Enzymatic Synthesis of Cell-surface Modifying Agents to Improve the Efficiency of Stem Cell Therapies

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A Thesis
in
Department of Biology

Presented in Partial Fulfilment of the Requirements
For the Degree of Master of Science (Biology)

At Concordia University
Montreal Quebec, Canada

July 2021

CONCORDIA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

This is to certify that the thesis prepared

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Entitled: Enzymatic Synthesis of Cell-surface Modifying Agents to Improve the Efficiency of Stem Cell Therapies

and submitted in partial fulfillment of the requirements for the degree of

Master of Science (Biology)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

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Haoyu Wu

Mesenchymal Stem cells (MSCs) are multipotent stromal cells that have a great potential to treat incurable diseases such as skeletal diseases and cardiovascular diseases. One of the challenges in MSCs therapy is to deliver MSCs to target tissues like heart and bone by systemic infusion. Some studies revealed that MSCs' low engraftment efficiency may be due to the lack of relevant cell-surface carbohydrates such as sialyl Lewis X (SLe^X), which act as selectin ligands, and which have been shown to enhance the targeted migration of MSCs in previous research. Thus, we aimed to develop a methodology to synthesize a cell-surface-attachable SLe^X conjugate and anchor it on the surface of live cells *via* linkage to cell-surface proteins. In this study, we employed an enzymatic synthesis pathway and successfully produced a SLe^X-PEG_3-Azide conjugate. We also added a cell surface labeling group NHS-ester on the azide tail of the SLe^X-PEG_3-Azide conjugate using click chemistry which is bio-orthogonal and compatible with living cells. Through our study, we built up a new method of synthesis of cell surface adhesive agent SLe^X and carried out preliminary tests towards establishing a cell-labeling method to attach the molecule onto cell-surfaces. Compared with previous studies, our methodology is not restricted to existing precursors of SLe^X on cell surfaces and it is also more economical by using enzymatic synthesis with glycosyltransferases that are easily produced by recombinant expression in *E. coli*.

ACKNOWLEDGEMENTS

I am grateful to the Department of Biology, Faculty of Arts and Science, Concordia University for cultivating me, providing me with valuable experimental conditions and learning opportunities, allowing me to master the relevant knowledge of this major, and lay a solid foundation for future scientific research.

My sincere thanks to Dr. David Kwan for his supervising, who provided opportunities and help for my project. I believe that your guidance will make me progress faster and achieve higher achievements in the future research and studies.

Thanks to my committee members Dr. Alisa Piekny and Dr. Christopher Brett for providing me valuable advice on my project and thesis. Thanks to Dr. David Walsh for your time for being my external examiner.

I would like to thank Kevin Larocque, Nhat Phi Pham and other members from Piekny lab for your valuable help on cell biology experiments.

I am also thankful to my colleagues: Lan Huong Thi Nguyen, F. Ifthiha Moheeden, Mohamed Nasr, Jamaac Ahmed, Trisha Gosh, Maxim Soroko and other members from Kwan lab for their selfless help. Thanks to you all for sharing your research experience which means a lot to me. I sincerely wish everyone a smooth progress and excellent scientific research achievement!

CONTRIBUTIONS

This project was conceived by Dr. David Kwan[1]. The HP0826 and NmGalT glycosyltransferase are expressed and purified by Max Soroko[1]. The A549 cell line used in this project is provided by Piekny lab, and the cell-based experiments are done with the help of Kevin Larocque[1] and Nhat Phi Pham[1] from Piekny lab. All other experiments were designed, performed, and analyzed by Haoyu Wu[1].

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CMP	Cytidine monophosphate
CMP-Sia	Cytidine 5′-monophosphate-sialic acid
DBCO	Dibenzocyclooctyne
E. coli	Escherichia coli
FPLC	Fast protein liquid chromatography
GalT	Galactocyltransferase
GDP	Guanosine diphosphate
GDP-Fuc	Guanosine 5′-diphosphate-β-L-fucose
MSCs	Mesenchymal Stem cells
NHS	N-Hydroxysuccinimide
PCR	Polymerase chain reaction
SLeX	Sialyl Lewis X
TLC	Thin layer chromatography
UDP	Uridine diphosphate
UDP-Gal	Uridine 5′-diphosphate-galactose

Chapter1: Introduction

Mesenchymal Stem cells (MSCs) are connective tissue progenitor cells that are responsible for cartilage and bone formation. They also hold an ability as a kind of multipotent stem cells that can differentiate into different cell types including osteoblasts, chondrocytes, myocytes, neural cells, adipocytes, and hematopoietic-supporting stroma. This ability gives MSCs the potential to treat diseases such as skeletal diseases and cardiovascular diseases. But the application of mesenchymal stem cells to treat cardiovascular diseases is still in the preliminary stage. One of the challenges is to deliver sufficient induced MSCs to the target like heart and bone by systemic infusion. Some studies revealed that MSCs' low engraftment efficiency may be due to the lack of relevant cell-surface ligands, and researchers have tried to apply different cell-surface modifications to improve that. Sialyl Lewis X, also known as Sialyl Le^X or SLe^X, is a tetrasaccharide carbohydrate that plays a role in mediating binding interactions between cells. It is an important ligand for the selectins that bind leukocytes and endothelial cells to induce a rolling adhesion response in the bloodstream. According to this feature, it should also be able to help induced stem cells to bind with endothelial cells at the target. Thus, we considered SLe^X as an ideal modifying agent to help to increase the efficiency of MSCs therapies. Through this project, we devised an approach to synthesis SLe^X in vitro by enzymatic modification and linked SLe^X chemically with a surface protein-reacting group to anchor this modification onto the cell surface. We hope our work could contribute to the improvement of MSCs therapy efficiency.

1.1 Stem Cells and Stem Cell Therapy

Stem cells are defined as precursor cells, they are undifferentiated or partially differentiated cells so that they can self-renew and to differentiate to various cell types[1]. Self-renew means they could go through numerous cycles of cell growth and division. Their ability of differentiation is called potency and the more cell types a cell can differentiate into, the greater its potency. The highest-level potency is called totipotency, then pluripotency, multipotency, oligopotency, and the least unipotency. Among them, pluripotent and multipotent stem cells are used in stem cell therapies most frequently. A lot of the applications in stem cell therapy are multipotent stem cells such as hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

Stem cell therapy provides unique efficacy to deal with functional restoration of tissues or organs for patients suffering from severe injuries or chronic disease[2]. One of the most typical applications of stem cell therapy which has a history of more than 50 years is hematopoietic stem-cell transplantation (HSCT). HSCT is the transplantation of multipotent hematopoietic stem cells derived from the autologous or allogeneic source. The allogeneic source is from unrelated donor tissues (bone marrow, peripheral blood, umbilical cord blood) and the autologous source is from the patient himself- or herself. [3-5]. Autologous transplants have a

lower risk of immune problems while allogeneic cells can be manufactured in large batches. HSCT could be used to treat diseases such as sickle cell anemia, leukemia, and other cancers.

