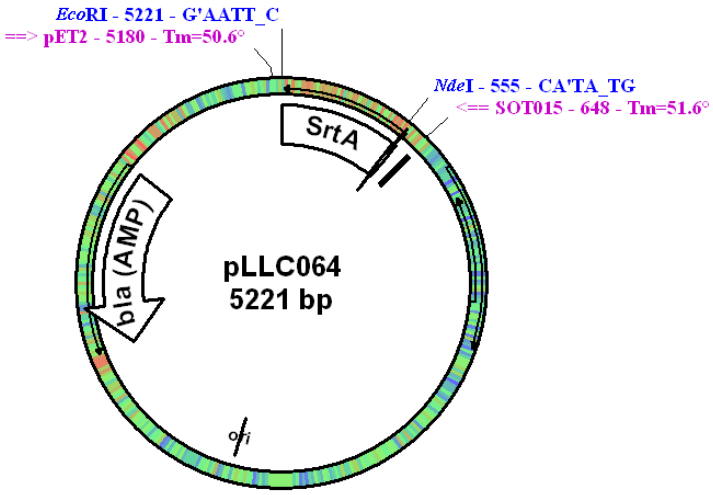

Figure B 2 Plasmid map of pLLC064 (*his_6-srtA*).

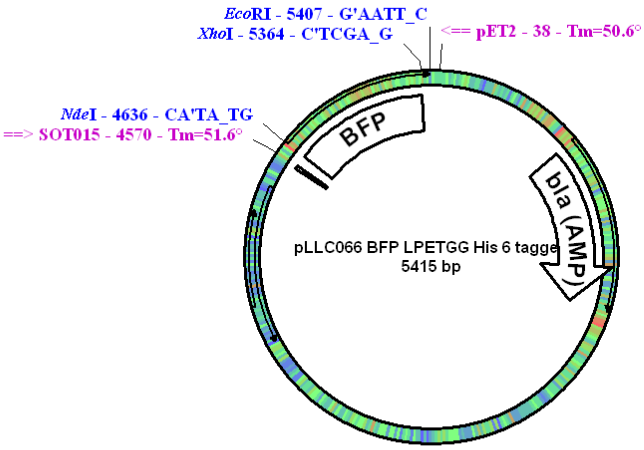

Figure B 3 Plasmid map of pLLC066 (*bfp-lpetgg-his*$_6$).

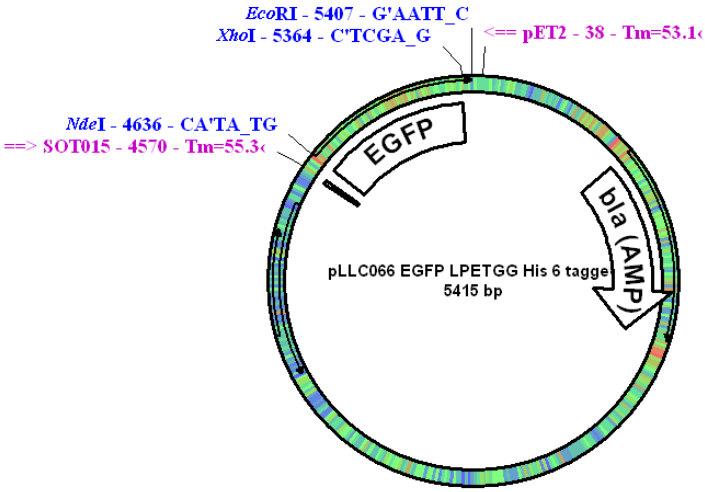

Figure B 4 Plasmid map of pLLC146 (*egfp-lpetgg-his*$_6$).

