

UNIVERSITY OF CALIFORNIA,
IRVINE

Optimizing Recombinant Adhesion Molecule Constructs for Targeted Therapeutic
Nanoparticles

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

Yuting Ji

Thesis Committee:
Assistant Professor Jered B. Haun, Chair
Assistant Professor Wendy Liu
Associate Professor James P. Brody

2016

DEDICATION

To

my parents, friends, and Mr. Liu,

in recognition of their love, support, trust, and help.

TABLE OF CONTENTS

	Page
LIST OF FIGURES	v
LIST OF TABLES	vii
ACKNOWLEDGMENTS	viii
ABSTRACT OF THE THESIS	ix
INTRODUCTION	1
CHAPTER 1: Enzyme-Mediated Conjugation by Lipoic Acid Ligase	5
1.1 Background	5
1.2 Bacterial expression and purification of <i>E. coli</i> LplA	8
1.3 Measurement of LplA-catalyzed TCO ₂ and azide 9 Ligation to LAP2	10
CHAPTER 2: Site-Specific Bioconjugation Catalyzed by Sortase A	16
2.1 Background	16
2.2 Production of Sortase A	18
2.2.1 Protein Expression	18
2.2.2 Protein Purification	19
2.3 Modification of Sortase A substrates	20
2.4 Protein-Protein Ligation	22
CHAPTER 3: Recombinant Plasmid Construction of Inserted Domain	26
3.1 Background	26
3.2 Materials and Methods	28
2.2.1 Reagents, Materials and Kits	28
2.2.2 Strains and Media	28
2.2.3 Primer and Plasmid Design	29
3.3 Results	32
CHAPTER 4: Yeast Surface Display of Protein Expression	37
4.1 Overview	37
4.2 Materials and Methods	38
4.2.1 Reagents	38
4.2.2 Strains and Media	39
4.2.3 Transformation	39
4.3 Yeast surface display	40
CHAPTER 5: Summary and Conclusions	42

REFERENCES	44
APPENDIX A: Gene Cloning and Preparation	48
APPENDIX B: Yeast Culture and Recombinant Protein Production	52

LIST OF FIGURES

		Page
Figure 1	Passive and active targeting strategies	2
Figure 2	Nanoparticles with numerous targeting ligands can bind to surface cells multivalency with high receptor density	3
Figure 3	Model of targeted delivery system	4
Figure 4	Construct of scFv with peptide linkers	4
Figure 1.1	Scheme for LplA-catalyzed fluorescent labeling	6
Figure 1.2	Model of LAP recombinant construct	7
Figure 1.3	TCO2 and azide 9 reaction scheme	8
Figure 1.4	SDS-PAGE result of expressed LplA	10
Figure 1.5	LplA reaction results	13
Figure 1.6	Mass spectra of LAP2-TCO2 and LAP2-azide 9	14
Figure 2.1	Sortase A structure and reaction scheme	17
Figure 2.2	SDS-PAGE result of expressed Sortase A	20
Figure 2.3	Mass spec result of Fmoc-GGGK and GGGK	21
Figure 2.4	Mass spectra of LPETG(GGS) ₃	22
Figure 2.5	MALDI-TOF result of ligated conjugate	24
Figure 3.1	Protein sequence of I domain and its mutants	27
Figure 3.2	Insert sequence of pCT-4420	29
Figure 3.3	Insert sequence of pCT-I domain and construct of wt I domain-mCherry- AviTag-6His-cymc.	30
Figure 3.4	Ligation model	31
Figure 3.5	Gel results of digestion	32

Figure 3.6	Sequencing results for I domain and its mutants	35
Figure 4.1	Map of plasmid pCT302	38

LIST OF TABLES

		Page
Table 1.1	LplA reaction system	10
Table 3.1	Reagents, materials and kits applied in plasmid construction	28
Table 3.2	Concentrations of digested inserts and vectors	33
Table 3.3	Ligation reaction system	33
Table 3.4	Colony PCR reaction system	34
Table 4.1	Components list for yeast transformation	40

ACKNOWLEDGMENTS

I would like to express the deepest appreciation to my committee chair, Professor Jered Haun, who is always patient to my every question and give me the most courage to conquer every difficulty. Without his guidance and persistent help this dissertation would not have been possible.

I would like to thank my committee members, Professor Wendy Liu and Professor James Brody, for their valuable knowledge and insights, which greatly enrich my work.

I would also like to thank Rajesh Kota, Esther Yu-Tin Chen, Phinix Dominh, Maha Rahim and Xiaolong Qiu for the instruction they have given to me about every equipment that I began to learn and use.

And thank you to all Haun labmates for their help and support during my entire thesis work.

ABSTRACT OF THE THESIS

Optimizing Recombinant Adhesion Molecule Constructs for Targeted Therapeutic Nanoparticles

By

Yuting Ji

Master of Science in Biomedical Engineering

University of California, Irvine, 2016

Professor Jered B. Haun, Chair

Nanoparticle-mediated drug delivery system is applied in order to provide specific nanoparticle-cell surface interactions and combat the downfalls of traditional drug delivery methods. In order to optimize the adhesion efficacy of nanoparticles to corresponding ligand substrates, we developed the targeting receptor by introducing an inserted domain (I domain) from Lymphocyte function-associated antigen-1, inserting it into pCT302 and pRS314 expression vector, then producing protein by yeast surface display and yeast secretion. Meanwhile, two enzymes, lipolic acid ligase (LplA) and Sortase A (SrtA), were produced for site-direct conjugation of targeting receptor to nanoparticles. As a result, I domain wild-type, I domain mutant F265S, I domain mutant K287C/294C were successfully cloned to construct pCT and pRS based plasmids, then transformed into yeast cells and made protein production. Besides, LplA and Sortase A were confirmed to catalyze the conjugation of aizde-9 to TCO2, and LPETG to GGGK, respectively. The establishment of I domain targeting protein platform and LplA/SrtA enzyme-mediated conjugation system will provide a promising and efficient method for optimizing adhesion of targeted nanoparticles.

INTRODUCTION

Targeted drug delivery is a method of delivering medication to a patient in a manner that increases the concentration of the medication in some parts of the body relative to others. The goal of a targeted drug delivery system is to prolong, localize, target and have a protected drug interaction with the diseased tissue. The delivery is largely founded on nano-medicine, which plans to apply nanoparticle-mediated drug delivery in order to combat the downfalls of conventional drug delivery. Nanoparticles would be loaded with drugs and targeted to specific parts of the body where there is solely diseased tissue, thereby avoiding interaction with healthy tissue.¹

Ideally, for anticancer drugs to be effective in cancer treatment, they should first be able to reach the desired tumor tissues through the penetration of barriers in the body with minimal loss of their volume or activity in the blood circulation. Second, after reaching the tumor tissue, drugs should have the ability to selectively kill tumor cells without affecting normal cells with a controlled release mechanism of the active form. These two basic strategies are also associated with improvements in patient survival and quality of life by increasing the intracellular concentration of drugs and reducing dose-limiting toxicities simultaneously. Increasingly, nanoparticles seem to have the potential to satisfy both of these requirements for effective drug carrier systems.²

Nanoparticle therapeutics are typically particles comprised of therapeutic entities, such as small-molecule drugs, peptides, proteins and nucleic acids. The targeting ligands that provide specific nanoparticle-cell surface interactions can play a vital role in the ultimate location of the nanoparticle. For example, nanoparticles can be targeted to cancer cells if their surfaces contain moieties such as small molecules, peptides, proteins or

antibodies. These moieties can bind with cancer cell-surface receptor proteins, such as transferrin receptors, which are known to be increased in number on a wide range of cancer cells.³ By using both passive and active targeting strategies, nanoparticles can enhance the intracellular concentration of drugs in cancer cells while avoiding toxicity in normal cells (Figure 1).⁴⁻⁵

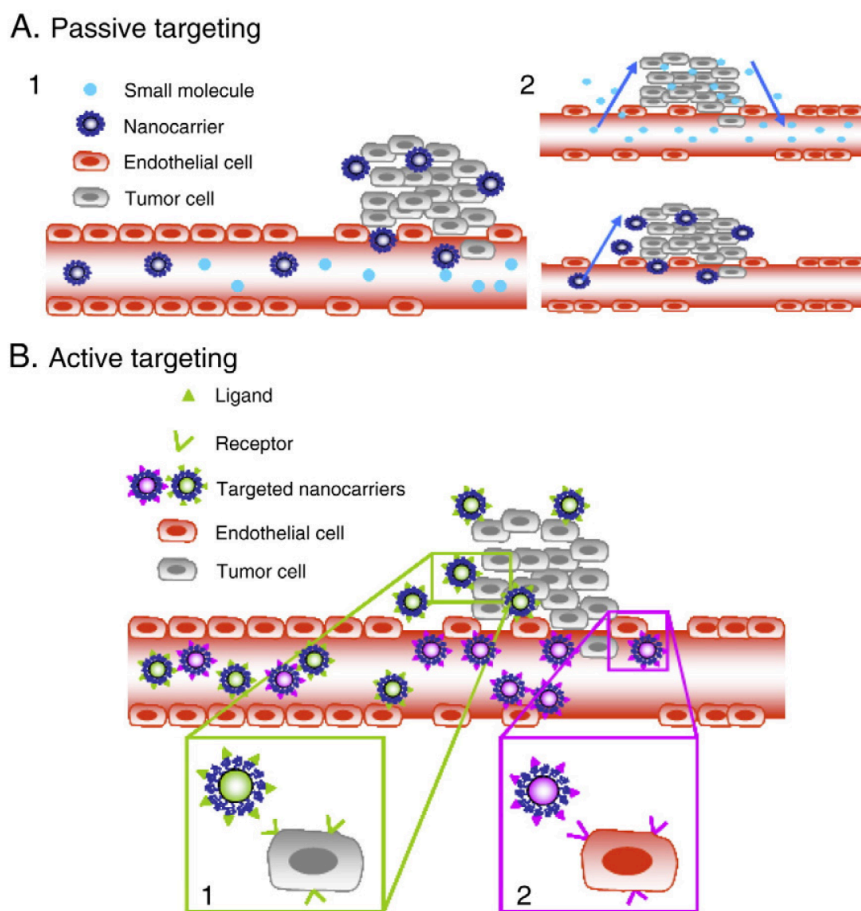


Figure 15. A. Passive targeting of nanocarriers. (1) Nanocarriers reach tumors selectively through the leaky vasculature surrounding the tumors. (2) Drugs alone diffuse freely in and out the tumor blood vessels because of their small size and thus their effective concentrations in the tumor decrease rapidly. By contrast, drug-loaded nanocarriers cannot diffuse back into the blood stream because of their large size, resulting in progressive accumulation. B. Active targeting strategies. Ligands at the surface of nanocarriers bind to receptors overexpressed by (1) cancer cells or (2) angiogenic endothelial cells.

There two parameters of nanoparticles for tuning the binding to target cells: the affinity of the targeting moiety and the density of the targeting moiety (Figure 2).⁶ The multivalency effects can lead to high effective affinities when using arrangements of low-affinity ligands.⁷ Thus, the receptors of molecules that can be used as targeting agents are greatly expanded as many low-affinity lands that are not sufficient for use as molecular conjugates can now be attached on nanoparticles to create higher affinity via multivalent binding to cell-surface receptors.

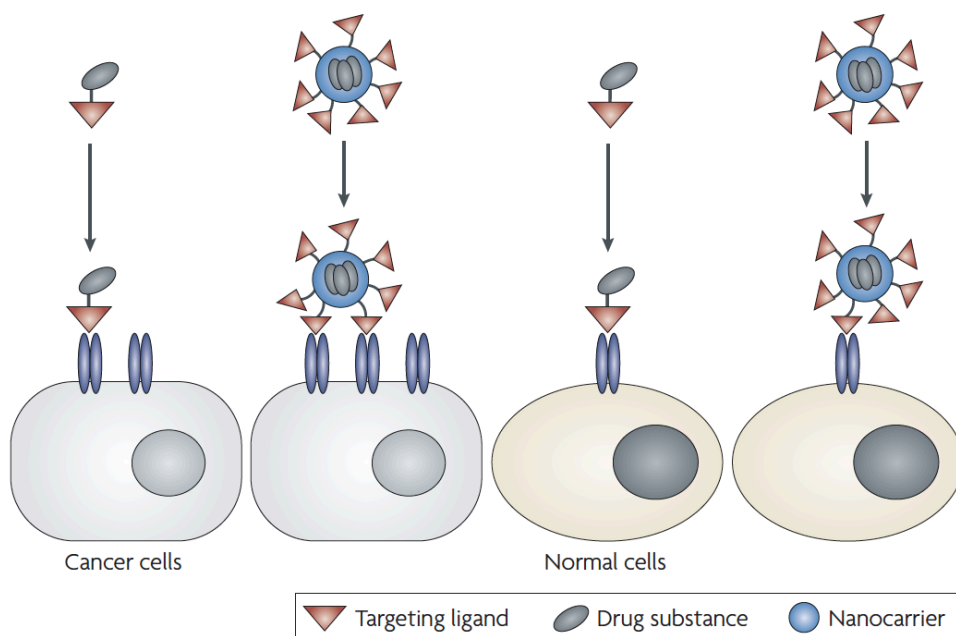


Figure 2. Nanoparticles with numerous targeting ligands can bind to the surface cells multivalency with high receptor density.⁶

When the surface density of the receptor is low on normal cells, the molecular conjugation of a single targeting ligand and a targeted nanoparticle can compete equally since only one ligand-receptor may occur. However, when there is a high surface density of the receptors on cancer cells, the targeted nanoparticle can engage numerous receptors simultaneously to provide enhanced interactions over the one ligand.

We have been previously established a platform of single-chain antibody (scFv) for targeted delivery nanoparticle therapies, in which the scFvs are fused with fluorescent

protein (eGFP or mCherry), biotin acceptor protein (AviTag), six-histidine tag (6His), c-myc epitope. The recombinant antibodies as targeting receptors were biotinylated and then were functionalized directly onto avidin-coated nanoparticles. Attachment and detachment of scFvs and paired ligands were then performed using a flow chamber (Figure 3).⁸

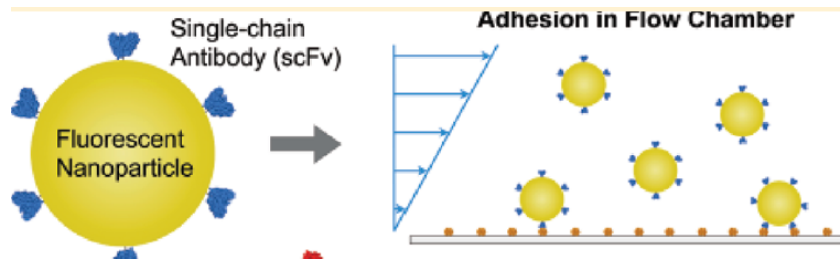


Figure 3. Model of targeted delivery system. ⁸

In this study, we developed the targeting receptor by introducing an inserted domain (I domain) from Lymphocyte function-associated antigen-1, inserting into pCT302 and pRS314 expression vector based on previous studies (Figure 4), then producing by yeast surface display and yeast secretion. At the meanwhile, two enzymes LplA and Sortase A were produced for site-direct conjugation of targeting receptor to nanoparticles. Thus to optimize the binding efficiency between nanoparticles and corresponding ligand substrates.