A very important factor of HSCT and other stem cell transplantation is engraftment. Engraftment is the process by which hematopoietic stem cells make their way (homing) to free bone marrow (BM) niches where they can find optimal conditions to survive and proliferate[6]. The transplanted stem cells can produce new blood cells only after engraftment. This important process needs the help of some adhesin reaction in the bone marrow. Researchers found that in endothelial selectin-deficient (lacking both P- and E-selectin) mice, the baseline levels of circulating hematopoietic progenitor cells (HPCs) are increased[7]. As we discuss in 1.2.1, the adhesin function of selectin family needs not only their protein ligands but also the co-expression of some glycans conjugated to those ligands. This may reveal the function of glycans involved in the engraftment process.

Stem cell therapy also has many other challenges, such as the lack of detectable stem cells for certain organs (brain, spinal cord, heart, kidney) and some unexpected side effects[8]. More research is needed to expand the potential therapeutic utility of stem cell therapy.

1.1.1 Mesenchymal Stem Cell Therapies

Another member of the stem cell family that we mainly care about is mesenchymal stem cells, also called mesenchymal stromal cells (MSCs). MSCs were first discovered in 1968 by Friedenstein and were considered as an adherent, fibroblast-like population in the adult bone marrow that is capable of differentiating into osteoblasts, chondrocytes, adipocytes, tenocytes, myotubes, and neural cells[9, 10], providing the supportive niche for hematopoietic stem cells[11, 12]. After decades of study of their biological characteristics and therapeutic potential, the application of MSCs in cell-based therapy has been explored. As a multipotent cell, MSCs have the potential to cure cardiovascular disease, brain and spinal cord injury, stroke, diabetes, cartilage, and bone injury. This medical potential is unique but the treatment of these diseases using MSCs is still in preliminary development[13]. In addition, MSCs are extensively expanded *in vitro* for clinical use[14]. Another interesting characteristic of MSCs is that they also have immunoregulatory properties. It has been demonstrated by research that MSCs can inhibit the effects of cytotoxic T cells (CTLs), not through inducing cell apoptosis but by inhibiting cell division[15].

There is also interest in MSCs' potential for treating cardiovascular diseases. Myocardial infarction (MI), commonly called heart attack, is the permanent damage of heart muscle cells due to lack of blood supply. The lack the endogenous repair mechanisms because cardiac stem cells were not found. Therefore, many cell lines were tried for cardiomyoplasty - the repair of failing hearts using healthy muscle cells from other tissues[16]. Among these cells, MSCs were highly evaluated because they can differentiate into a cardiomyocyte-like phenotype when implanted in healthy myocardium[17]. They could also be allogeneic and can be delivered systemically[16]. These features made MSCs a very good tool in cardiomyoplasty.

When using MSCs for cellular therapy, a critical step for clinical success is the efficient cell delivery of stem cells to their affected region. Infused MSCs are thought to have the capability to home and engraft as well, but some research found that MSCs lose engraftment ability following *in vitro* cultures. After 24 hours culture the seeding fraction of murine in the bone

marrow is reduced to 10%, and almost no MSC was detected in the lymphohematopoietic organs after transplantation of 48 hours cultured primary MSC[18]. This has to be overcome before applicate MSCs in stem cell therapies for illnesses like cardiovascular diseases.

1.2 Glycobiology

Glycans (carbohydrates including oligosaccharides and polysaccharides) are considered the fourth major class of cellular macromolecules besides nucleic acids, proteins, and lipids[19]. They serve not only as a source of energy and as components of cellular structure, but they also act as signaling effectors, and recognition markers. Glycobiology is the study of glycan structure, biosynthesis, biology, and evolution, also includes the study of how glycans interact with other natural biological molecules[20].

Glycans are sugar polymers. The basic building blocks of glycans are monosaccharides. All monosaccharides consist of a chain of chiral hydroxymethylene units with a hydroxymethyl group at its end and the other end attached with either an aldehyde group (aldoses) or an α-hydroxy ketone group (ketoses)[21]. Monosaccharides can also form a ring rather than a chain. Glycans in the human body are commonly assembled from 9 kinds of monosaccharides: glucose (Glc), N-Acetyl-glucosamine (GlcNAc), Galactose (Gal), N-Acetyl-galactosamine (GalNAc), Mannose (Man), Xylose (Xyl), Glucuronic acid (GlcA), Fucose (Fuc), and Neuraminic acid (NeuAc).

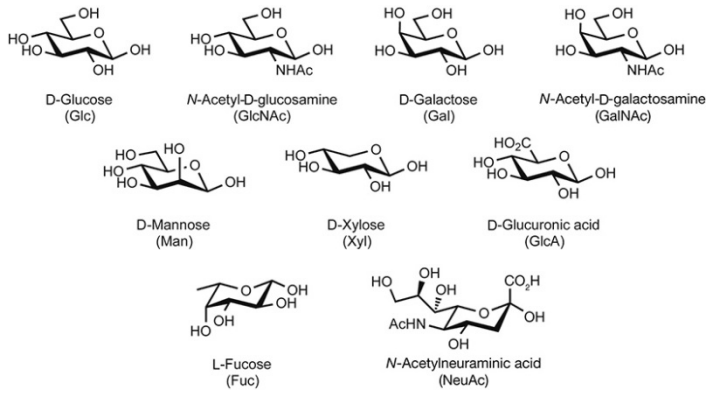


Figure 1. Monosaccharide units found in major human glycans and glycoconjugates.

1.2.1 Oligosaccharides and Glycan Diversity

As described in the previous section, glycans are often oligomers or polymers rather than monosaccharides. In living organisms, a monosaccharide is normally linked to a lipid or a protein as a glycoconjugate, then other sugars join together to make a more complex structure called an oligosaccharide (usually less than a dozen monosaccharides) or a polysaccharide (usually more than a dozen monosaccharides, typically in a repeating sequence). These sugars are covalently linked with each other through glycosidic linkages between the anomeric carbon

of the added sugar and the hydroxyl oxygen of the preceding sugar. Owing to the many possible different combinations of sugars and the way that the sugars are linked, oligosaccharides and polysaccharides' structures exhibit a large diversity that results in glycans playing roles in a broad range of functions[22]. Forming linkages between sugars is an enzymatic process that is catalyzed by glycosyltransferases, which are enzymes that transfer a sugar unit from an activated sugar donor to an acceptor molecule, exhibiting high specificity towards their donor and acceptor substrates, and towards the position and stereospecificity of the linkage. This characteristic of glycosyltransferases allows us to use them as biocatalytic tools for the enzymatic synthesis of a desired glycan structure.