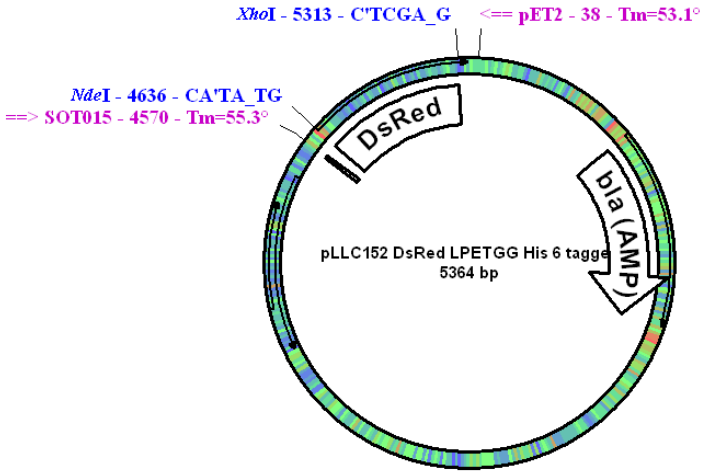

Figure B 5 Plasmid map of pLLC152 (*dsred-lpetgg-his*$_6$).

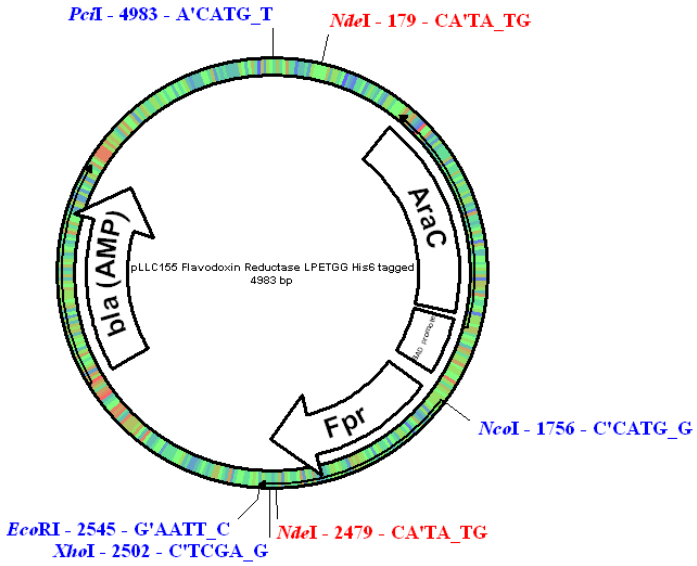

Figure B 6 Plasmid map of pLLC155 (*fpr-lpetgg-his*$_6$).

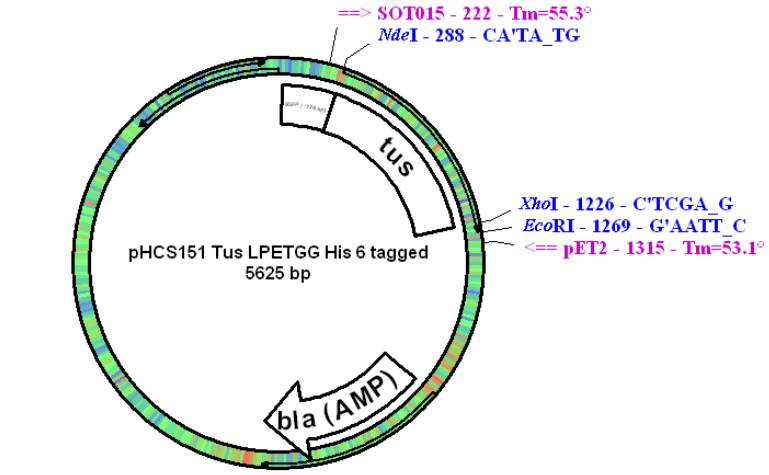

Figure B 7 Plasmid map of pHCS151 (*tus-lpetgg-his*$_6$).