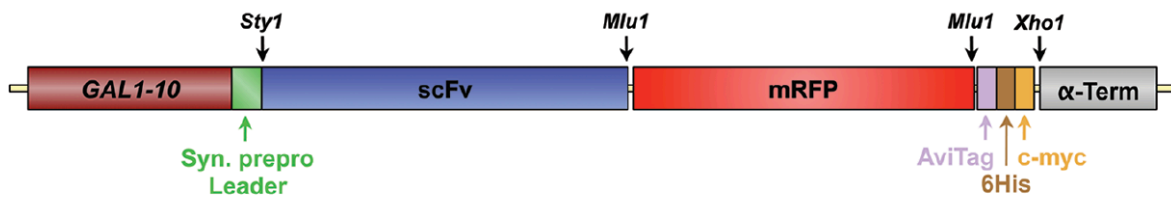


Figure 4. Construct of scFv with peptide linkers. ⁸

Construct including GAL1-10 as promoter, synthetic prepro leader as sequence to direct the protein, mRFP as fluorophore, AviTag as biotin acceptor protein, 6His tag used for protein purification, c-myc as epitope tag, and untranslated region.

CHAPTER 1 Enzyme-Mediated Conjugation by Lipoic Acid Ligase

1.1 Background

Fluorescent labeling of cell surface proteins enables imaging of the trafficking and function of receptors, channels and transporters. Many protein labeling methods have been developed in recent years,⁹ but none currently allows the covalent attachment of small fluorophores onto cell surface proteins modified only by a small peptide tag, with short labeling times and with extremely high specificity over a wide range of expression levels and labeling conditions. Previous studies have performed to develop an approach to the targeting problem called probe incorporation mediated by enzymes (PRIME).¹⁰ In PRIME, a mutant of *Escherichia coli* (*E. coli*) enzyme lipoic acid ligase (LplA) is used to catalyze the covalent conjugation of chemical probes to recombinant proteins fused to a 13-amino acid LplA acceptor peptide (LAP). The naturally high sequence-specificity of LplA makes this targeting method highly specific. In comparison to other intracellular post-translational protein labeling methods—SNAP, HaloTag, and FLaSH—PRIME uniquely offers the combination of a small peptide-directing sequence and high labeling specificity.¹¹⁻¹³

The natural function of LplA is to catalyze ATP-dependent, covalent ligation of lipoic acid onto specific lysine side chains of three *E. coli* proteins involved in oxidative metabolism (E2p, E2o and H-proteins).¹⁴ There are mainly two steps in the site-specific fluorescence labeling of proteins by LplA (Figure 1.1).¹⁵

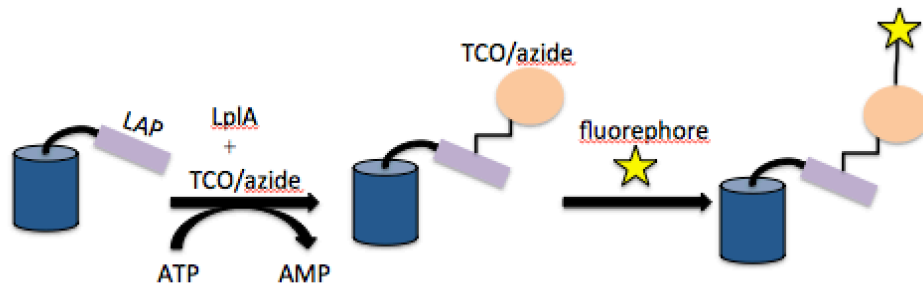


Figure 1.1 Scheme for LplA-catalyzed fluorescent labeling

In the first step, the LplA ligates *trans*-cyclooctenes TCO or azide onto LplA acceptor peptide (LAP), which is fused to the protein of interest. In the second step, ligated *trans*-cyclooctenes is chemoselectively derivatized with a fluorephore.

The technology for labeling cell surface proteins fused to a 22-amino acid recognition sequence for *E. coli* LplA. Small, non-cross-linking probes such as Cy3, Alexa Fluor and biotin can be conjugated site-specifically and covalently to the LAP peptide in as little as 20 min. An important feature of this technique is its generality, potentially a wide variety of cell surface proteins in diverse cell types can be labeled with a wide range of chemical probes linked to cyclooctynes or azide. Many new protein labeling methods have been developed in recent years, those techniques have tried to bridge the requirements of small tag size and high labeling specificity, by making use of enzyme ligases.

By replacing Avitag (Figure 4) by LAP, and modifying nanoparticles with azide or TCO coat, the intrinsic sequence-specificity of enzyme catalysis of LplA could achieve highly specific probe conjugation, and greatly improve the binding efficiency between nanoparticle and receptor proteins, thus to increase the robustness of the targeted nanoparticle delivery platform (Figure 1.2).

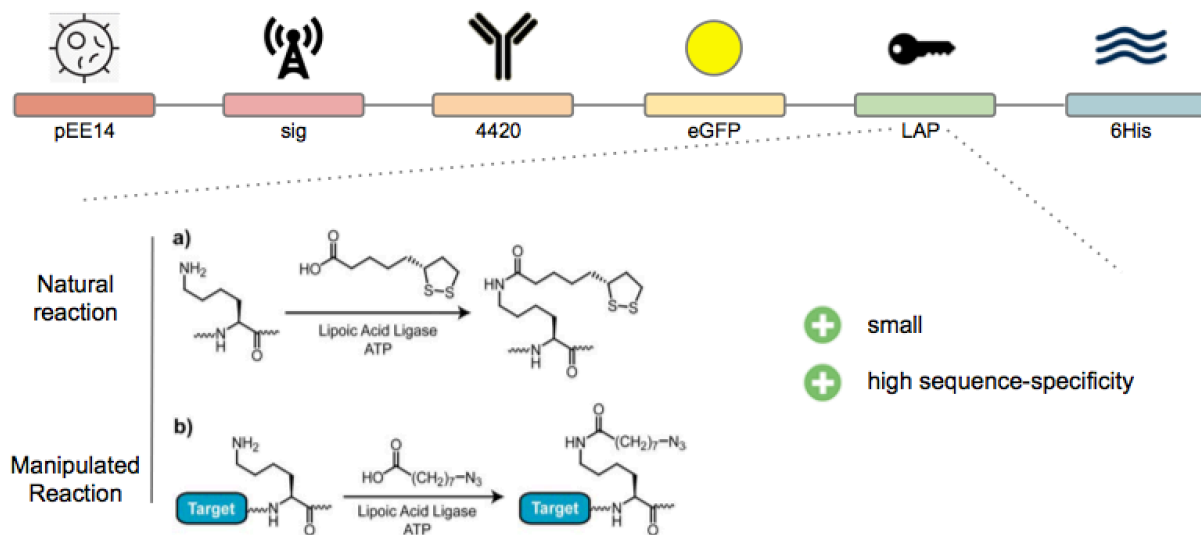


Figure 1.2 Model of LAP recombinant construct

In previous study, researchers have evolved a novel peptide substrate for LplA called LAP2 (GFEIDKVWYDLDA), with kinetic properties comparable to those of LplA's natural protein substrates.¹⁶ LAP2 has the ability to label peptide-tagged cell surface receptors with unnatural probes, even at low or medium receptor expression levels. Besides, it also allows fluorophore tagging of intracellular proteins. Also, LAP2 is shorter than natural LAP (13 amino acids instead of 17-22 amino acids) and can be recognized by LplA at the N-terminus, C-terminus, and internally.¹⁷

Several small-molecule structures were considered in previous work to replace lipoic acid. Direct ligation of a fluorophore would offer a simpler and shorter labeling procedure, but incorporation of a 'functional group handle' is more feasible due to the small size of the lipoate binding pocket, and it provides greater versatility for subsequent incorporation of probes with diverse structures.¹⁸ Many functional group handles have been used in chemical biology, including ketones, organic azides and alkynes.¹⁹ Organic

azides are the most suitable for live cell applications, because the azide group is both abiotic and nontoxic in animals and can be selectively derivatized under physiological conditions with cyclo-octynes, which are also unnatural. Here in this paper, we engineered an azide 9 substrate to test whether it can be used efficiently in place of lipoic acid. It is noted that the *trans*-cyclooctene moiety is stable in aqueous solution and require less re-engineering of LplA, among different types of *trans*-cyclooctene, TCO2 can be ligated to LAP efficiently, the kinetics of which is comparable to that of the fastest unnatural probe and only 2-fold slower than ligation of the natural substrate lipoic acid by the same enzyme. Thus in this paper, we engineered with LAP2, azide 9 and TCO2 to test their ligation by LplA (Figure 1.3).

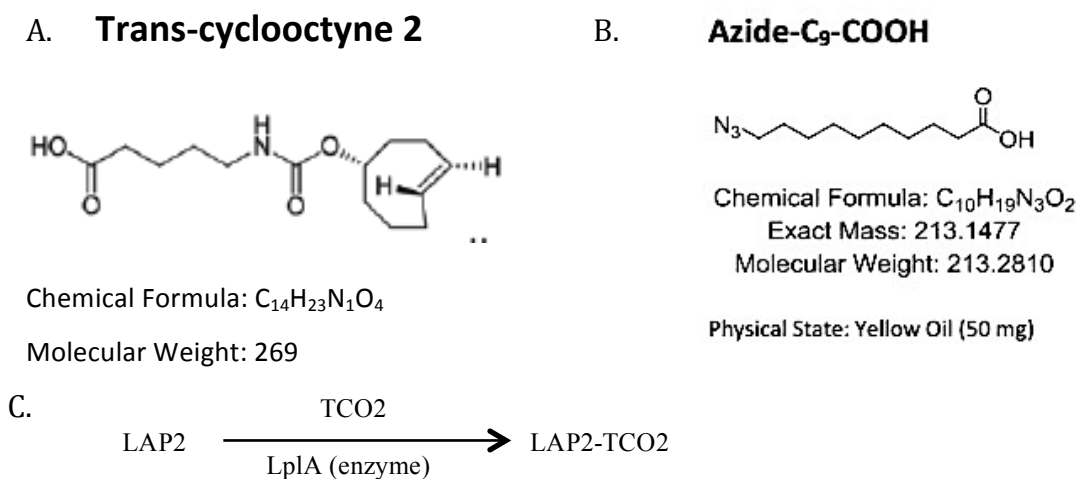


Figure 1.3 A. TCO2 chemical form; B. Azide 9 chemical form; C. reaction formula of LAP2-TCO2.

1.2 Bacterial expression and purification of *E. coli* LplA

In this paper, LplA was expressed from the *E. coli* expression plasmid pYFJ16 (Addgene). The plasmid was first transformed into *E. coli* BL21 (DE3) cells: transferring 2 μ L plasmid miniprep collections to 50 μ L *E. coli* BL21 (DE3) competent cell mixture, pipette

to mix. Placed on ice for 30 min and heat shock on 42 °C for exactly 10 s, then immediately placed back on ice for 5 min. Pipetted 950 µL SOC culture broth into the mixture, slightly pipette. Incubate in 37 °C shaker with 250 rpm, for 60 min. Then spin samples at 3,000 rpm by centrifuge for 1 min, remove 800 µL supernatant culture broth, resuspend cells by pipetting in the left 200 µL mixture, plated on LB agar plates (50 µg/mL ampicillin) for 16 hours. Colonies were picked and amplified in LB media supplemented with 50 µg/mL ampicillin at 37 °C , 250 rpm shaker for overnight. Transferring the cells to 50 mL scale LB media according to the ratio 1:100. Cells were grown at 37 °C while shaking at 200 rpm until OD₆₀₀ 0.5-0.9. Enzyme expression was induced with 200 µg/mL IPTG for 5 hours at 37 °C.

Cells were harvested by centrifugation (3,000 rpm, 10 minutes, 4 °C), the pellet was washed by PBS, and resuspended in lysis buffer (50 mM Tris base, 300 mM NaCl) containing 2.5 mM protease inhibitor cocktail. Thereafter, cells were lysed by ultrasonic treatment (six 15-second bursts, with 1 minute of cooling on ice between bursts). The extract was cleared by centrifugation (14,000 rpm, 10 minutes, 4 °C).

The supernatant portion with His₆-tag was purified using Ni-NTA (GE Healthcare Life Science).

Fractions of both supernatant and pellet were mixed 1:1 with running dye (2-mercaptoethanol and bromophenol Blue), heated in 95 °C for 10 min and then put on ice for 10 min. Then analyzed by 12% SDS-PAGE followed by Coomassie staining (Figure 1.4).

LplA was then collected and buffer exchanged to PBS by Ultra-4 centrifugal concentrator. The concentration of LplA product was tested by NanoDrop 2000, the yield reached to 1 mg/mL

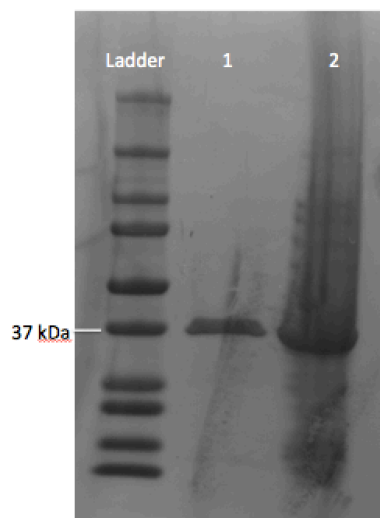


Figure 1.4 SDS-PAGE result of expressed LplA. Band (1) is the supernatant after purification; Band (2) is the pellet after ultrasonic treatment.

1.3 Measurement of LplA-catalyzed TCO2 and azide 9 ligation to LAP2

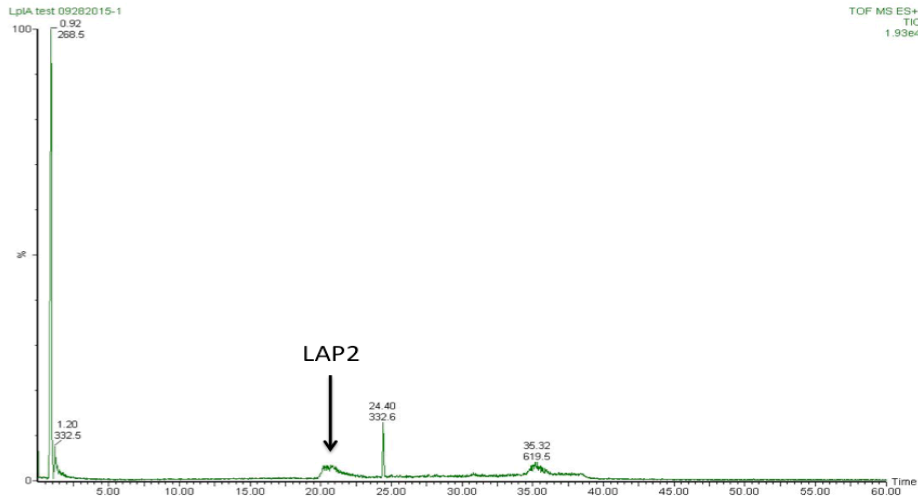
To measure the LplA-catalyzed ligation of *trans*-cyclooctenes TCO2 and azide 9 to LAP2, the reaction conditions were as follows: 5 μ M LplA, 200 μ M LAP2, 500 μ M TCO2 or azide 7, 1 mM ATP, and 5 mM magnesium acetate in sodium phosphate pH 7.0. The reaction was initiated with addition of 2.5 mM ATP. The mixture was incubated at 30 $^{\circ}$ C, and quenched with 50 mM EDTA (Table 1.1).