1.2.2 Glycosyltransferases and other Enzymes in Glycosylation

As we introduced, glycosyltransferases are important in glycan diversity, which also makes them powerful tools for enzymatic synthesis owing to their high efficiency and specificity. In addition, research on bacterial glycosyltransferases has shown a broad range of substrates: they can not only work with bacterial glycans but also mammal glycans such as human glycans. This is because many commensal and pathogenic bacteria need to mimic host cell surface antigens. Therefore, many bacteria in the human microbiome can produce the same glycans as their human host cells. Another advantage of bacterial glycosyltransferases is they are easy to obtain in the lab by recombinant cloning and protein expression in *E. coli*. In this project, we applied multiple bacterial enzymes in our synthesis pathway, such as β-1,4-Galactosyltransferase Hp0826, and an α-1,3-fucosyltransferase FucT originally from *H. pylori*. *H. pylori* is the pathogen of gastritis and its later stages involving gastric cancer. It produces a lipopolysaccharide terminating in fucosylated oligosaccharides to mimic Lewis antigens. Several prior reports have demonstrated its use for *in vitro* enzymatic synthesis[23, 24]. The α-1,3-fucosyltransferase FucT in *H. pylori* is the key enzyme for the final step of natural antigens formation. Like the enzyme Hp0826, FucT is also important for the pathogenic pathway of *Helicobacter pylori*. This bacterial human pathogen can mask itself from the host immune detection by mimicking the host cell Le^X antigen. Through extensive research, the FucT enzyme has been characterized and developed for laboratory use for biocatalysis[25, 26]. Another kind of glycosyltransferase is α-2,3-sialyltransferase Cst-I from *C. jejuni*. Sialic acids play important roles in mammalian cell-cell recognition. *C. jejuni* displays mimics of sialylated human glycan structures on their cell surfaces and the role of these carbohydrates in pathogenesis has been demonstrated[27].

Bacteria also express other enzymes or kinase to synthesize sugar donors for glycosylation. Galactokinase GalK from *Streptococcus pneumoniae* TIGR4 is an important enzyme in the conversion of exogenous galactose (Gal) to UDP-galactose through the Leloir pathway. It catalyzes the phosphorylation of the C-1 hydroxyl group of α-D-Gal to yield galactose-1-phosphate (Gal-1-P)[28]. UDP-sugar pyrophosphorylase (AtUSP) from *Arabidopsis thaliana* was a precedent use to synthesize Gal-1-P[29]. *Neisseria meningitidis* CMP-Neu5Ac synthetase NSY and *E. coli* cytidylate kinase CMK. Recombinant NSY had been shown to be expressed in E. coli and optimized for a high-level expression[30]. L-fucokinase and L-fucose-1-P guanylyltransferase FKP is responsible of the synthesis of Fuc-1-P and GDP-Fuc. It is an enzyme from *Bacteroides fragilis* that plays a role in cell surface fucosylation to mimic the

glycosylation pattern of human host cell surfaces[31] and its usage for in vitro synthesis of GDP-L-Fucose and Lewis antigen has been reported[32]. With the help of these enzymes, we could synthesize a complex glycan conjugate much easier than chemical synthesis.

1.3 Cell Surface Glycans and Their Functions

After more than 3 billion years of evolution, a common feature among the cells of all living organisms is that they are covered with dense and complex cell surface glycans. Many of these glycans are the result of posttranslational modification leading to glycosylation on cell membrane proteins. In eukaryotes, membrane proteins usually go through glycosylation in the ER–Golgi pathway before they are transported to their position on the on cell membrane[33]. Proteins can be N-glycosylated or O-glycosylated to form N-glycan or O-glycan posttranslational modifications. An N-glycan makes a glycosidic bond with the side-chain nitrogen of an asparagine residue that is a part of a consensus peptide sequence NX(S/T). An O-glycan makes a glycosidic bond with the terminal oxygen on the side-chain of a serine or threonine residue[34]. Cell surface glycans provide different functions such as protection, stabilization, and (of particular interest) mediating cell-cell interactions. Most of their functions are mediated via recognition by glycan-binding proteins (GBPs). GBPs can be generally divided into two groups: lectins and glycosaminoglycan-binding proteins. Lectins are known to have a carbohydrate-recognition domain to bind specific terminal aspects of glycan chains. Glycosaminoglycan-binding proteins tend to specifically recognize sulfated glycosaminoglycans (a type of anionic polysaccharide)[35]. Serving as ligands or receptors for GBPs, glycans are important in cell signaling, recognition and adhesion. One example providing evidence that glycans can regulate cell recognition and adhesion is the high affinity binding between P-selectin and P-selectin glycoprotein ligand (PSGL-1) which promotes the adhesion of leukocytes to endothelial surfaces. Research has proved that the binding of P-selectin to PSGL-1 needs the co-expression of specific glycosyltransferases that generate core 2-type O-glycans and a sialyl Lewis X (SLe^X) glycan [36]. The same investigators also determined that the high affinity recognition of PSGL-1 needs peptide, tyrosine sulfate, and O-glycan determinants. Through this investigation, it was determined that there are multiple factors necessary for functional recognition: the glycoprotein peptide sequence, glycan position within a peptide portion of a glycoprotein, the structure of the glycans, and other posttranslational modifications of a glycopeptide domain[36]. There are also studies that have shown that specific glycans on surface glycoproteins are important for cell signaling. As early as in 1960, P. C. Nowell discovered that phytohemagglutinin, a plant lectin extracted from red kidney bean *Phaseolus vulgaris*, could be used as an initiator of mitosis in cultures of human leukocytes by binding specific glycans[37].

1.3.1 Cell Surface Glycans in Immune System

Due to their functions in adhesion, recognition and signaling, cell surface glycans are involved in many immune processes. Bacteria and viruses often use their GBPs, such as hemagglutinins and adhesins, to adhere to host cells' surface glycans. One example is the

influenza virus hemagglutinin, which was the first GBP isolated from a microorganism. It binds to sialic acid on the host cell membrane[35]. Many immune cells also need lectin-glycan binding interactions to activate/inactivate their function. One important example is the rolling response of leukocytes, in which glycosylated ligands displayed on leukocytes bind to selectins displayed on endothelial cells of blood vessels. The important selectin ligands are PSGL-1, CD44 and ELS-1. PSGL-1 is the major endothelial selectin ligand on leukocytes which consists of a glycosylated type I membrane protein. PSGL-1 can bind to P-selectin, E-selectin, and L-selectin as blood cells flow through vasculature. Besides mediating leukocyte tethering and rolling response, PSGL-1 also play an important role in the signaling of rolling leukocytes and leukocytes decorated with platelets. CD44 is a common vertebrate cell transmembrane glycoprotein involved in cell growth, differentiation, and motility. In the rolling response of leukocytes, CD44 serves as an E-selectin ligand with post-translational modification that is decorated with sialylated, α1,3-fucosylated, N-linked glycans. E-selectin ligand-1 (ESL-1) is another transmembrane glycoprotein to bind E-selectin and its interaction with E-selectin is facilitated by α-1,3 fucosylation[38]. A very important discovery of leukocyte rolling is that P- and E-selectin need not only the binding of PSGL-1, but also the help of sialyl Lewis X (SLe^X) containing O-glycan. Notably, E-selectin and x-ray crystal structure of P- and E-selectin shows a sialyl Lewis X binding site[39].The function of SLe^X inspired us that engineering cell surface glycans may be a solution to the lack of these molecules on some cell types that are used for therapeutics.