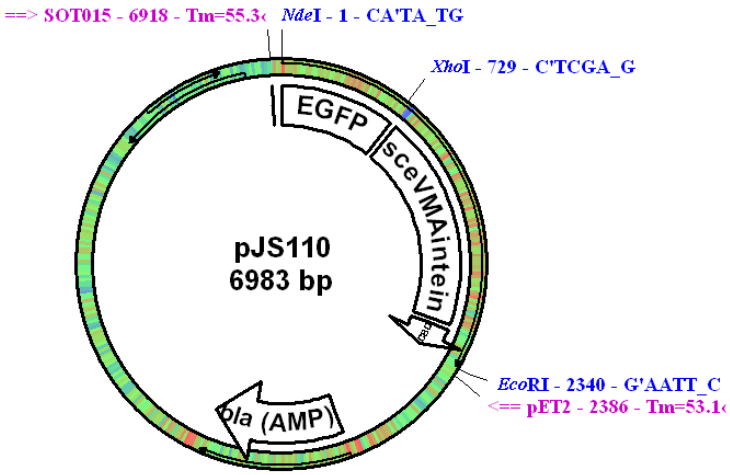

Figure B 8 Plasmid map of pJS110 (*egfp-intein-his*$_6$).

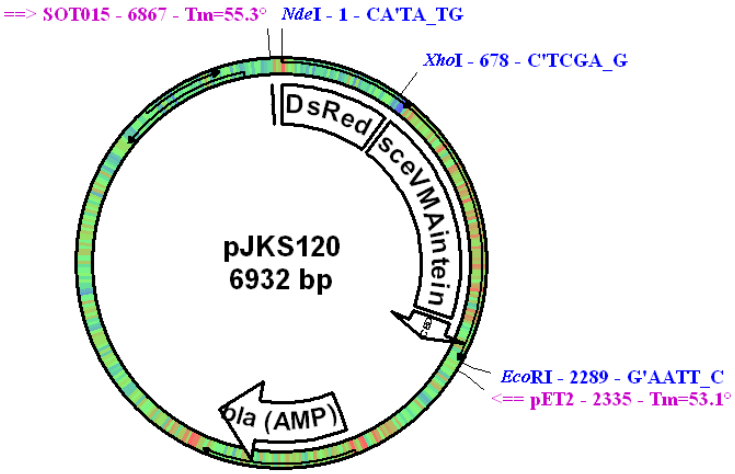

Figure B 9 Plasmid map of pJKS120 (*dsred-intein-his*$_6$).

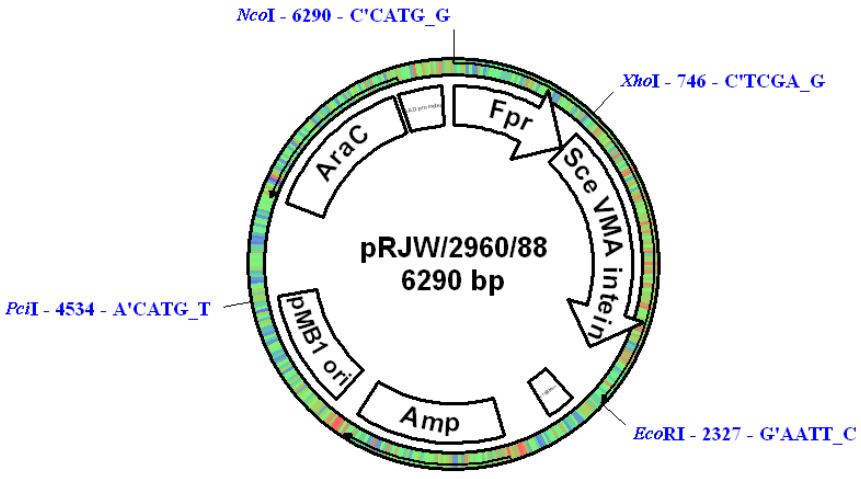

Figure B 10 Plasmid map of pRJW/2960/88 (*fpr-intein*).