Table 1.1 Reaction system

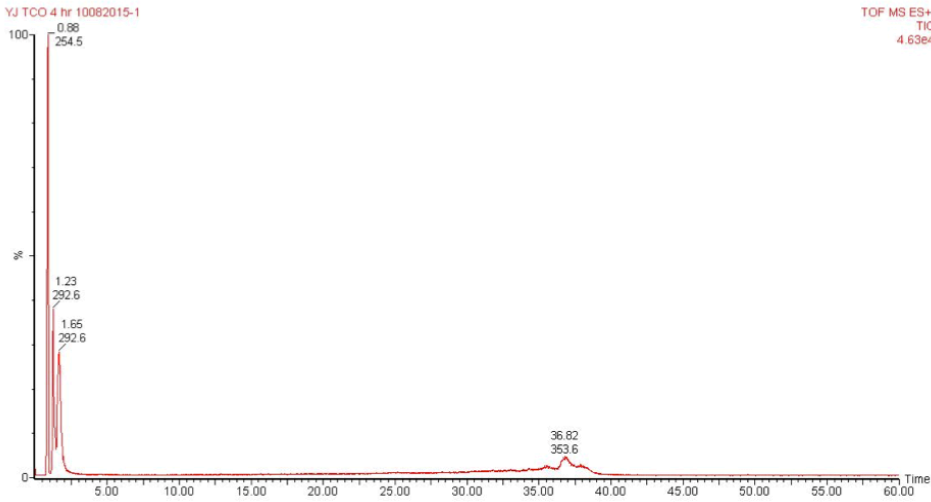
	LplA	Peptide	Substrate	ATP	Mg	Sodium phosphate	Additional reagent	Reaction Temp	Reaction time	Quench
Ctrl	--	LAP2	--	2.5 mM	5 mM	DPBS	10% Glycerol	30 $^{\circ}$ C	--	EDTA
1.	5 μ M	LAP2	TCO2	2.5 mM	5 mM	DPBS	10% Glycerol	30 $^{\circ}$ C	4 hour	EDTA
2.	5 μ M	LAP2	TCO2	2.5 mM	5 mM	DPBS	10% Glycerol	30 $^{\circ}$ C	12 hour	EDTA
3.	5 μ M	LAP2	Azide 9	2.5 mM	5 mM	DPBS	10% Glycerol	30 $^{\circ}$ C	4 hour	EDTA
4.	5 μ M	LAP2	Azide 9	2.5 mM	5 mM	DPBS	10% Glycerol	30 $^{\circ}$ C	12 hour	EDTA

The reaction was measured by liquid chromatography-mass spectrometry (LC-MS) (Figure 1.5, Figure 1.6), which can be used for generally detection and identification of chemicals of particular masses in complex mixtures. LC-MS combines the physical separation capabilities of liquid chromatography (HPLC) with the mass analysis capabilities of mass spectrometry (MS), is a powerful technique that has very high sensitivity, making it useful in many applications. LC-MS is very commonly used in pharmacokinetic studies of pharmaceuticals and is thus the most frequently used technique in the field of bioanalysis. These studies give information about how quickly a drug will be cleared from the hepatic blood flow, and organs of the body. MS is used for this due to high sensitivity and exceptional specificity compared to UV (as long as the analyte can be suitably ionised), and short analysis time. The major advantage of MS is the use of tandem MS-MS. The detector may be programmed to select certain ions to fragment. The process is essentially a selection technique, but is in fact more complex. The measured quantity is the sum of molecule fragments chosen by the operator. As long as there are no interferences or ion suppression, the LC separation can be quite quick. LC-MS is also used in proteomics where again components of a complex mixture must be detected and identified in some manner. The bottom-up proteomics LC-MS approach to proteomics generally involves protease digestion and denaturation followed by LC-MS with peptide mass fingerprinting or LC-MS/MS to derive sequence of individual peptides.²⁰

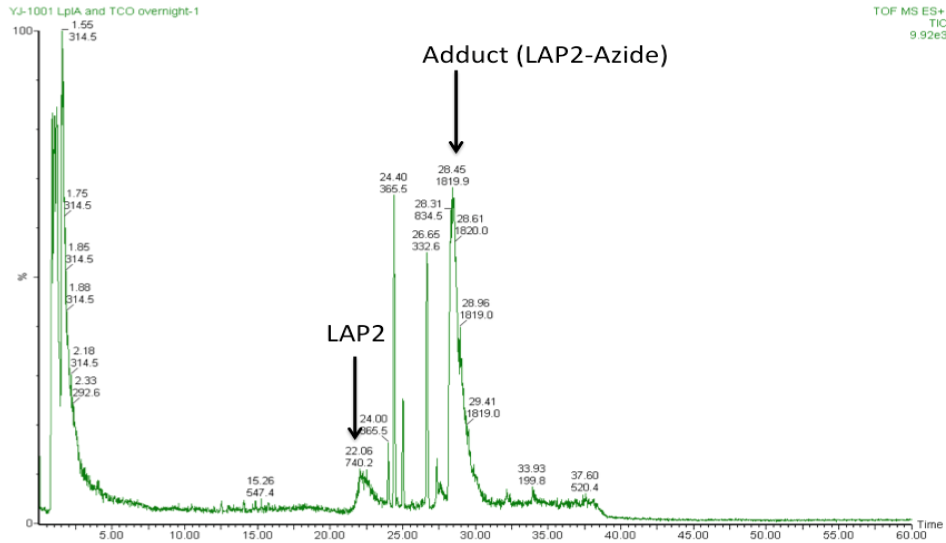
Control.



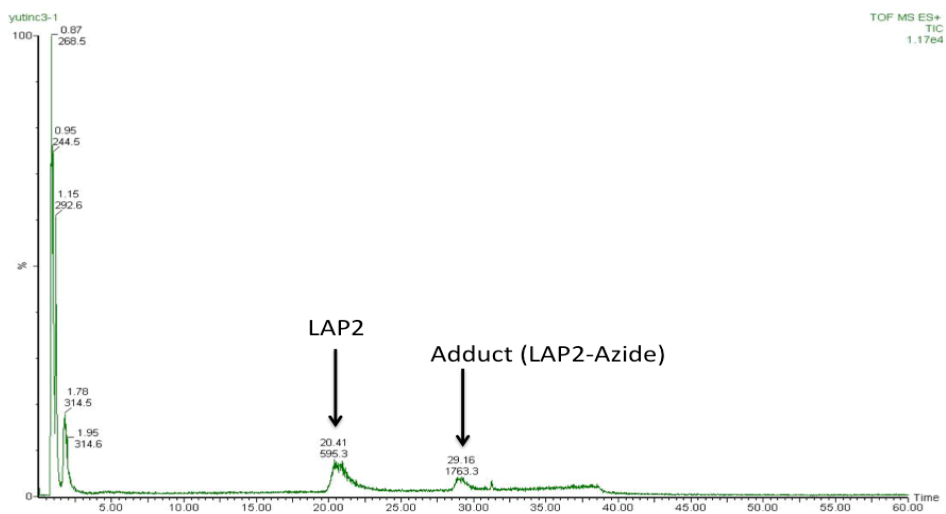
1.



2.



3.



4.

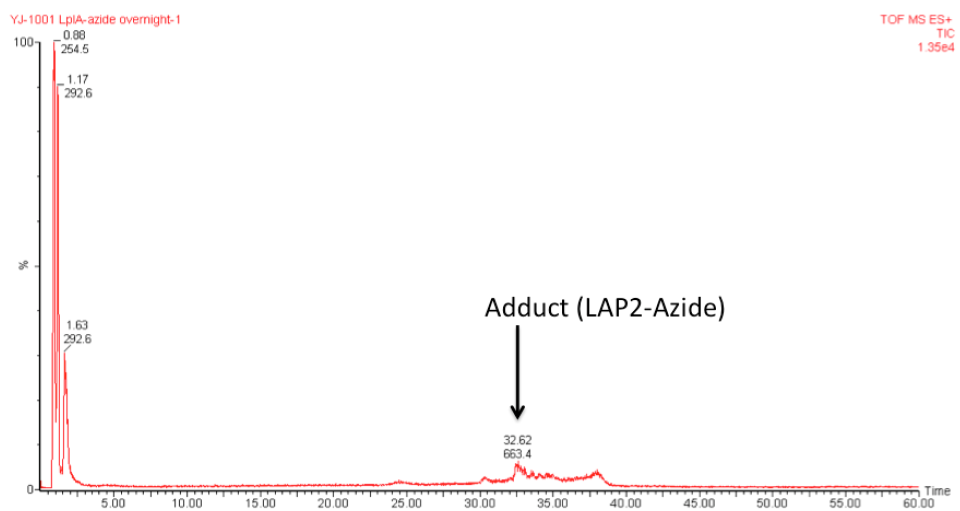


Figure 1.5 Control: by adding in everything but LpIA and substrate, the LC-MS test showed only a LAP2 peak labeled in the figure; (1) and (2) are 4 hours and 12 hours reaction of LAP2 and TC02, respectively, in which 4 hours one showed with non-sense (maybe junk) peak, and 12 hours one showed peak of LAP2 and peak of conjugation (LAP2-TC02); (3) and (4) are 4 hours and 12 hours reaction of LAP2 and azide 9 respectively, which both showed a clear adduct peak (LAP2-azide), and in 12 hours situation, LAP2 was almost consumed by the reaction.

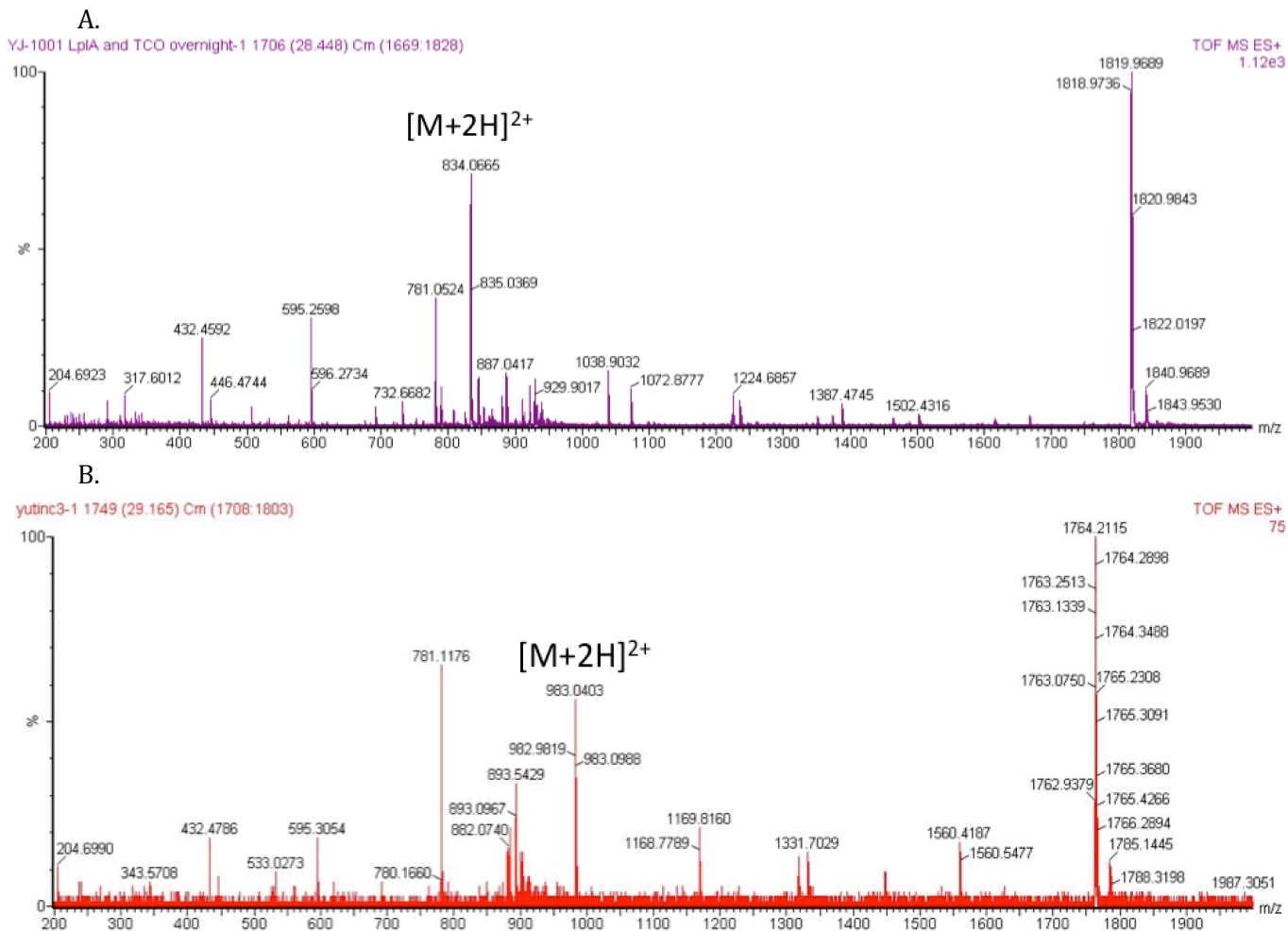


Figure 1.6 Mass spectra of LAP2-TCO2 (A) and LAP2-Azide 9 (B) conjugates.

From Figure 1.6, the mass spectrometry of adduct LAP2-TCO2 and LAP2-azide 9 were shown. The mass about 1820 in Figure 1.6 (A), is the sum of LAP2 molar mass (1568) and TCO2 (269) then subtracting a hydroxide (17). The mass about 1764 in Figure 1.6 (B), is the sum of LAP2 molar mass (1568) and azide 9 (213) and then subtracting a hydroxide (17).

Results from LC-MS showed, the LplA enzyme mediated site-specifically conjugation of peptide LAP2 and TCO2 is less robust than azide 9. First, the reaction of LAP2 with TCO2 is much slower than that of azide 9, the reaction only showed adduct after 12-hour

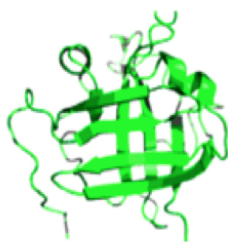
incubation (Figure 1.5 (1) and (2)). Whereas the reaction of azide 9 is already functional at 4 hours. Second, in Figure 1.5 (2), the peak of peptide LAP2 should show up even there is no reaction to conjugate, this means a low robustness of TCO₂-LAP2 ligation system. Thirdly, after 12-hour reaction, in LAP2-azide case the peptide LAP2 was almost consumed by the reaction system, while the LAP2-TCO₂ system still showed with LAP2 peak. The existence of peptide will weaken the stability of the ligated system. Thus in this case, azide 9 would be more ideal for LplA mediated-conjugation system, and we may apply this feature to further use for our recombinant protein platform, hence increase the robustness of nanoparticle and recombinant protein binding capability. To test the exact functional time, we will further make a time-scale setting for example, 10 min as interval, to test the kinetic efficiency of azide 9 ligation.