1.3.2 Surface Glycan of Stem Cells

Glycans also play a very important role in stem cell biological activities. They are not only involved in the intracellular maturation of many glycoproteins that are essential for stem cell viability, but they also form a dense glycocalyx on the surface, which is optimally positioned to help the stem cell interact with its environment and interact with other cells or receive signals[19] that are quite important in stem cell signaling of proliferation and differentiation. Lack of these cell surface glycans sometimes can cause embryonic lethality[40]. An example of how cell surface glycan functions in cell growth is that fibroblast growth factor (FGF), a growth factor found in brain and pituitary, must bind to both high affinity FGF receptors and lower affinity heparan sulfate proteoglycans (HSPGs) on cell surface to perform its function. It regulates the self-renewal and proliferation of stem cells. Another example of growth/differentiation factor is the Wnt family. Besides HSPGs, Wnt also binds to Lewis X glycans for signaling[19]. These surface glycans provide stem cells many markers for their signaling, specific for different types of stem cells. Certain types of stem cells or induced pluripotent stem cells (iPSCs) may lack some of the cell surface glycan structures, which are desirable for cell therapies as targeting agents. For example, in hematopoietic stem cell transplantation (widely known as bone marrow transplantation) sialyl $Lewis^X$ glycans plays in important role in the homing and engraftment of transfused stem into the bone marrow through interaction with E-selectins on endothelial cells in the bone marrow vessels[41]. Lack of these glycans on other types of stem cells, such as mesenchymal stem cells (MSCs) weaken their homing ability and limits their potential in various cell therapies like bone marrow transplantation because cellular recruitment to bone needs the help of interactions with E-

selectin expressed by specialized marrow vessels.

Researchers also demonstrate that stem cells could remodel their surface glycans during differentiation. In spontaneous differentiation of menstrual blood-derived mesenchymal cells, they found the reason of a typical phenomenon during ectoderm differentiation, the enhanced binding of *Peanut agglutinin* (PNA), can be attributed to an increased level of the cell surface saccharides containing terminal β-galactopyranoside (β-Gal*p*) residues which could be recognized by PNA. They also found that cell differentiation of some stem cells can be induced by the removal of sialic acids residues of cell surface glycans[42]. These studies suggest that if it is possible to control the type and amount of glycans displayed on cell surface, we can regulate many bio-functions of stem cells.

1.3.3 Sialyl Lewis^X

Sialyl Lewis^X (sLe^X) is a tetra-saccharide carbohydrate which is usually attached to O-glycans on the surface of cells. It is also referred to as cluster of differentiation 15s (CD15s). SLe^X is commonly found on the surfaces of leukocytes. When leukocytes flow through the blood stream they interact with three kinds of selectins: E-, P- and L-selectins to bind themselves to the endothelial wall and roll along the endothelial tissue. Following this rolling adhesion, the leukocytes may interact more tightly with integrins which determines whether they come to an arrest in the bloodstream and exit circulation (extravasate) to get into tissue where inflammation or injury has occurred. The sLe^X epitope has been reported as a ligand for all three kinds of selectins and its function as a general selectin glycoprotein ligand makes it an important factor in leukocyte rolling response. sLe^X is also found on the surface of other kinds of cells such as activated platelets and tumor cells, where they mediate cell adhesion.

1.4 Surface Glycan Engineering

Functions of cell surface glycans provide us the possibility to enhance cells' capacity or give them new abilities which could make them work better through surface glycans engineering. Researchers had already made attempts on cell surface glycan engineering. Genetic approaches are available to adjust cell surface glycans through manipulating the expression of glycosyltransferases[43]. Biochemical and chemical approaches offer us a chance to modify cell surface glycans, and even enable the introduction of unnatural groups such as fluorophores[43]. But both genetic and chemical pathways have limitations. Genetic ways are limited by combinatorial nature of glycan biosynthesis and the functional redundancy of glycosyltransferase genes, while chemical methods are restricted by synthetic challenges resulting from the structural complexity as well as potential safety concerns[43]. Other strategies are developed to overcome these disadvantages, such as chemo-enzymatic synthesis methods. The characterization of different glycotransferases guaranteed the diversity of glycans synthesized. Compared with other strategies, chemo-enzymatic approaches offer high stereoselectivity and economic efficiency[44].

1.4.1 Stem Cell Surface Engineering

Some research has found that the absence of cell surface glycans is responsible for their low homing ability[45]. In some studies, efforts had been made to overcome this shortcoming of MSCs through engineering cell surface glycans. Sackstein and his group developed an *ex vivo* engineering strategy for living cells[45]. They treated MSCs with an α-1,3-fucosyltransferase preparation at specifically designed condition to convert CD44 surface antigens into hematopoietic cell E-selectin/L-selectin ligands because constitutively expressed E-selectin on vascular cell surfaces is responsible for recruitment in marrow vessels. They observed that intravenously infused engineered MSCs infiltrated marrow within hours of infusion. Another study led by Sarkar *et al*. modified MSCs using SLe^X. They immobilized biotin onto the cell membrane then attached biotinylated SLe^X through streptavidin (however, streptavidin is a bacterial protein so it could be reacted by immune system when applicated *in vivo)*. The rolling response of modified MSCs was observed in a chamber containing P-selectin coated substrate[46]. These two studies clearly indicate that cell surface engineering is compatible with stem cells. But their work still has room for improvement. CD44 also plays a part in cell adhesion and migration and glycotransferase treatment of the cells may also work on other cell-surface antigens besides CD44. Inspired by their work, we attempted to design a stable, living cell compatible surface engineering pathway to anchor synthesized surface agent on cell surface. We also hope our modification could restrict the influence on other surface agents.

1.5 Objectives

The major purpose of this project is to build an applicable approach to anchor SLe^X on cell surface which is capable to help MSCs engraftment. Our project could be mainly divided into three steps to reach this goal:

1. Design a one-pot three step synthesis pathway of a SLe^X conjugate that contains an azide group which makes it possible perform through further chemical modifications. The chemoenzymatic method make the synthesis easier than chemical strategy.
2. Link a cell surface anchoring group to the SLe^X glycoconjugate. The linkage should be uncomplicated and spontaneous. Since we leave an azide group, Click-Chemistry which includes an azide-alkine reaction would be a very good choice. This kind of approach is modular, universal, give very high yields, and generate only inert byproducts. It is quite suitable for our objective after some optimization.
3. Attach the complete structure on cell surface. The cell anchoring group should be easy to perform and provide high stability. N-hydroxysuccinimide (NHS-ester) could be our choice. It is a widely used reagent in peptide synthesis or protein labeling. The ability of NHS-ester which include reaction with the primary amines of protein or biomolecule to form N-Acylamides structure. These primary amino groups are present in proteins and peptides as ε-amino groups of lysines and N-terminal amino groups. Another advantage of NHS ester derivative is they process well in aqueous environment and do not need any catalysts, which allow us to do the direct treatment on living cells.

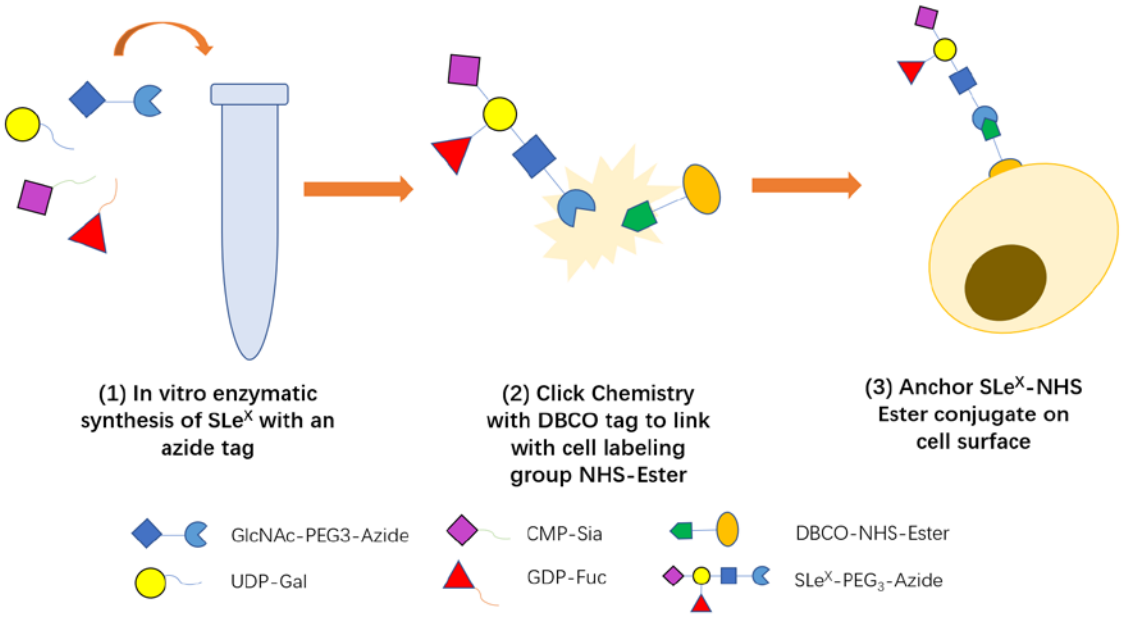


Figure 2. The whole pathway of Cell Labeling Approach: The whole pathway is generally divided in three steps. (1) A one-pot enzymatic synthesis reaction to synthesis SLe^X has an azide tag on the end. (2) The cell labeling NHS-Ester group has DBCO tag that can bind with azide through click chemistry. (3) NHS-Ester group could react with cell surface proteins to anchor the SLe^X conjugate on cell surface.

Chapter 2: Materials and Methods

2.1 PIPE Cloning

Primer design for CMK gene and pET28 vector:
CMK forward:
CTGGTGCCGCGCGGCAGCCATATGACGGCAATTGCCCCGGTTATTACCATTGATGG
CMK reverse:
GTCGACGGAGCTCGAATTCGGATCCTTATGCGAGAGCCAATTTCTGGCGCGCGTAT
TG
pET28 forward:
CAATACGCGCGCCAGAAATTGGCTCTCGCATAAGGATCCGAATTCGAGCTCCGTCG
AC
pET28 reverse:
CCATCAATGGTAATAACCGGGGCAATTGCCGTCATATGGCTGCCGCGCGGCACCAG

PCR amplification was set with 2.5µl template DNA, 2.5µl 10µM forward primer, 2.5µl 10µM reverse primer, 2.5µl 10mM dNTP, 3µl DMSO, 1µl Phusion polymerase, 12.5µl 5x GC buffer, 23.5µl dH_2O. The PCR program setting showed below:

For CMK Gene		For pET28 Vector		
98℃	2 minutes	98℃	2 minutes	
98℃	30s	98℃	30s	
Gradient	45s	Gradient	45s	25x
72℃	30s	72℃	2.5 minutes	
4℃	Hold	4℃	Hold	

Table 1. PCR Program for CMK Gene and pET28 Vector. In 'Gradient' we set a series temperature of 62℃, 65℃, 68℃, 71℃, 74℃ to find the best condition.

2.2 Enzyme Expression and Purification

2.2.1 General Method of Protein Expression

E. coli strain BL21(DE3) was used for expression strain except were noted otherwise, transformed with the expression vector encoding protein of interest, plated on LB plate with

proper antibiotics at 37℃ overnight. Single colony from the plate was cultured in 50ml LB overnight at 37℃, shaking at 220 rpm to get starter culture. 800mL LB broth containing proper antibiotics (specific for the different expression vector) was inoculated by the starter culture and grown overnight at 37℃ in shaking incubator. This main culture was induced with IPTG to a final concentration of 1 mM at log phase (OD600nm = 0.4-0.6). Induced culture was grown overnight, at 18˚C in shaking incubator. The overnight grown cultures were harvested by centrifuging at 4200g, the pellet was resuspended in 15 ml of lysis/wash buffer (50 mM sodium phosphate pH 7.4 – 8.0, 500 mM sodium chloride and 0–20 mM imidazole) with DNAse I, RNAse A and lysozyme at final concentration of 5 µg/mL with the addition of one Roche protease inhibitor tablet per 30 ml of resuspended cells. The cells were lysed by sonication and centrifuged to remove cell debris. The crude lysate was and filtered sterilized use 22 µM filter. This crude lysate could be kept at 4℃ before further purification.

2.2.2 Immobilized metal ion affinity chromatography (IMAC) purification

1ml Ni-NTA resin (bind ~ 60 mg of 6xHis-tagged protein per milliliter of resin) column was used, the column was equilibrated with 10 column volumes (10 mL) lysis/wash buffer. Then crude lysate was loaded to the column and then washed with lysis/wash buffer. All the flow through was collected for later test. His-tagged protein fractions were eluted by AKTA-FPLC with elution buffer (50 mM sodium phosphate pH 7.4 – 8.0, 500 mM sodium chloride and 250–500 mM imidazole). Fractions including flow through were analyzed by SDS-PAGE electrophoresis. The confirmed fractions were pooled and concentrated using Vivaspin concentrator spin columns and stored in storage buffer. The final concentration of purified protein was determined by BCA assay. Purified protein was aliquoted to 100µl small fractions and was flash frozen using liquid nitrogen then stored at -80℃.

2.2.3 Expression of Cst-I

The pET28-CstI plasmid (from Kwan Lab plasmid library) was used to transform E. coli BL21(DE3), and the C. jejuni sialyltransferase, Cst-I, was expressed following the general protocol in 2.1.1 with 50 µg/mL kanamycin in each culture. Enzyme was purified as described in 2.1.2.

2.2.4 Expression of FucT

pET21-FucT plasmid (from Kwan Lab plasmid library) was used to transform E. coli BL21(DE3), and the *H. pylori* fucosyltransferase, FucT, was expressed following the general protocol in 2.1.1 with 100 µg/mL ampicillin in each culture. Enzyme was purified as described in 2.1.2

2.2.5 Expression of HP0826

The H. pylori galactosyltransferase (HP0826) was prepared by M. Soroko. Briefly, pCW-HP0826 plasmid (from Kwan Lab plasmid library), was used to transform *E. coli* AD202 strain, and HP0826 followed the general protocol in 2.1.1 using 100 μg/mL ampicillin in each culture with some specific modifications: In incubation step, the main culture was induced with IPTG to a final concentration of 0.3 mM at log phase (OD600nm = 0.4-0.6) at 25℃, shaking at 220rpm for 18h. Enzyme was not purified, the crude cell lysate was used for enzyme reaction.