CHAPTER 2 Site-Specific Bioconjugation Catalyzed by Sortase A

2.1 Background

In recent years, the development of chemical biology has been increasing focusing on efficient and mild methods for chemo-selective ligations and site-specific proteins labeling and modifications. These methods have ability to obtain large amount of post translational modifications and artificial proteins, which could not be acquired by using traditional gene cloning and recombinant protein expression strategy. In late 1990s, expressed protein ligation (EPL) has developed and garnered interest as chemo-selective bioconjugation strategy that allows for site-specific coupling reactions.²¹ EPL refers to a native chemical ligation whereby a recombinant protein with a C-terminal thioester is ligated to a second molecule containing an N-terminal cysteine and the cargo of interest. Recently, a new transpeptidase Sortase A was isolated from *Staphylococcus Aureus*, which can be used to modify proteins bearing a short recognition sequence (most usually as LPXTG, where X represents any amino acid).²² The active-site Cys residue of Sortase A can cleave between LPXT and G residue and catalyzes to produce a thioester intermediate, which can react with a nucleophile containing 1 to 5 Gly to afford the ligation product. (Figure 2.1)

A



Sortase sequence:

```
MQAKPQIPKDKSKVAGYIEIPDADIKEPVYPGPATPEQ  
LNRGVSF AEENESLDDQNISIAGHTFIDRPNYQFTNLK  
AAKKGSMVYFKVGNETRKYKMTSIRDVKPTDVGVLDE  
QKGKDKQLTLITCDDYNEKTGVWEKRKIFVATEVK
```

B

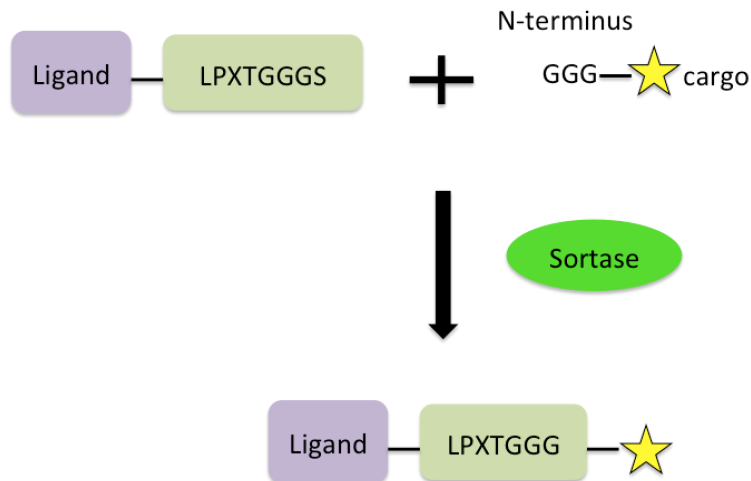


Figure 2.1 (A) The structure representation and sequences of Sortase A.²³ (B) Scheme of Sortase A mediated reaction fusing two peptides.

The Sortase-mediated ligation has been successfully applied to many fields such as C-terminals protein modification, labeling and protein semisynthesis with high efficiency. Compared to traditional chemical modification, Sortase catalyzed semi-chemical synthesis method can preferably address the size problem of protein chemical synthesis, and also applicable for immobilization of proteins to solid surfaces, for cell-surface abelling of living organisms, for protein circularization, and for post-translational incorporation of novel functionality in proteins which cannot be genetically encoded and thus are not accessible via molecular cloning techniques.

Sortase recognizes solvent-exposed LPXTG recognition motifs, proteins with an accessible LPXTG recognition motif can be used as substrates. The target peptides carry one (or more) solvent exposed N-terminal glycine residues, serving as nucleophile. Efficient targets are oligoglycine probes, which may carry C-terminal functional groups, such as aizde and cyclooctyne.²⁴

An LPXTG motif is followed by a hydrophobic domain and a tail of mostly positively charged residues. Highly conserved proline (P) and threonine (T) residues of LPXTG motif are held in position by hydrophobic contacts, whereas the glutamic acid residue (E) at the X position can point out into the solvent. The scissile T-G peptide bond is positioned between the active site 184-Cys and 197-Arg residues of Sortase A, and at a greater distance from the imidazolium side chain of 120-His. In previous study,²⁵ The sortase-tag expressed protein ligation (STEPL) technique has been developed and linked protein purification and conjugation into a single step. A (GGG)₅ linker is attached downside LPETG to give the sortase domain the conformational freedom to recognize the LPETG in a unimolecular reaction. Addition of calcium and any protein/peptide with an N-terminal glycine activates the sortase domain, ligating the protein of interest to peptide while simultaneously cleaving it from the rest of the sortase chimera.

In this study, two peptides LPETG(GGG)₃ and GGGK were used to validate Sortase A enzyme-mediated conjugation, in which GGGK is modified with Fmoc at N-terminus, in case further modify azide or cyclooctyne on C-terminus of K to apply the efficiency of the bioconjugation to nanoparticle delivery platform.

1.2 Production of Sortase A

1.2.1 Protein Expression

In this study, Sortase A was produced from pGMBCS-SrtA (Addgene). Constructs were first transformed into BL21 (DE3) cell line. Inject 1 µL plasmid solution to 50 µL BL21 competent cells, sit on ice for 30 min, then heat shock for 10 s under 42 °C. Transfer the cell mixture to 950 µL SOC, grow in 200 rpm, 37 °C shaker for 1 hour. Centrifuge the cell media

for 1 min under 3,000 rpm, transfer 200 μ L mixture to LB growth plate, culture for overnight. Pick a single colony from the growth plate to 2 mL LB-Ampicillin broth, grow overnight in 200 rpm, 37 °C shaker. Use 1:100 ratio transfer cell mixture to 100 mL LB-Ampicillin and grown to an OD600 of 0.8-1. And then induce with 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Cultures were allowed to express for 5 hours at 37 °C. Cells were then harvested by centrifugation for 15 min at 3,000 rpm, and resuspended in 2 mL lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH7.5) with 40 μ L protease inhibitor. Lysates were then sonicated and separated by centrifugation under 4 °C, 13,000 rpm for 10 min.

1.2.2 Protein Purification

Transfer the supernatant from last step to a clean vial, then Sortase-His6 was purified from supernatant using Ni-NTA (GE Healthcare Life Science). Invert and shake the His SpinTrap column repeatedly to resuspend the medium, place the column in a 2 mL microcentrifuge tube and centrifuge for 30 s at 100 rcf. Then apply 600 μ L binding buffer (20 mM Tris-HCl, 8 M urea, 500 mM NaCl, 5 mM imidazole, pH 8.0) to the column, centrifuge for 30 s at 100 rcf. The supernatant was then applied to the column 600 μ L each time, centrifuge for 30 s at 100 rcf. Wash with binding buffer and collect with elution buffer (20 mM Tris-HCl, 8 M urea, 500 mM NaCl, 500 mM imidazole, pH 8.0). The purified sortase enzyme was then buffer exchanged to filtered PBS by Amicon Ultra – 0.5 mL centrifugal concentrator. The enzymes were analyzed by 12% SDS-PAGE followed by Coomassie staining (Figure 2.2). The concentration of Sortase enzyme was tested by NanoDrop 2000, the yield reached to 1 mg/mL.

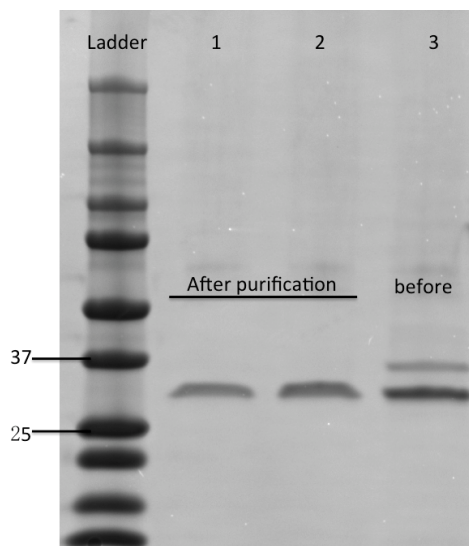


Figure 2.2 Columns (1) and (2) are enzyme Sortase A after purification, the size is shown correct at 29 kDa. Column (3) is enzyme Sortase A before purification.

2.2 Modification of Sortase A substrates

LPETG(GGS)₃ and GGGK were synthesized by Biomatik, where the peptide GGGK is modified with an fmoc modification at N-terminus. Fmoc can thus block with the N-terminus of the first Glycine, to make the N-terminus of Lysine free to be further used for azide or cyclooctyne modification.

In this paper, fmoc is first removed to create an original reaction between LPETG and GGGK. 20% piperidine was applied to 1 mg fmoc-GGGK, avoid the light and react on shaker for 20 min. Weight a clean centrifuge tube, then transfer the fmoc-GGGK with piperidine solution and dry for 6 hours by vacuum. Weight to calculate the compound amount, and add ultrapure water to create 10 mM GGGK solution. 500 uL Ethyl Acetate was added to wash the piperidine-fmoc mixture out, remove the upper layer. Fmoc was thus knocked off and obtain the purified GGGK solution (Figure 2.3).

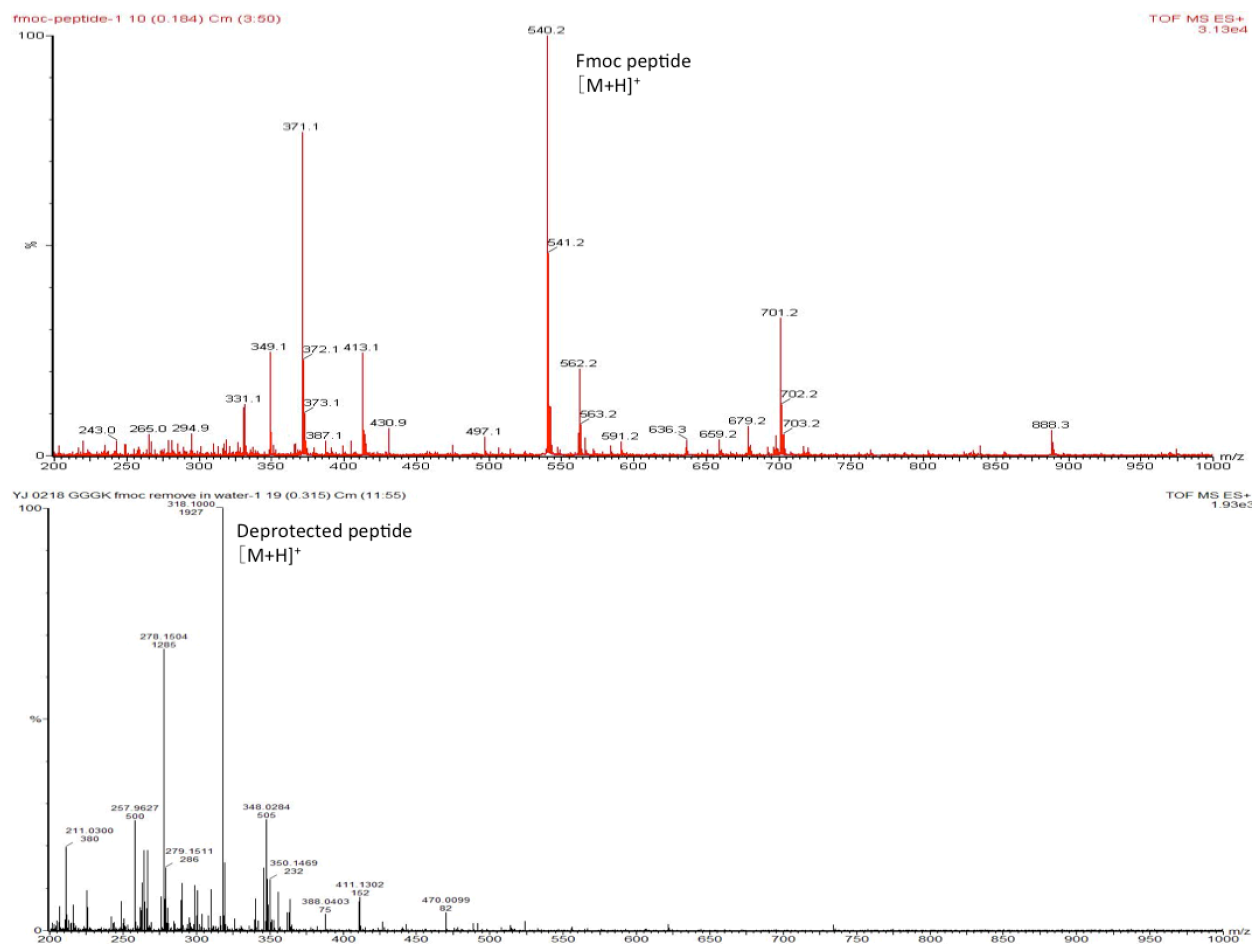


Figure 2.3 Mass spec result of Fmoc-GGGK (MW 540) and GGGK (MW 318).

LPETG(GGS)₃ was prepared for desalting before the reaction by ZipTip (Fisher Scientific). First pipetting 10 μ L wetting solution (100% acetonitrile) into ZipTip, and equilibrate the ZipTip by washing solution (0.1% trifluoroacetic acid in HPLC water). Apply 10 μ L LPETG(GGS)₃ water solution to ZipTip, aspirate and dispense 7-10 cycles to ensure the maximum binding of the sample and filter. Similarly aspirate and dispense appropriate wash solvent to remove contaminants. The concentrated, purified sample is eluted in 5 μ L elution solvent (50% acetonitrile, 0.1% trifluoroacetic acid) and transferred to a mass spectrometer vial to measure the peptide (Figure 2.4).

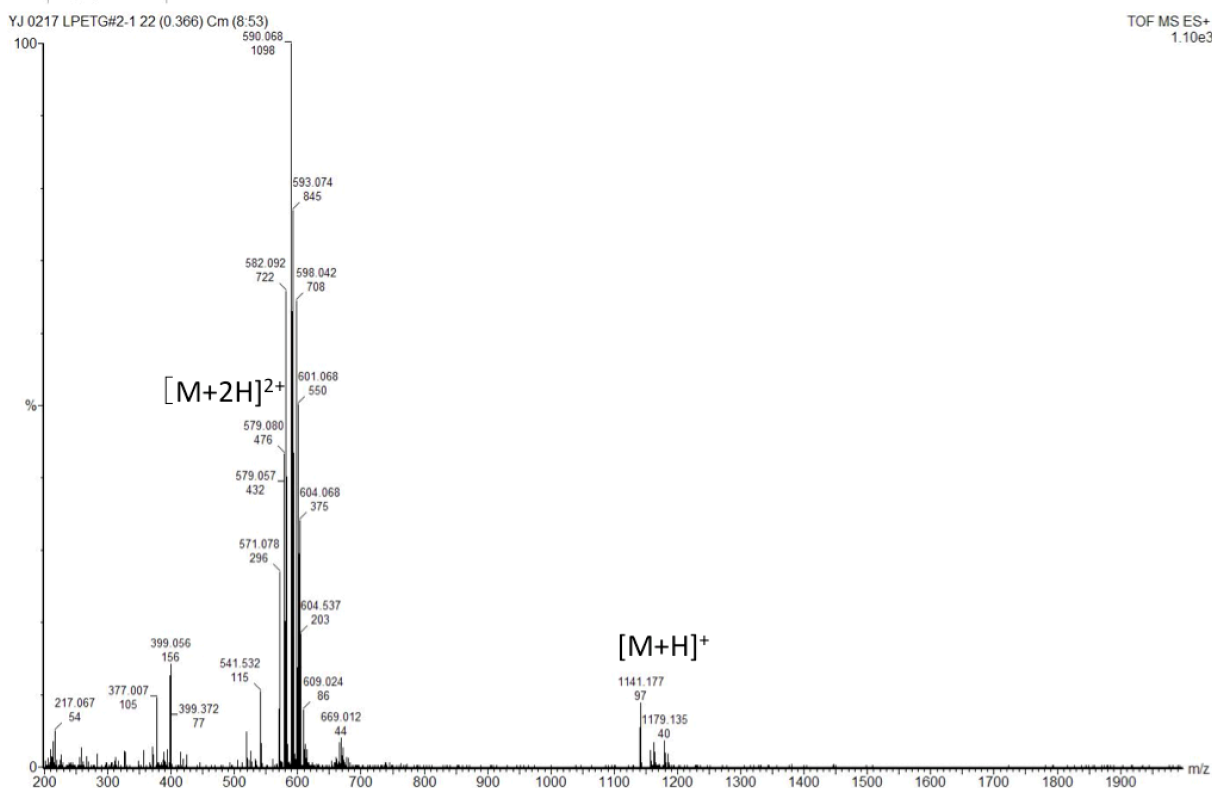


Figure 2.4 The peak about 579 is the mass of double charged LPETG(GGS)₃ plus Na⁺, The peak about 1141 is the mass of single charged LPETG(GGS)₃ plus Na⁺.