2.2.6 Expression of NSY

pCW-NSY05 plasmid (from Kwan Lab plasmid library) was used to transform E. coli strain AD202, and the *Neisseria meningitidis* CMP-sialic acid synthetase NSY05, was expressed following the general protocol in 2.1.1 with 100 μg/mL ampicillin in each culture. Enzyme was purified as described in 2.1.2 except using Q-resin instead of Ni-NTA resin.

2.2.7 Expression of CMK

pET28-CMK plasmid was used to transform E. coli strain BL21(DE3), and the *E. coli* cytidylate kinase CMK, was expressed following the general protocol in 2.1.1 with 100 μg/mL ampicillin in each culture. Enzyme was purified as described in 2.1.2.

2.2.8 Expression of FKP

pET15-FKP plasmid (from Kwan Lab plasmid library) was used to transform E. coli BL21(DE3), and the *Bacteroides fragilis* L-fucokinase/GDP-fucose pyrophosphorylase, FKP, was expressed following the general protocol in 2.1.1 with 100 μg/mL ampicillin in each culture. Enzyme was purified as described in 2.1.2

2.3 Enzyme Reactions

2.3.1 Galactosylation

A small-scale 20 μl reaction was performed to test the first step of synthesis, the transfer of a galactose unit to the GlcNAc conjugate in a β-1,4-linkage. Briefly, 5mM substrate GlcNac-PEG-Azide and sugar donor UDP-gal were incubated with 5mM β-mercaptoethanol for avoiding oxidation and 5mM $MgCl_2$. 50 mM Tris-HCl was used for maintaining pH at 7.5. 0.2mg/ml MtGalT or same volume HP0826 cell lysate was used for the best condition. The whole reaction was incubated at 25℃ for 2h, the result was checked by TLC plate with the solvent containing methanol, ethyl acetate, and ddH2O in a 4:2:1 ratio.

2.3.2 Sialylation

The second step was carried out right after the galactosylation step. 5mM CMP-sia and 0.2 mg/ml Cst-I enzyme were added to the reaction with 5mM $MnCl_2$ while maintaining the whole reaction volume at 20 µl. The whole reaction was incubated without adding Cst-I enzyme at 25℃, 2h for the first step, then add Cst-I and continued incubating at 37℃ for 30min. The result was checked by TLC with the mobile phase containing methanol, ethyl acetate, and ddH2O in a 3:2:1 ratio.

2.3.3 Fucosylation

The fucosylation reaction was carried out right after the sialylation step. 5mM GDP-Fuc and 2.46 mg/ml FucT enzyme were added to the reaction with 5mM $MnCl_2$ while maintaining the whole reaction volume at 20 µl. The whole reaction was incubated without adding FucT enzyme at 25℃, 2h for the first step, then add FucT enzyme and continued incubating at 37℃ for 2 hours. The result was checked by TLC with the mobile phase containing methanol, ethyl acetate, and ddH2O in a 3:2:1 ratio.

2.4 Click-Chemistry

A 20µl reaction was made with SLeX-PEG3-Azide conjugate and DBCO-sulfo-NHS ester concentration at 1:1 ratio. The reaction is performed at room temperature, gently turned over for 1 hour. The result was checked on TLC plate with the solvent contained methanol, ethyl acetate, and ddH2O in a 3:2:1 ratio.

2.5 Cell Labeling

A549 lung cancer cell line was chosen to test the cell labeling because its low expression of CD15s(SLe^X). Cells are cultured on coverslips in the wells of a 6-well plate for 24 hours then washed with 2ml PBS. Before fixation the cell was treated with 150µl DBCO-NHS-sulfo ester for 15 minute and 150µl SLe^X for another 15 minutes at 37℃. Cultured labeled cells are fixed using 4% Formaldehyde Fixative Solution incubate for 20 minutes at room temperature, then washed with PBS for three times. Fixed cells are blocked using 150 µL of blocking buffer to each coverslip and incubate for 45 minutes at room temperature, then washed three times with 150 µL of wash buffer. Diluted antibody (Alexa Fluor® 488 Mouse Anti-Human CD15s) was added to cover the coverslip and incubated at room temperature for 2 hours.

Coverslip were mount onto a microscope slide with one drop of anti-fade mounting medium. Slides were visualized using fluorescence microscope.

2.6 Dot Blot Assay for BSA labeling

100µl 0.1% BSA was treated with 10µl 10mM NHS-ester for 15min, then 10µl 10mM SLe^X-

PEG^3-Azide for 15min. The reaction was incubated at room temperature. Other three group of 100µl 0.1% BSA was treated by only ester, only SLe^X-PEG^3-Azide and negative control with only ddH_2O.

1 µL of each sample was loaded onto an Immobilon-E PVDF membrane, dried for on the 1.5 hours. The membrane was blocked with 1% BSA in PBS-T buffer, washed three times with PBS-T after blocking. Blocked membrane was incubated with 2 µg/mL of biotinylated Aleuria aurantia lectin in PBS for 30 minutes at room temperature, washed another three times with PBS-T.

After that the membrane was incubated with a 1:3000 dilution of HRP-Streptavidin (Sigma, RABHRP3) for 1 hour at 4 °C, washed three times with PBS-T, then imaged using enhanced chemiluminisence (ECL) reagent (Cytiva RPN2235).

2.7 Western Blot Assay for Cell Labeling

Confluent A549 culture was trypsinized on a 10 cm dish and re-suspend in 10 mL of fresh media (F12 w/ PBS), then seeded in 6-well plates. Allow the cells to grow at 37 °C in the cell culture incubator to 70-80% confluency (about 1 day).

After incubation, cells were washed with 2 mL of PBS buffer pre-warmed to 37 °C, treated with 150 µL of 1 mM DBCO-NHS-sulfo ester in PBS for 15 minutes at 37 °C (for control use PBS) and washed with 2 mL of PBS buffer pre-warmed to 37 °C. Then the cells were treated with 150 µL of 1 mM sialyl $Lewis^X$-PEG_3-azide dissolved in PBS for 15 minutes at 37 °C (for control use PBS), washed with 2 mL of PBS buffer after treatment.

Fully treated cells were harvested with 100 µL of Lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X100, 1X protease inhibitor) and pipetted into a microfuge tube on ice. The lysate was clarified by centrifuging at ~10,000 rpm for 5 minutes at 4 °C and supernatant was kept. Protein concentration of the supernatant was measured by Bradford microplate assay to decide the volume of 4X SDS protein buffer added.

The samples were boiled at 95 °C for 3 minutes, spined down and loaded on an 10% SDS-PAGE gel, run electrophoresis at 200 V for ~30-40 minutes. After running proteins were transferred onto a PVDF membrane, run overnight with low voltage (30 mV) at 4 °C.

The membrane was blocked with 1% BSA in PBS-T (PBS + 0.1% Tween buffer), then washed the membrane three times with PBS-T buffer before Incubated with 2 µg/mL biotinylated Aleuria aurantia lectin (AAL) in PBS (10 mL volume) for 30 minutes at room temperature, then was washed three times again. Incubated with a 1:3000 dilution (in PBS; 10 mL volume) of HRP-streptavidin (Sigma, RABHRP3) for 1 hour at 4 °C and Wash three times with PBS-T buffer. The result was imaged with enhanced chemiluminesence (ECL) reagent (Cytiva RPN2235) and analyzed by Fiji ImageJ2 program.

Chapter 3: Results

3.1 Enzymatic Synthesis of a SLe^X Glycoconjugate

SLe^X is a glycan that acts as an important cell surface adhesive agent which can help to increase stem cell trafficking. Previous work of Sackstein *et al.* [45] and Sarkar *et al.* [46] showed the feasibility of engineering cell surface glycans to reach that goal. Sackstein *et al.* developed a method that uses recombinant human enzymes to act directly on the surface of stem cells, *ex vivo*, adding sugar residues to existing glycans to form SLe^X. This approach can only add sugar units to glycans that are already present on the cells and requires the use of difficult-to-express human enzymes. The strategy that Sarkar *et al.* reported uses a synthetic biotin-tagged SLe^X conjugate to label cell surfaces (bridged through streptavidin and a biotinylated adapter). However, this approach relies on a synthetic SLe^X conjugate, which although commercially available (from Sigma Aldrich) is costly, and otherwise difficult to synthesize through conventional means. We hoped instead to modify cell surfaces without relying on existing surface glycans of the cell, nor rely on procedures that involve expensive chemical reagents to attach glycans to cell surfaces by complex linkages. We explored approaches to more efficiently modify cell surfaces that involved using bacterial glycosyltransferases as biocatalytic tools for the effective *in vitro* enzymatic synthesis of SLe^X conjugates that could easily be attached to cells. Here, we designed an enzymatic synthesis pathway for a SLeX-PEG3-Azide conjugate (Figure 3). The azide end of the conjugate allows us to process simple linkage for cell labeling or further modification, like the NHS attach structure used in D. Sarkar's study. We started with GlcNAc-PEG3-Azide as our first substrate. With different bacterial glycosyltransferases, we are able to transfer different sugar groups onto the substrate at particular position. The glycosylation should be performed step-by-step to add the monosaccharide units in a specific order, following the substrate specificity of each enzyme to ensure maximum product yield and avoid dead-end reactions that form shunt products not recognized by enzymes in the subsequent steps.

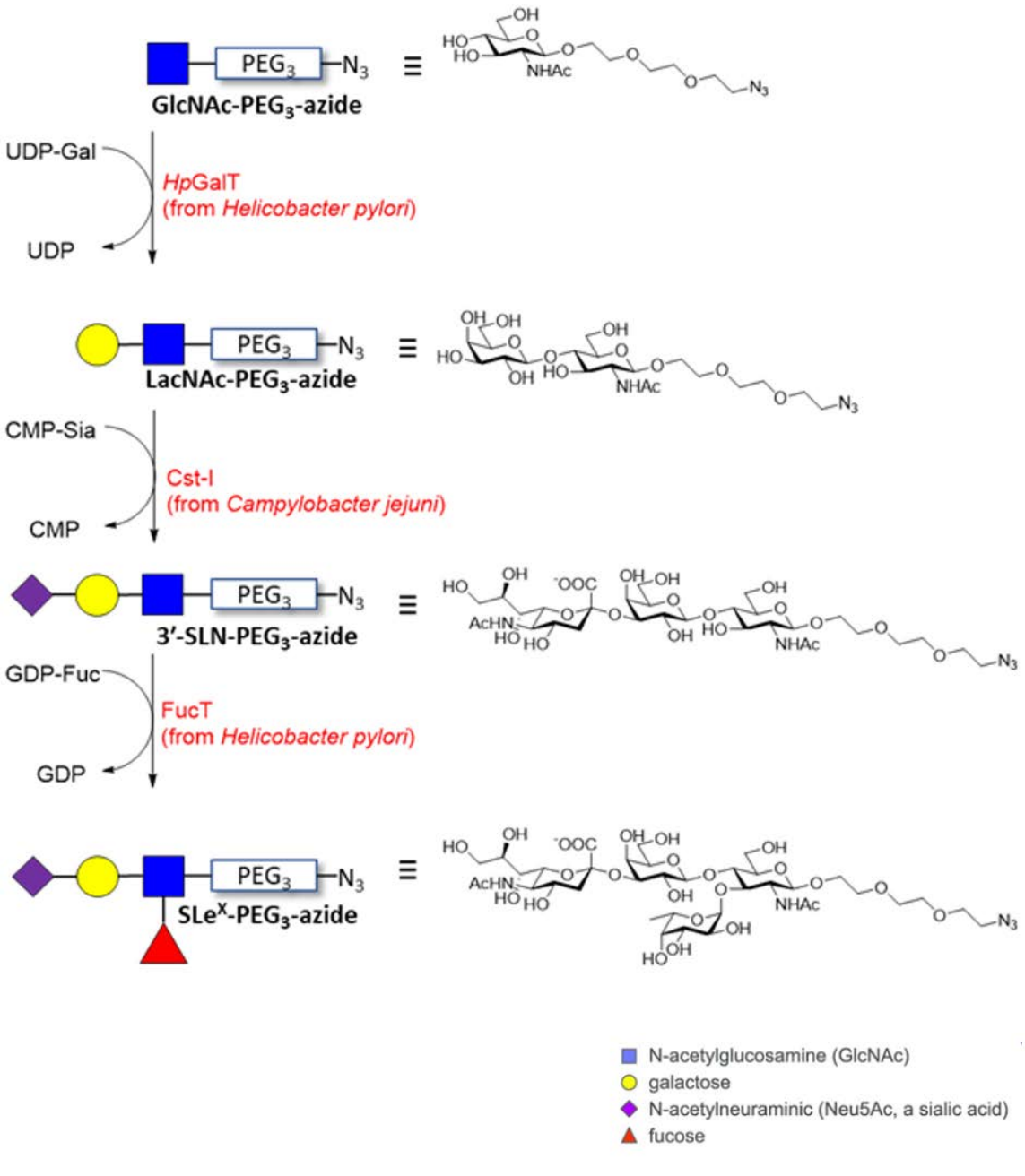


Figure 3. Synthesis pathway of cell surface attachable SLe^X-PEG$_3$-Azide conjugate: a one-pot three step synthesis pathway through glycosylation. Reaction started with GlcNac-PEG$_3$-Azide, bacterial glycosyltransferases (*Hp*GalT, Cst-I, FucT) would transfer galactose, sialic acid and fucose to the substrate from sugar donors (UDP-Gal, CMP-Sia, GDP-Fuc). After three steps of glycosylation, SLe^X-PEG$_3$-Azide glycan conjugate was synthesized.