2.3 Protein to Protein Ligation

Sortase A mediated protein ligation requires a substrate protein with C Terminal LPET motif and a target protein determined for ligation with 2-3 N-terminal glycine residues. Both termini must be solvent-exposed and must be sterically accessible to Sortase A. The reaction was set in sortase buffer (20 mM Tris-base, 50 mM NaCl, pH 7.5), and since the Sortase A is a calcium-assisted transpeptidase, 100 μM Ca²⁺ was also added in the reaction solution. The reaction conditions were as follow: 100 uM LPETG(GGS)₃, 1 mM GGGK, 5 μM Sortase A enzyme, 100 μM CaCl₂. The mixture was incubated at 37 °C for overnight and protected from light.

The reaction was desalted as described in Chapter 2.2, the purified sample was eluted with elution solvent with matrix (1 mg α -Cyano-4-hydroxy-cinnamic acid dissolved in 50% ACE and 0.1% TFA), and direct transfer to a clean mass spectrometer vial. In this study, Matrix-assisted laser desorption/ionization (MALDI) was used to measure the ligation product. MALDI is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules and large organic molecules, which tend to be fragile and fragment when ionized by more conventional ionization methods. The sample was first applied to a metal plate. Then a pulsed laser irradiated the sample, triggering ablation and desorption of the sample and matrix material. The analyte molecules are ionized and then accelerated into a mass spectrometer, the result was shown in Figure 2.5(B).

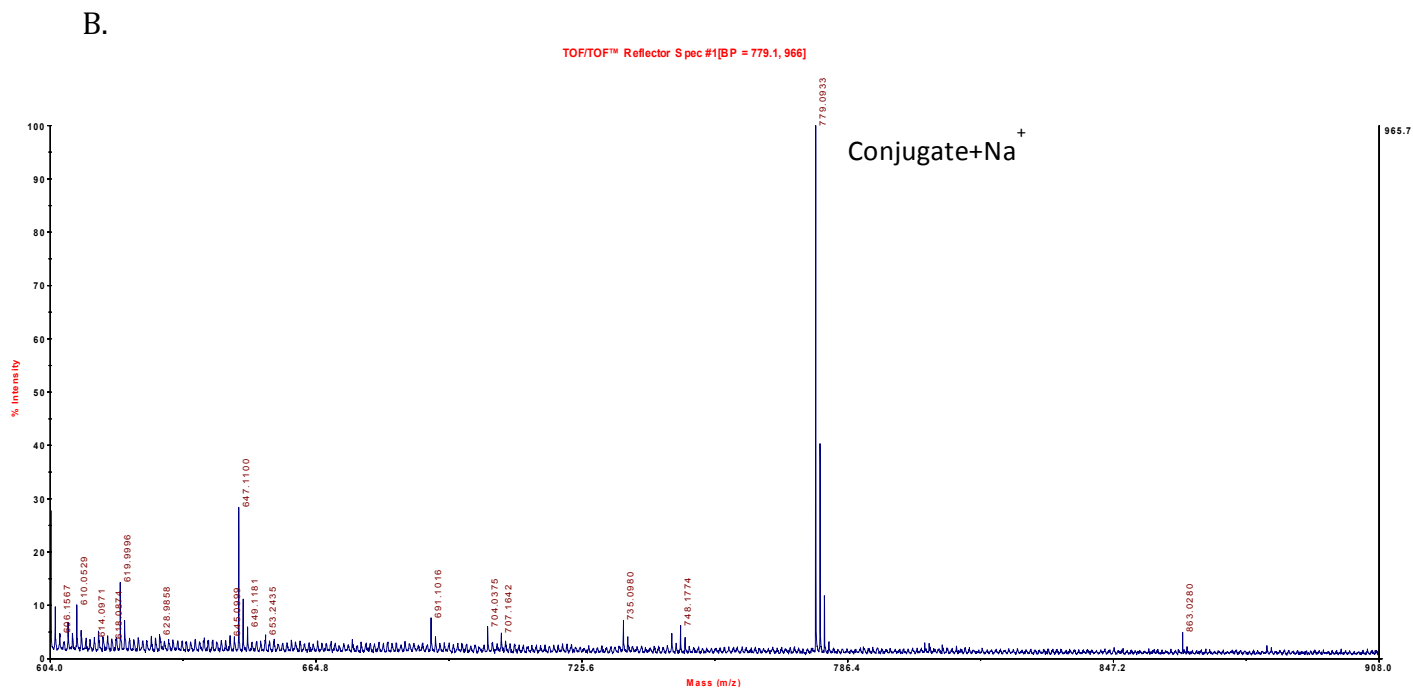
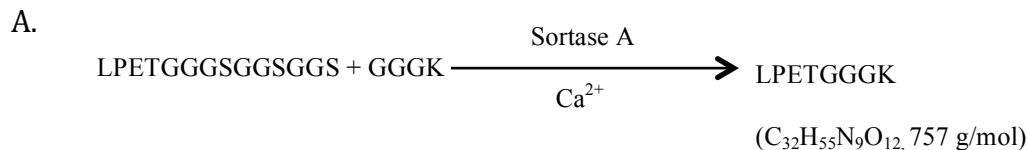


Figure 2.5 (A) Sortase A-mediated reaction scheme. (B) MALDI-TOF result of ligated conjugate

As Figure 2.5 (A) shown, the enzyme Sortase A cleaves the peptide bond between the amino acids threonine and glycine, within the motif LPETG, the product remains transiently attached through the active cysteine residue of Sortase A, until the N-terminal glycine from GGGK displaces the cysteine residue and forms a new peptide bond between the two peptide chains. The new peptide conjugate has a molar mass of 757 g/mol, if this conjugate plus Sodium, the mass is about 780 g/mol, which has been shown in Figure 2.5 (B). This result indicates that our production Sortase A can work to make site-specific bioconjugation of two peptides happen, which can be a very favorable and promising approach for bioconjugation reactions. The site-specific functionalization can be applied for

protein-drug conjugates, as it allows for a conjugation of cargo to attach to targeting ligands. Besides, the site-specific attachment of targeting ligands or proteins can be applied for nanoparticles by such a reaction, which would provide a higher efficiency than randomly labeled targeting ligands.

CHAPTER 3 Recombinant plasmid construction of inserted domain

3.1 Background

Integrins are a family of noncovalently associated, $\alpha\beta$ heterodimeric transmembrane molecules that mediate cell-cell and cell-extracellular matrix adhesion. Lymphocyte function-associated antigen-1 (LFA-1, $\alpha L\beta 2$) is an integrin that is critically important in antigen-specific responses and homing by lymphocytes and together with other $\beta 2$ integrins in diapedesis by monocytes and neutrophils at inflammatory sites.²⁶⁻²⁷ $\alpha L\beta 2$ recognizes intercellular adhesion molecules (ICAMs), of which ICAM-1 is the most biologically important.²⁸

Although the extracellular domains of αL and $\beta 2$ are each large and structurally complex, the ligand binding site of ICAM-1 is contained solely within the 180 residue inserted domain (I domain) of αL .²⁹ Crystal structures of integrin I domains reveal a dinucleotide binding or Rossmann fold, with a central hydrophobic β sheet surrounded by seven amphipathic α helix.³⁰

Allostery is important in many signaling proteins, In the I domain, present in some integrin α subunits, allosteric activation pathways have been studied structurally and functionally.³¹⁻³³ Several researches have described evolutionary mutants applied to the integrin αL I domain for studying allostery by using yeast surface display systems.³⁴⁻³⁵ Many hot spots for activation are identified, and some single mutants exhibit remarkable increases of 10, 000-fold in affinity for ICAM-1. The location of activating mutations traces out an allosteric interface in the interior of the inserted domain that connects the ligand binding site to the α helix. The activating mutation F265S led to a 1,000 fold decrease in dissociation rate and a 10,000-fold increase in affinity to ICAM-1, in which the slower

association rate of this mutant may indicate some subtle improvements in the ligand-bound structures, and as a consequence, a lower free energy of the complex. The combination of point mutations of K287C and K294C, each of which were mutated to stabilize the closed conformations of the I domain respectively, were also determined for high-affinity.³⁵

In this study, we cloned wild-type I domain, F265S mutant I domain and K287C/K294C mutant I domain, which are received as a gift from Dr. Eric Boder. The peptide sequences are shown with the secondary-structure assignment (Figure 3.1).

1.

```

1 CIKGNVDLVFLFDGSMQLQP
21 DEFQKILDFMKDVMKCLSNT
41 SYQFAAVQFSTSYKTEFDFFS
61 DYVKWKDPDALLKHVKHMLL
81 LTNTFGAINYVATEVFREEL
101 GARPDATKVLIIITDGEATD
121 SGNIDAAKDIIRYIIGIGKH
141 FQTKESQETLHKFASKPASE
161 FVKILDTFEKLKDLFTELQK
181 KIYVIEG

```

2.

```

1 NVDLVFLFDGSMQLQPDEFQ
21 KILDFMKDVMKCLSNTSYQF
41 AAVQFSTSYKTEFDFFSDYVK
61 WKDPDALLKHVKHMLLLTNT
81 FGAINYVATEVFREELGARP
101 DATKVLIIITDGEATDSGNI
121 DAAKDIIRYIIGIGKH[S]QTK
141 ESQETLHKFASKPASEFVKI
161 LDTFEKLKDLFTELQKKIY

```

3.

```

GNVDLVFLFDGSMQLQPDEF
QKILDFMKDVMKCLSNTSYQ
FAAVQFSTSYKTEFDFFSDYV
KWKDPDALLKHVKHMLLLTN
TFGAINYVATEVFREELGAR
PDATKVLIIITDGEATDSGN
IDAAKDIIRYIIGIGKHFQT
KESQETLHKFASKPASEFV[C]
ILDTFE[C]LKDLFTELQKKIY

```

Figure 3.1 (1) Wild-type I domain sequence is shown from residue 125-311; (2) Single mutant F265S sequence is shown from residue 129-307, in which the 265th amino acid mutated from Phenylalanine (F) to Serine (S) (highlight in red); (3) Combination mutants K287C/K294C sequence is shown from residue 128-307, in which the 287th and 294th amino acids mutated from Lysine (K) to Cysteine (C)(highlight in red).

3.2 Materials and Methods

3.2.1 Reagents, Materials and Kits

All materials and reagents used in this paper were listed in table 3.1

Table 3.1 Reagents, materials and kits applied in plasmid construction

Reagents	Company
Restriction enzyme NheI	New England Biolabs
Restriction enzyme XhoI	New England Biolabs
Restriction enzyme NheI	New England Biolabs
Restriction enzyme NheI	New England Biolabs
100 bp DNA ladder	New England Biolabs
1 kb DNA ladder	New England Biolabs
T4 DNA ligase	New England Biolabs
Vent DNA polymerase	New England Biolabs
SOC outgrowth medium	New England Biolabs
Gel loading dye	New England Biolabs
Agarose	Invitrogen
TAE	Invitrogen
Ethidium bromide	Invitrogen
LB Broth Base	Invitrogen
LB Agar powder	Invitrogen
ampicillin	Invitrogen
PCR tubes	Fisher Scientific
Razor blades	Fisher Scientific
QIAquick Gel Extraction Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen

3.2.2 Strains and Media

Escherichia coli strain DH5 α (New England Biolabs) was used for recombinant plasmids gene cloning. Luria-Bertani (LB) medium (10.0 g/L tryptone, 5.0 g/L yeast extract,

10.0 g/L NaCl, pH7.5, supplemented with 50 mg/ml ampicillin) was used for bacteria growth and plasmid amplification. Luria-Bertani (LB) agar plate (10.0g/L peptone, 5.0g/L yeast extract, 5.0g/L NaCl, 12.0g/L agar, supplemented with 50 mg/ml ampicillin) was used for bacteria growth and selection.

3.2.3 Primer and Plasmid Design

Three I domain sequences (wild-type, F265S mutant, K287C/K294C) were amplified by PCR using sense (GAGGAGGCTAGCGGCAACGTAGACCTGGTATTTTC) and antisense (CTCCTCACGCGTATAGATCTTCTTCTGCAGCTCAGT) primers, which introduced *Nhe1* and *Mlu1* restriction sites (underlined) at the 5' and 3' ends, respectively, to facilitate subcloning. All three I domain sequences were similarly amplified and inserted into previous pCT-4420 plasmid used for yeast surface display (Figure 4, Figure 3.2) and pRS314-based expression vector for yeast secretion. The expected pCT-wt I domain and pRS-wt I domain plasmid constructs are predicted in Figure 3.3.

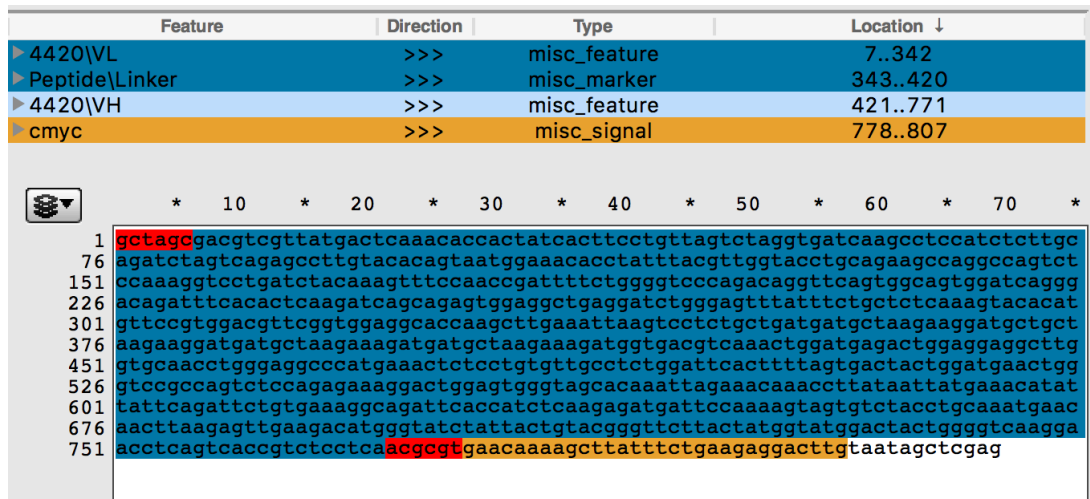


Figure 3.2 Insert sequence of pCT-4420. Forward restriction site *Nhe1* and reverse restriction site *Mlu1* are in highlight red.

A.



B.

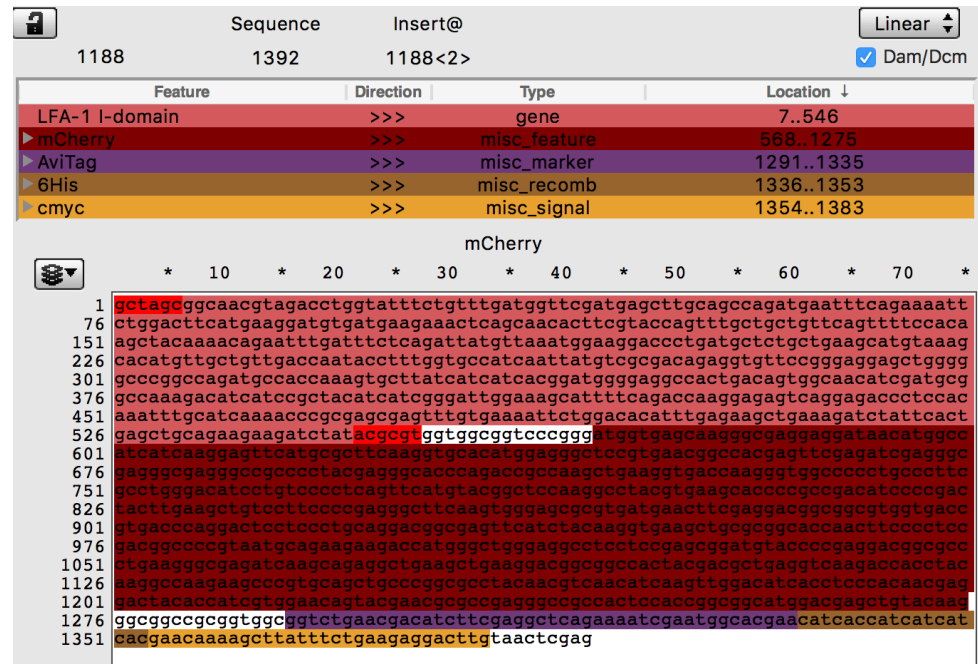


Figure 3.3 (A) Inserted sequence in pCT-wt I domain. (B) Construct of inserted sequence wt I domain-mCherry-AviTag-6His-cmyc. The introduced two restriction sites *Nhe1* and *Mlu1* are highlighted in red.

PCR product was resolved by electrophoresis on a 1% agarose gel, and all products approximately 560 bp in size were extracted. Digested on both insert DNA and vector DNA extraction using *Nhe1* and *Mlu1* restriction enzymes at 37 °C. NEBuffer 2.1 was applied in digestion reaction system to keep >50% activity of the enzymes to achieve maximal success in the restriction endonuclease reactions. 1 ng DNA fragments were added in the reaction, mixed with 1 unit restriction enzymes and 10X NEBuffer, ultrapure water was

added to reach the total volume 50 μ L reaction. The reaction mixture was incubated in 37 $^{\circ}$ C for 12 hours.

T4 DNA Ligase was used for ligating insert DNA into pCT and pRS cloning vector by catalyzing the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini. The restriction enzyme *Nhe1* and *Mlu1* will create sticky ends during digestion, and T4 ligase will joint the two cohesive ends in duplex DNA (Figure 3.4). The ligation reaction required a molar ratio 3:1 of inserts and vectors, used 1 μ L of T4 ligase in a 20 μ L reaction for 4 hours under room temperature.

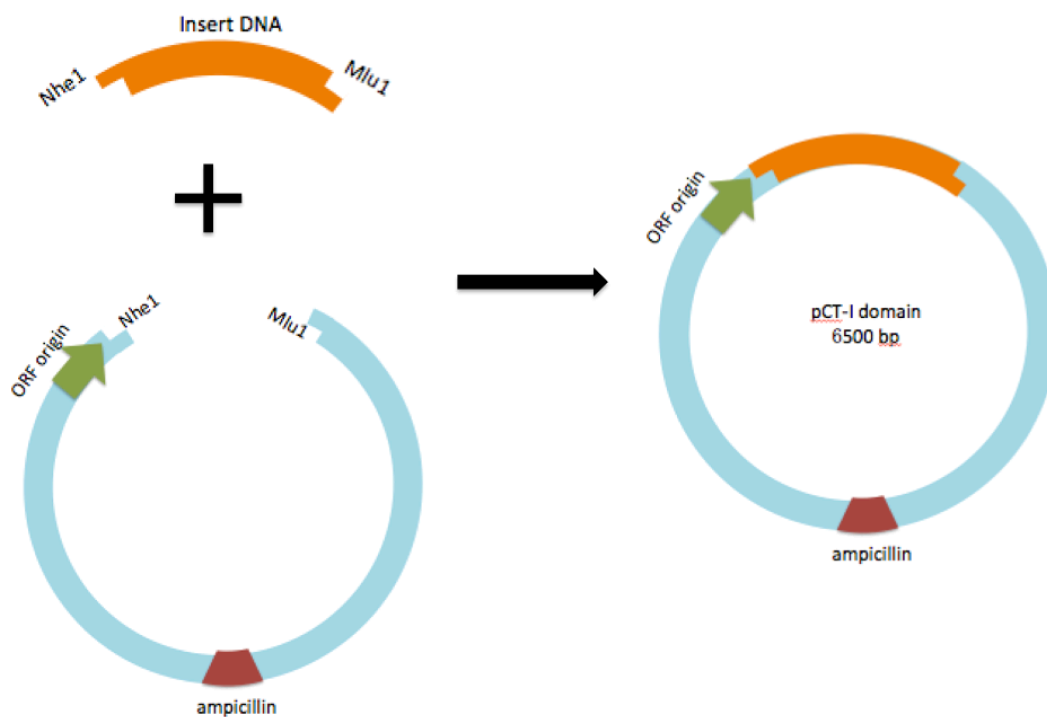


Figure 3.4 Ligation model

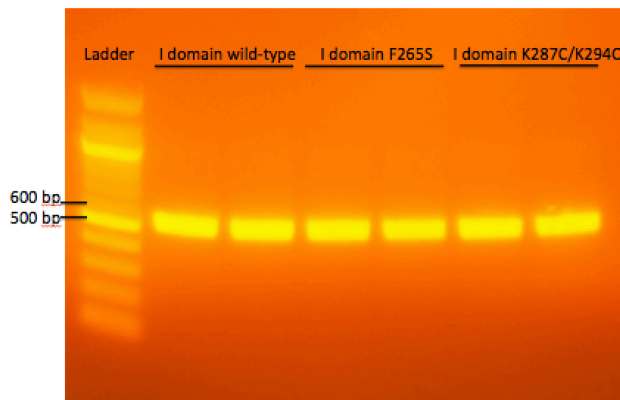
Thereafter, the complete plasmids were transformed into *E. coli* strain DH5 α by heat shock. 2 μ L plasmid was added to 50 μ L DH5 α cells on ice. After 30 min, the DH5 α cells were heat shocked for 30 sec at 42 $^{\circ}$ C, and immediately placed back on ice for 5 min, followed by addition of 0.95 ml SOC media (BioLabs). The cells were incubated at 37 $^{\circ}$ C for

1 hour and then centrifuged at 13,000 x RPM for 1 min. 800 μ L of the supernatant was discarded, while the cells were resuspended in the rest 200 μ L media and all plated onto an LB-Ampicillin (LB-Amp) plate. The cells were grown overnight at 37 $^{\circ}$ C and the ampicillin resistant transformants were selected the after 16 hours. The products were then confirmed by DNA sequencing (GENEWIZ).

3.3 Results

According to Figure 13, the wild-type I domain sequence is about 540 bp, and we applied same primers for all three types I domain, thus the mutant F265S and K287/K294C were same length with wild-type. After restriction enzymes digestion for both inserts and plasmids for overnight at 37 $^{\circ}$ C, the electrophoresis agarose gel is shown in Figure 3.5.

A.



B.

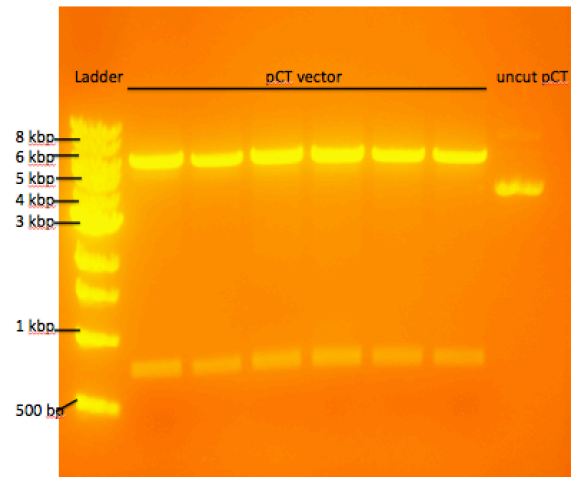


Figure 3.5 Gel results of digestion for (A) all 3 types I domain; (B) pCT vector.

According to Figure 3.2, the insert 4420 is about 750 bp, in Figure 3.5(B), there are bands between 500 bp to 1k bp, which are the insert 4420 cut off from the plasmid. The

uncut pCT in Figure 3.5(B) is way below the cut pCT, this is because the circular plasmid is usually running faster than linear ones.

The digested I domains and pCT backbones were collected by gel extraction kit (QIAGEN) and concentrations were tested by Nanodrop 2000 (Thermo Scientific).

Table 3.2 Concentrations of digested inserts and vectors

I domains	Concentration	pCT	Concentration
Wild-type	13.3 ng/ μ L	No.1	8.8 ng/ μ L
F265S	15.9 ng/ μ L	No.2	12.2 ng/ μ L
K287C/K294C	9.2 ng/ μ L	No.3	8 ng/ μ L

The collected fragments were then ligated using the molar ratio 3:1 for inserts and vectors, the ligation systems are shown in Table 3.3.

Table 3.3 Ligation reaction system

I domain wild type		I domain F265S		I domain K287C/K294C	
Insert	3 μ L	Insert	2 μ L	Insert	3 μ L
Vector	14 μ L	Vector	15 μ L	Vector	14 μ L
T4 ligase	1 μ L	T4 ligase	1 μ L	T4 ligase	1 μ L
T4 buffer	2 μ L	T4 buffer	2 μ L	T4 buffer	2 μ L
Total volume 20 μ L		Total volume 20 μ L		Total volume 20 μ L	

The fragments were ligated follow the scheme in Figure 8 for 4 hours under room temperature. Then *E.coli* DH5 α was transformed with ligated plasmids by heat shock. and plated on LB plates (ampicillin) as described in Appendix A. After 16 hours growth, colonies shown in plates were mostly with resistance of ampicillin, pick 6-10 colonies to run colony PCR (Table 3.4) using the primers in section 3.2.3. Each single colony was scraped using a scrapper ring and dipped into 50 μ L ultrapure water. The colony

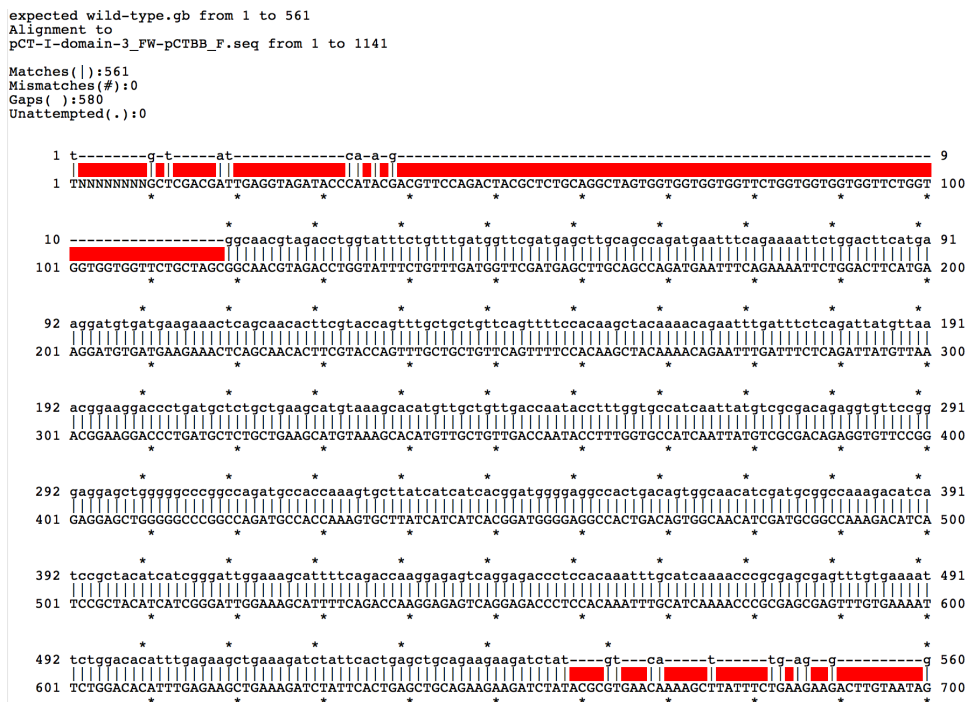
suspension can be stored in 4 °C for one week. Result from colony PCR was checked by 1% agarose gel.

Table 3.4 Colony PCR reaction system

Thermopol buffer	2.5 µL
dNTP	0.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
Cell suspension	5 µL
Vent polymerase	0.5 µL
Ultrapure water	15.5 µL
Total reaction volume 25 µL	

The colony suspensions with positive results were replated onto new LB-Amp plates and minipreped for storage and sequencing. The correction of inserts wild-type I domain, F265S I domain, and K287C/K294C I domain were confirmed by DNA sequencing (Figure 3.6).

A.



B.

```
expected F265S.gb from 1 to 537
Alignment to
pCT-I-domain-2_FW-pCTBB_F.seq from 1 to 1110
Matches( ):537
Mismatches(#):0
Gaps( ):573
Unattempted(.):0

1 ----- 1
1 TNNNNNNNGCTCGACGATTGAGGTAGATACCCATACGACGTTCCAGACTACGCTTCGAGGCTAGTNGGTGGTGGTTCGGTGGTTCCTCG 100
  * * * * *
1 -----aacgtagacctggtattctgtttgatggttcgatgagcttgcagccagatgaatttcagaaaaattctggactcatg 78
201 TGGTGGTGGTTCCTGCTAGCGCAACCTAGACCTGGTATTCTGTGTTGATGGTTCGATGAGCTTGCAGCCAGATGAATTCAGAAAATTCGGACTTCATG 200
  * * * * *
79 aaggatgtgatgaagaactcagcaaacacttctaccagtttgctgctgttcagttttccacaagctacaaaacagaatttgatttctcagattatgta 178
201 AAGGATGTGATGAAGAAACTCAGCAACACTTCGTACCAAGTTTGTCTGTTCAGTTTCCACAAGCTACAAAACAGAAATTGATTTCTCAGATTATGTTA 300
  * * * * *
179 aacggaagaccctgatgctctgctgaagcatgtaaacacatggtgctgttgaccaatacctttggtccatcaattatgctcgacagaggtgttccg 278
301 AACGGAAGACCCTGATGCTCTGCTGAAGCATGTAAAGCACATGTTGCTGTTGACCAATACCTTTGGTGCCATCAATATATGTCGCGACAGAGGTGTCCG 400
  * * * * *
279 ggaggagctggggcccgccagatgccaccaagtgttatcatcatcacggatggggaggccactgacagtggcaacatcgatgcgcccaagacatc 378
401 GGAGGAGCTGGGGCCCGCCAGATGCCCAAAGTGCTTATCATCATCAGGATGGGAGGCCACTGACAGTGGCAACATCGATGCGGCCAAAGACATC 500
  * * * * *
379 atccgctacatcatcggttggaaagcatAGtcagaccaaggagatcaggagaccctccacaaattgcatcaaaaccgagcagattttgtgtaaaa 478
501 ATCCGCTACATCATCGGGATTGGAAGCATAGTCAGACCAAGGAGAGTCAGGAGACCCTCCACAAATTTGCATCAAAACCCGAGCGAGTTTGTGTA 600
  * * * * *
479 ttctggacacatttgagaagctgaaagatctattcactgagctgcagaagaagatctat----- 537
601 TTCTGGACACATTTGAGAAGCTGAAAGATCTATTCACTGAGCTGCAGAAGAAGATCTATACGCGTGAACAAAAGCTTATTCTGAAGAAGACTTGTAA 700
  * * * * *
```

C.

```
expected K287C:294C.gb from 1 to 540
Alignment to
pCT-I-domain-4_FW-pCTBB_F.seq from 1 to 1136
Matches( ):540
Mismatches(#):0
Gaps( ):596
Unattempted(.):0

1 ----- 1
1 TNNNNNNNGCTCGACGATTGAGNTAGATACCCATACGACGTTCCAGACTACGCTTCGAGGCTAGTNGGTGGTGGTTCGGTGGTTCCTCG 100
  * * * * *
1 -----ggcaacctagacctggtattctgtttgatggttcgatgagcttgcagccagatgaatttcagaaaaattctggactcatg 81
201 TGGTGGTGGTTCCTGCTAGCGCAACCTAGACCTGGTATTCTGTGTTGATGGTTCGATGAGCTTGCAGCCAGATGAATTCAGAAAATTCGGACTTCATG 200
  * * * * *
82 aaggatgtgatgaagaactcagcaaacacttctaccagtttgctgctgttcagttttccacaagctacaaaacagaatttgatttctcagattatgta 181
201 AAGGATGTGATGAAGAAACTCAGCAACACTTCGTACCAAGTTTGTCTGTTCAGTTTCCACAAGCTACAAAACAGAAATTGATTTCTCAGATTATGTTA 300
  * * * * *
182 aacggaagaccctgatgctctgctgaagcatgtaaacacatggtgctgttgaccaatacctttggtccatcaattatgctcgacagaggtgttccg 281
301 AACGGAAGACCCTGATGCTCTGCTGAAGCATGTAAAGCACATGTTGCTGTTGACCAATACCTTTGGTGCCATCAATATATGTCGCGACAGAGGTGTCCG 400
  * * * * *
282 ggaggagctggggcccgccagatgccaccaagtgttatcatcatcacggatggggaggccactgacagtggcaacatcgatgcgcccaagacatc 381
401 GGAGGAGCTGGGGCCCGCCAGATGCCCAAAGTGCTTATCATCATCAGGATGGGAGGCCACTGACAGTGGCAACATCGATGCGGCCAAAGACATC 500
  * * * * *
382 atccgctacatcatcggttggaaagcattttcagaccaaggagatcaggagaccctccacaaattgcatcaaaaccgagcagattttgtgta 481
501 ATCCGCTACATCATCGGGATTGGAAGCATTTTCAGACCAAGGAGAGTCAGGAGACCCTCCACAAATTTGCATCAAAACCCGAGCGAGTTTGTGTA 600
  * * * * *
482 ttctggacacatttgagTGTctgaaagatctattcactgagctgcagaagaagatctat----- 540
601 TTCTGGACACATTTGAGTGTCTGAAAGATCTATTCACTGAGCTGCAGAAGAAGATCTATACGCGTGAACAAAAGCTTATTCTGAAGAAGACTTGTAA 700
  * * * * *
```

Figure 3.6 Sequencing results for (A) wild-type I domain, (B) F265S I domain, and (C) K287C/K294C I domain.

The results showed, all the base groups paired successfully, thus wild-type I domain, F265S I domain and K287C/K294C I domain have been inserted in pCT and pRS, the

recombinant plasmids have been confirmed to be structured. The confirmed colonies were picked into LB-Amp broth to culture overnight, and stored with 50X glycerol under -80 °C. Both stored cell culture and clonal population will be further used for making protein production and testing binding assay.

CHAPTER 4 Yeast Surface Display of Protein Expression

4.1 Overview

Many platforms are available for the construction of peptide and antibody libraries, allowing directed functional genomics studies. Yeast surface display is well suited to engineer extracellular eukaryotic proteins such as antibody fragments, cytokines, and receptor ectodomains. A further advantage of yeast surface display is that soluble ligand-binding kinetics and equilibria may be measured in the display format, and as a result quantitatively optimized screening protocol may be designed. Using such optimal screening conditions, numerous mutants with small improvements may be finely discriminated with high statistical certainty, and further recombination may be used to achieve greater improvements.³⁶⁻³⁷

A given protein may be displayed on the surface of yeast by expression as a protein fusion to the Aga2p mating agglutinin protein. We have constructed the pCT302 plasmid in previous study (Figure 4.1) for expression of such fusions under control of the GAL1-10 galactose-inducible promoter. Because the Aga2p protein is tethered in the cell wall via disulfide bridges to the Aga1p protein, we have constructed yeast strain EBY100, in which Aga1p expression is inducible from a single, integrated open reading frame (ORF) downstream of the GAL1-10 promoter.

The presence of the fusion protein on the cell surface may be detected independently of its ligand-binding activity by immunofluorescent labeling of epitope tags. Quantitative detection of fusion polypeptide surface levels via epitope tag labeling is important to normalize for random variation in surface expression levels when measuring ligand-binding kinetics and equilibria. Two epitope tags are used in the fusion, one N

terminal (hemagglutinin, HA) and one C terminal (c-myc) to the protein of interest. Both tags are of value: immunofluorescent detection of the c-myc epitope on the cell surface confirms the presence of the full-length polypeptide, and the quality control apparatus of the endoplasmic reticulum provides high confidence that the protein on the surface is correctly folded. The HA tag serve as a useful internal control should the fusion be displayed in a partially proteolyzed form.³⁸⁻³⁹

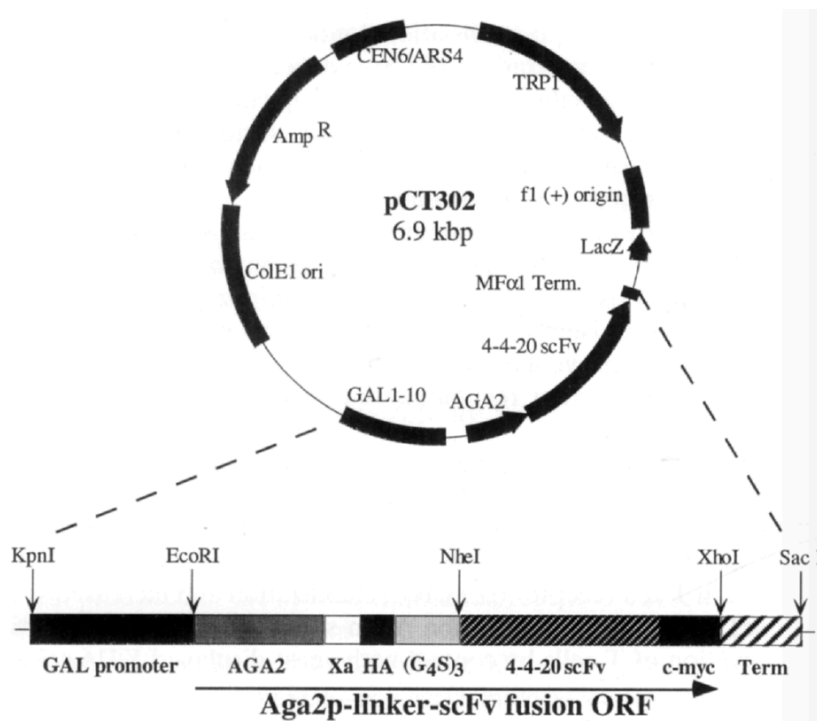


Figure 4.1.⁴⁰ Map of plasmid pCT302 for expression of Aga2p fusion proteins. In this paper, the scFv 4-4-20 is replacing of all three types of I domains as protein of interest.

4.2 Materials and Methods

4.2.1 Reagents

Lithium Acetate (1.0 M) was made by dissolving 5.1 g of lithium acetate dehydrate in 50 mL water, sterilized by autoclaving for 15 min, and stored at room temperature. Polyethylene Glycol 3350 (50% w/v) was made by dissolving 50 g of PEG 3350 in 30 mL

distilled, deionized water. Single-stranded carrier DNA (2.0 mg/mL) was made by dissolving 200 mg of salmon sperm DNA in 100 mL TE buffer (10 mM Tris-HCl, 1 mM Na₂EDTA, pH 8.0), stirring at 4 ° for 1-2 hr. Store 1.0 mL samples at -20 °. Denature the carrier DNA in a boiling water bath for 5 min and chill in ice/water before use.

4.2.2 Strains and Media

Saccharomyces cerevisiae strain EBY100 containing an integrated copy of Aga1p under GAL1-10 promoter control was used for cell surface display of the tree types of I domains. EBY100 yeast were grown in complex media YPD (10.0 g/L yeast extract, 20.0 g/L peptone, 20.0 g/L dextrose) and minimal SD-CAA medium (20.0 g/L dextrose, 6.7 g/L yeast nitrogen base, 5.0 g/L casamino acids, 5.4 g/L Na₂HPO₄, 7.46 g/L NaH₂PO₄). Expression of scFv protein was induced using SG-CAA medium (20.0 g/L galactose instead of dextrose), which was supplemented with bovine serum albumin (BSA) at 1 mg/L as a nonspecific carrier.

4.2.3 Transformation

The introduction of exogenous DNA into yeast by transformation has become an essential technique in molecular biology. Intact yeast cells can be transformed by a number of procedures: the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/SS-Carrier DNA/PEG) method,⁴¹ electroporation,⁴² agitation with glass beads,⁴³ and bombardment with DNA-coated microprojectiles.⁴⁴ In this paper, we conduct the transformation of yeast cells by the LiAc/SS-Carrier DNA/PEG method since it is the most widely applicable.

Incubated the yeast strain in 3 mL YPD with 2 μ L EBY 100 and grown overnight. Spin the overnight cultured yeast at 3000 rpm for 1 min, discard the supernatant, and resuspend in 1 mL ultrapure water and transfer to a microcentrifuge tube. Pellet cells at 13,000 rpm and discard the supernatant. Add the following components to the cell pellet in the order listed:

Table 7. Components list for yeast transformation

Component	Volume (μ L)
PEG 3350 (50%, w/v)	240
Lithium acetate 1.0 M	36
Boiled SS-Carrier DNA (2 mg/mL)	50
Plasmid DNA (1 μ g) plus water	34

Vortex to mix, and resuspend the cells by pipetting. Incubate the mixture at 42 °C for 1hr, and then centrifuge at 3000 rpm, aspirate the transformation mix with a micropipettor. Add in 1 mL ultrapure water, resuspend the cell by stirring with a micropipette tip and then plate 100 μ L on SDCAA plates. Incubate at 30 °C, check the plate after 3-4 days, pick one single colony and stripe onto a new plate to create a clonal population. Store in 4 °C for future use.

4.3 Yeast surface display

The Aga2p protein fusion is expressed on the cell surface by induction in galactose-containing medium. From SDCAA (containing 1 M sorbitol) plates, EBY100 transformants colonies were picked in 3 mL liquid SDCAA culture, inoculated and grown at 30 °C for 12 hours. Culture grown to stationary phase ($OD_{600} >10$) may be induced for surface expression, the stationary-phase liquid cultures may be reprepared for optimal surface

display by pelleting cells, resuspending in fresh SD medium, and grown for 6-10 hours at 30 °C. The culture was centrifuged for 1 min at 3,000 rpm, and resuspended in SGCAA media, which is identical to SD media except that glucose is replaced by galactose as the carbon source. Surface expression cultures were inducted at 30 °C for 20-24 hours.

CHAPTER 5 Summary and Conclusions

To optimize the adhesion of therapeutic nanoparticle system, I domain wild-type, I domain mutant F265S, and I domain mutant K287C/294C were introduced to yeast recombinant protein platforms and developed as targeting receptors of nanoparticles. The structure of recombinant plasmids was confirmed by sequencing. And the protein of I domain recombinant platform was produced by transforming it into *Saccharomyces cerevisiae* strain EBY100. Based on the conformation, I domain and its mutants will provide an increased affinity for protein or ligand and that disulfide bond compared to other nanoparticle receptors.²⁹

In the meanwhile, lipoic acid ligase (LplA) and Sortase A enzyme-mediated conjugation system were primarily validated by ligation reaction test in this study. First we successfully produced LplA and Sortase A enzyme in a 100 mL scale by introducing the plasmids into *E. coli* BL 21 (DE3) cell line and IPTG induction, and the production yield was able to reach a relatively high concentration, 1 mg/mL. And then we demonstrated a conjugation of LAP2 to azide-9 and TCO2 via LplA catalyzed mechanism, and a ligation between two single peptides LPETG and GGGK via Sortase A cleavage and mediation, where the lysine in GGGK will be further modified with azide or TCO at the N-terminus. Thereafter, new targeting protein platform pCT-I domain-mCherry-LAP2-His₆ and pCT-I domain-mCherry-LPETG-His₆ were designed to achieve a higher efficacy of nanoparticle delivery system. The nanoparticles modified with azide coat or TCO coat can conjugate with the receptor proteins above through LAP2-TCO, LAP2-azide, LPETGGGK-TCO and LPETGGGK-azide ligation by enzyme LplA and Sortase A.

To further achieve the optimization of the adhesion between nanoparticles and protein platform, the enzyme ligation reaction will be set for a time scale from 1 hour to 4 hours, gradually decreasing the time intervals to find a desired reaction time, which can consume the substrate completely to make a maximum extent of reaction. The ZipTip method of desalting can also be applied to LplA system to create a robust and stable mass spectrometer test sample.

Moreover, the transformed yeast pCT proteins will be further measured on binding with CHO-ICAM1 and CHO-K1 cells to confirm the I domains working capacity, then make pRS-I domain-mCherry-LAP2-His₆ and pRS-I domain-mCherry-LPETG-His₆ platform yeast secretion from successful recombinant proteins to achieve a scaled-up production for targeted delivery of nanoparticle therapies.

Reference

1. Muller, Rainer H., and Cornelia M. Keck. "Challenges and solutions for the delivery of biotech drugs—a review of drug nanocrystal technology and lipid nanoparticles." *Journal of biotechnology* 113.1 (2004): 151-170.
2. Cho, Kwangjae, et al. "Therapeutic nanoparticles for drug delivery in cancer." *Clinical cancer research* 14.5 (2008): 1310-1316.
3. Gatter, KEVIN C., et al. "Transferrin receptors in human tissues: their distribution and possible clinical relevance." *Journal of clinical pathology* 36.5 (1983): 539-545.
4. Allen, Theresa M. "Ligand-targeted therapeutics in anticancer therapy." *Nature Reviews Cancer* 2.10 (2002): 750-763.
5. Danhier, Fabienne, Olivier Feron, and Véronique Pr at. "To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery." *Journal of Controlled Release* 148.2 (2010): 135-146.
6. Davis, Mark E., and Dong M. Shin. "Nanoparticle therapeutics: an emerging treatment modality for cancer." *Nature reviews Drug discovery* 7.9 (2008): 771-782.
7. Carlson, Coby B., et al. "Selective tumor cell targeting using low-affinity, multivalent interactions." *ACS chemical biology* 2.2 (2007): 119-127.
8. Haun, Jered B., et al. "Using engineered single-chain antibodies to correlate molecular binding properties and nanoparticle adhesion dynamics." *Langmuir* 27.22 (2011): 13701-13712.
9. Marks, Kevin M., and Garry P. Nolan. "Chemical labeling strategies for cell biology." *Nature Methods* 3.8 (2006): 591-596.
10. Uttamapinant, Chayasith, et al. "A fluorophore ligase for site-specific protein labeling inside living cells." *Proceedings of the National Academy of Sciences* 107.24 (2010): 10914-10919.
11. Keppler, Antje, et al. "A general method for the covalent labeling of fusion proteins with small molecules in vivo." *Nature biotechnology* 21.1 (2003): 86-89.
12. Los, Georgyi V., et al. "HaloTag: a novel protein labeling technology for cell imaging and protein analysis." *ACS chemical biology* 3.6 (2008): 373-382.

13. Griffin, B. Albert, Stephen R. Adams, and Roger Y. Tsien. "Specific covalent labeling of recombinant protein molecules inside live cells." *Science* 281.5374 (1998): 269-272.
14. Green, D. E., et al. "Purification and properties of the lipoate protein ligase of *Escherichia coli*." *Biochem. J* 309 (1995): 853-862.
15. Liu, Daniel S., et al. "Diels–Alder cycloaddition for fluorophore targeting to specific proteins inside living cells." *Journal of the American Chemical Society* 134.2 (2012): 792-795.
16. Puthenveetil, Sujiet, et al. "Yeast display evolution of a kinetically efficient 13-amino acid substrate for lipoic acid ligase." *Journal of the American Chemical Society* 131.45 (2009): 16430-16438.
17. Uttamapinant, Chayasith, et al. "A fluorophore ligase for site-specific protein labeling inside living cells." *Proceedings of the National Academy of Sciences* 107.24 (2010): 10914-10919.
18. Fernández-Suárez, Marta, et al. "Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes." *Nature biotechnology* 25.12 (2007): 1483-1487.
19. Prescher, Jennifer A., and Carolyn R. Bertozzi. "Chemistry in living systems." *Nature chemical biology* 1.1 (2005): 13-21.
20. Walch, Axel, et al. "Histochemistry and cell biology Volume: 130 ISSN: 0948-6143 ISO Abbreviation: Histochem. Cell Biol. Publication Date: 2008 Sep." *Detail*.
21. Muir, Tom W., Dolan Sondhi, and Philip A. Cole. "Expressed protein ligation: a general method for protein engineering." *Proceedings of the National Academy of Sciences* 95.12 (1998): 6705-6710.
22. Leung, Melissa KM, et al. "Bio-Click Chemistry: Enzymatic Functionalization of PEGylated Capsules for Targeting Applications." *Angewandte Chemie* 124.29 (2012): 7244-7248.
23. Xianglong, Tan, et al. "Applications of Transpeptidase Sortase A for Protein Modifications." *PROGRESS IN CHEMISTRY* 26.10 (2014): 1741-1751.
24. Wagner, Koen, et al. "Bispecific antibody generated with sortase and click chemistry has broad antiinfluenza virus activity." *Proceedings of the National Academy of Sciences* 111.47 (2014): 16820-16825.

25. Warden-Rothman, Robert, et al. "Sortase-tag expressed protein ligation: combining protein purification and site-specific bioconjugation into a single step." *Analytical chemistry* 85.22 (2013): 11090-11097.
26. Gahmberg, Carl G. "Leukocyte adhesion: CD11/CD18 integrins and intercellular adhesion molecules." *Current opinion in cell biology* 9.5 (1997): 643-650.
27. Larson, Richard S., and Timothy A. Springer. "Structure and function of leukocyte integrins." *Immunological reviews* 114.1 (1990): 181-217.
28. Dustin, M. L., and T. A. Springer. "Lymphocyte function associated-1 (LFA-1, CD11a/CD18)." *Guidebook to the extracellular matrix and adhesion proteins* 2 (1999): 228-232.
29. Shimaoka, Motomu, et al. "Reversibly locking a protein fold in an active conformation with a disulfide bond: integrin α L I domains with high affinity and antagonist activity in vivo." *Proceedings of the National Academy of Sciences* 98.11 (2001): 6009-6014.
30. Shimaoka, Motomu, Junichi Takagi, and Timothy A. Springer. "Conformational regulation of integrin structure and function." *Annual review of biophysics and biomolecular structure* 31.1 (2002): 485-516.
31. Li, Rui, et al. "Two functional states of the CD11b A-domain: correlations with key features of two Mn²⁺-complexed crystal structures." *The Journal of cell biology* 143.6 (1998): 1523-1534.
32. Huth, Jeffrey R., et al. "NMR and mutagenesis evidence for an I domain allosteric site that regulates lymphocyte function-associated antigen 1 ligand binding." *Proceedings of the National Academy of Sciences* 97.10 (2000): 5231-5236.
33. Jin, Moonsoo, Ioan Andricioaei, and Timothy A. Springer. "Conversion between three conformational states of integrin I domains with a C-terminal pull spring studied with molecular dynamics." *Structure* 12.12 (2004): 2137-2147.
34. Jin, Moonsoo, et al. "Directed evolution to probe protein allostery and integrin I domains of 200,000-fold higher affinity." *Proceedings of the National Academy of Sciences* 103.15 (2006): 5758-5763.
35. Shimaoka, Motomu, et al. "Structures of the α L I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation." *Cell* 112.1 (2003): 99-111

36. Stemmer, Willem P. "DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution." *Proceedings of the National Academy of Sciences* 91.22 (1994): 10747-10751.
37. Shusta, Eric V., et al. "Directed evolution of a stable scaffold for T-cell receptor engineering." *Nature biotechnology* 18.7 (2000): 754-759.
38. Hammond, Craig, and Ari Helenius. "Quality control in the secretory pathway." *Current opinion in cell biology* 7.4 (1995): 523-529.
39. Boder, Eric T., and K. Dane Wittrup. "[25] Yeast surface display for directed evolution of protein expression, affinity, and stability." *Methods in enzymology* 328 (2000): 430-444.
40. Boder, Eric T., and K. Dane Wittrup. "Yeast surface display for screening combinatorial polypeptide libraries." *Nature biotechnology* 15.6 (1997): 553-557.
41. Gietz, R. Daniel, and Robin A. Woods. "Yeast transformation by the LiAc/SS Carrier DNA/PEG method." *Yeast Protocol*. Humana Press, 2006. 107-120.
42. Becker, Daniel M., and Leonard Guarente. "High-efficiency transformation of yeast by electroporation." *Methods in enzymology* 194 (1991): 182-187.
43. Costanzo, Maria C., and Thomas D. Fox. "Transformation of yeast by agitation with glass beads." *Genetics* 120.3 (1988): 667-670.
44. Johnston, Stephen A., and Michael J. DeVit. "Biolistic transformation of yeasts." *Yeast Protocols*. Humana Press, 1996. 147-153.

Appendix A

Gene Cloning and Propagation

A.1 PCR (typical protocol using Vent Polymerase)

Prepare master mix (50 ml volume): 5 ml Thermopol buffer, 1 ml Vent polymerase, 1 ml dNTPs, 1 ml forward and reverse primers at 100 mM concentration, 1 ml plasmid DNA and 40 ml ultrapure water.

Thermocycling: 94°C for 2 min to denature, 25 cycles of 94°C for 30 sec, primer anneal (typically 55-62°C) for 30 sec and polymerization at 72°C for 45 sec, and 72°C for 5 min to ensure all copies are full length.

A.2 Creation of an Artificial Gene by Extension PCR (limited to approx 150-200 bp)

Round 1: generation of small amount product. Mix 2 ml of each oligo (at 0.1 to 10 mM) and add 1 ml to 5 ml Thermopol buffer, 1 ml Vent, 1 ml dNTPs and 42 ml DI. Thermocycling: 30 cycles of 94°C for 45 sec, anneal for 45 sec and 72°C for 30 sec, followed by 5 min at 72°C.

Round 2: amplify product from round 1. Combine 1 ml product taken directly from round 1, 5 ml Thermopol buffer, 1 ml Vent, 1 ml dNTPs, 1 ml each primer at 1-100 mM and 40 ml DI. Thermocycling: same as above.

A.3 Electrophoresis and Gel Extraction

Prepare 0.5 g agarose (or 1 g low-melting temperature agarose) in 50 ml TAE. Melt agarose in microwave, allow to cool and add 2.5 ml ethidium bromide. Pour into gel box and add comb.

Dilute sample with loading buffer and add to gel (up to 30 ml for 8 well, 10 ml for 15 well). Separate samples using an empty lane unless the product sizes are well separated.

Run at 105 V for 45 min (1%) or 1.5 hrs (2%).

Cut out bands with a clean razor blade, place in microcentrifuge tube and weigh.

Extract the bands using the Qiagen kit per instructions.

A.4 Plasmid Digestion

Prepare master mix (40 ml): 4 ml digestion buffer, 2 ml enzyme(s), 0.4 ml 100x BSA (if necessary) and 3.6 ml ultrapure water (or 5.6 ml if using only 1 enzyme). Add 28 ml insert from GE or 20 ml vector (+ 8 ml DI) from miniprep.

Update: digest 1000ng DNA, for each enzyme, add 10 Units.

Compare the enzymes' conditions, and choose the optimal buffer

Incubate at 37°C for 1 hour. (compare the enzymes' conditions, and pay attention to the temperature. For example, digest DNA with XbaI and BsiWI. BsiWI requires 55C. Set the temperature at 37C for 1hour and then increase it to 55C for another 1hr.)

Purify vector by electrophoresis as outlined above, except run the gel for at least 1 hour. Inserts can be purified by gel extraction or simply using a Qiagen PCR clean-up kit.

A.5 Ligation

Prepare master mix (20 ml): 4 ml Ligase buffer, 1 ml T4 DNA ligase, 9 ml ultrapure water, 1 ml digested vector and 5 ml digested insert. Ideally should have a 3:1 molar ratio of insert to vector.

Incubate at room temperature for 1-2 hrs (usually 1.5). Note recommended to use at lower temperature.(16C, overnight) Can be transformed directly into E. coli or frozen and used at a later time.

A.6 Transformation into E. coli (based on DH5a from Invitrogen)

Add 2 ml ligation mixture to 50 ml DH5a cells, let sit 30 min on ice.

Heat shock for 45 sec at 42°C, place back on ice for 2 min and then add 0.95 ml SOC media.

Incubate 1 hr at 37°C and plate cells onto LB-Amp. If using low transformation efficiency clones, spin sample and plate all cells. Grow overnight at 37°C.

A.7 Colocy PCR

Scrape a single colony using a pipet tip or small ring and dip into 50 ml ultrapure water.

Perform typical PCR except use ½ the volumes listed above and add 5 ml of the cells suspension. Run PCR as usual with annealing at 55°C. Check for product on a 1% agarose gel.

Positive samples can be plated (5 ml) and grown up (remaining 40 ml) in 3 ml LB-Amp for later use and sequencing, respectively.

Media Recipes

LB Broth:

Base media (for 1 L): 25 g LB powder, filter sterilize or autoclave.

Supplement: Ampicillin or other antimicrobial agent, typically used at 50 mg/ml

LB Plates:

40 g LB agar powder/L, autoclave.

Supplements: Add ampicillin or other antimicrobial agent (typically at 50 mg/ml) once the media has cooled to 50°C (touchable by bare hand).

Appendix B

Protocol: Yeast Culture and Recombinant Protein Production

B.1 Growth

Streak a single colony on YPD or SD-CAA plates and incubate at 30°C for 2 days.

Inoculate 3 ml liquid culture with a single colony and grow overnight (YPD) or for 1.5 days (SD-CAA) at 30°C while shaking at 200-225 RPM.

B.2 Transformation (Lithium Acetate method from Gietz RD and RA Woods, Methods in Enzymology, 2002)

Inoculate 3 ml YPD with BJ5464 (transformation with pRS plasmid) or EBY100 (pCT plasmid) and grow overnight.

Heat single-stranded carrier DNA (2 mg/ml) in a boiling water bath for 5 min and then place on ice.

Spin yeast at 3000 RPM for 1 min, resuspend in 1 ml ultrapure water and transfer to a microcentrifuge tube. Spin again and discard the supernatant.

Layer the following on top of the pellet: 240 µl PEG 3500 (50% w/v), 36 µl LiAc (1.0 M), 50 µl carrier DNA (after vortexing) and 34 µl plasmid DNA (0.1 to 1 mg mixed in ultrapure water, typically use 2 µl). Vortex to mix and resuspend cells (pipet if necessary).

Incubate at 42°C for 1 hr before centrifugation and aspiration of the transformation mix. Add 1 ml ultrapure water, resuspend and plate 100 µl on selective media (SD-CAA).

B.3 Surface Display of Recombinant Protein

Inoculate 3 ml of SD-CAA with EBY100 yeast transformed with a pCT-based plasmid. Grow overnight at 30°C while shaking at 225 RPM.

Measure concentration based on absorbance at 600 nm, which is typically 4-6 absorbance units for an overnight culture. 1 absorbance unit is approximately 107 cells/ml.

Transfer 3×10^7 cells to a microcentrifuge tube, centrifuge, resuspend in 1 ml SG-CAA media, spin, and resuspend in 3 ml SG-CAA.

Grow for 16 hours at 20°C while shaking at 225 RPM.

B.4 Soluble Expression of Recombinant Protein (small volume assessment).

Inoculate 3 ml of SD-CAA+Ura with BJ5464 yeast transformed with a pRS-based plasmid. Grow 24 hrs at 30°C while shaking at 225 RPM.

Centrifuge cells, resuspend in 1 ml SG-CAA media, spin, and resuspend in 3 ml SG-CAA+Ura+0.1% BSA.

Grow for 3 days at 20°C (alternatively grow 2 days at 30°C) while shaking at 225 RPM.

Recover supernatant by centrifuging at 3000xg for 10 min.

B.5 Soluble Expression of Recombinant Protein (scale-up).

Inoculate 50 ml of SD-CAA+Ura with BJ5464 yeast transformed with a pRS-based plasmid. Grow at least 24 hrs at 30°C while shaking at 225 RPM.

Centrifuge cells and resuspend in 1 L SD-CAA+Ura, continue to grow overnight (at least 16 hours).

Centrifuge cells and resuspend in 1 L SG-CAA+Ura+0.1% BSA. Grow for 3 days at 20°C (alternatively grow 2 days at 30°C) while shaking at 225 RPM.

Place on ice for at least 1 hour to aggregate ammonium sulfate. Recover supernatant by centrifuging at 3000xg for 10 min.

Media Recipes

Complex Media: YPD

Base media (for 1 L): 10 g yeast extract, 20 g peptone, 20 g dextrose

Filter sterilize

For plates: Autoclave bacto-agar (15 g/L) in 90% of the final volume. Filter i carbon source, yeast nitrogen base and amino acids in 10% of final volume and add to autoclaved mixture once it has cooled.

Minimal Media: SD-CAA and SG-CAA

Base media (for 1 L): 20 g dextrose or galactose, 6.7 g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids (-ADE, -URA, -TRP), 5.4 g Na₂HPO₄, 7.46 g NaH₂PO₄

Supplements: Uracil (20 mg/L), Tryptophan (20 mg/L), BSA (1 g/L)

Filter sterilize. Note that the buffer salts do not dissolve well, can autoclave in half of the total volume (subtract ~20 ml for the solids volume).

For plates: Autoclave buffer salts, agar (15-18 g/L) and sorbitol (182 g/L) in 80% of the final volume. Filter sterilize carbon source, yeast nitrogen base and amino acids in 10% of final volume and add to autoclaved mixture once it has cooled below 50°C (touchable by bare hand).

Strains

BJ5464 or BJ5465: supplement with uracil, as well as tryptophan unless transformed with a pRS-based plasmid.

EBY100: supplement with tryptophan unless transformed with a pCT-based plasmid.