The first attempt of our enzymatic pathway was carried out immediately after we obtained all of our recombinant glycotransferases (Appendix 1). We ran small-scale (10 to 20 µL) reactions first to test and find the best condition of each glycosylation step (Appendix 2). Once we found the suitable condition for reactions, we carried out a 2 mL volume synthesis, which included 10 µmoles of the key GlcNAc-PEG3-azide starting material, and 15 µmoles of each of the sugar donor substrates, UDP-Gal, CMP-Sia, and GDP-Fuc. The progress of the reaction

could be monitored by TLC assay as well. We took 0.5 µl from the reaction at each checkpoint (before adding the next enzyme, Figure 4). Before the reaction begins there are three clear spots showed on the TLC plate. We considered the yellow spot as our substrate GlcNAc-PEG^3-Azide and the spots below are sugar donors. We could see after each steps a new spot appeared (different migration from the last step) and the spot from last step disappeared, which means the reaction went forward and the substrate consumed completely. After the three steps, we got a clear spot of SLe^X-PEG^3-Azide showed and a very faint mark of the sugar donors. This is the similar results as the small-scale reaction in which we tried to find the best condition for synthesis (Supplementary Figure S1). Our synthesis pathway did work but detection of residual substrates and intermediates revealed the reaction was not totally efficient.

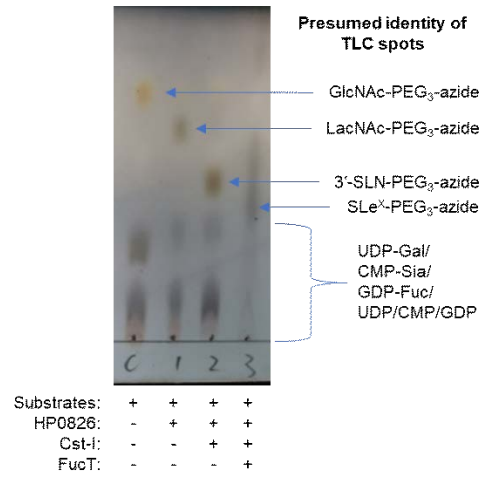


Figure 4. TLC assay monitored the synthesis progress: Spots showed the generation of each step product. Lanes from left to right represent the four check points of the reaction (1. before adding any enzyme, 2. galactosylation, 3. sialylation, 4. fucosylation). New spots appeared when compared each lane with its left lane, which means the generation of products. The disappear of spots from previous step means the substrates were completely consumed. Spots also showed that some unconsumed sugar-donors and nucleotides byproduct were in the mixture.

To obtain pure product, we carried out P2-size exclusion chromatography for purification. The compounds in the reaction mixture were separated by size and went into different fractions after size exclusion. These fractions were first spotted on a TLC plate, which was then developed with *p*-anisaldehyde stain to determine which fractions contained material (Figure 5 (A), from 28-46). Then we tested each fraction containing material to identify which fractions contain our product – each fraction was checked by TLC assay and compared with a SLe^X-PEG$_3$-Azide control. The fractions showed only a spot had same migration with the control would consider containing pure SLe^X-PEG$_3$-Azide (Figure 5 (A), from 28-31). We also checked them using mass spectrometry to get a confident result (Figure 5, (B-E)). We collected fraction 28-31 as our pure product for freeze drying, the yield was ~50% of the theoretical yield of a complete reaction. Considering loss in the reaction and purification, the yield is acceptable. The MS result also pointed out many substrates were not consumed, and the reaction thoroughly completed at the sialylation step.

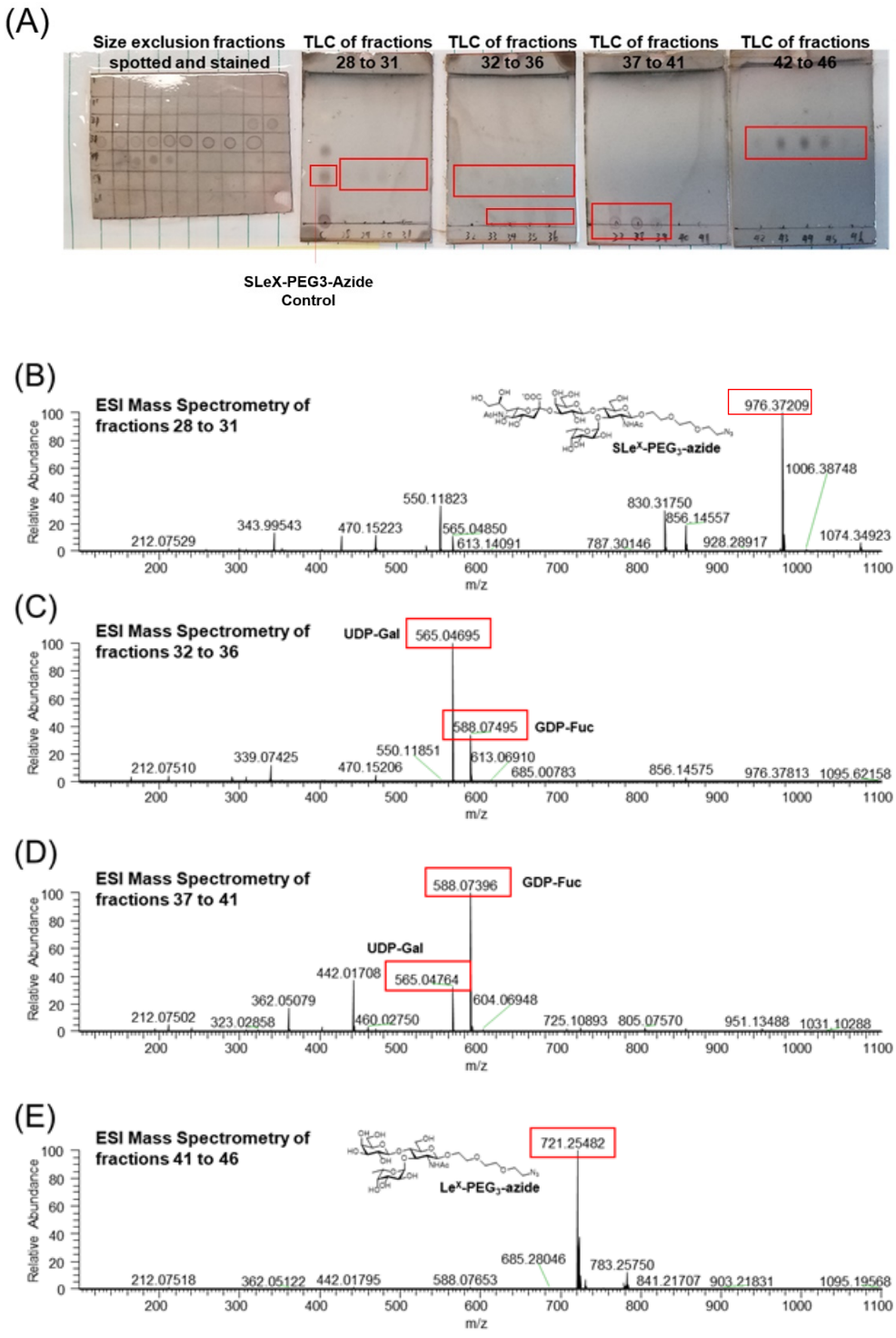
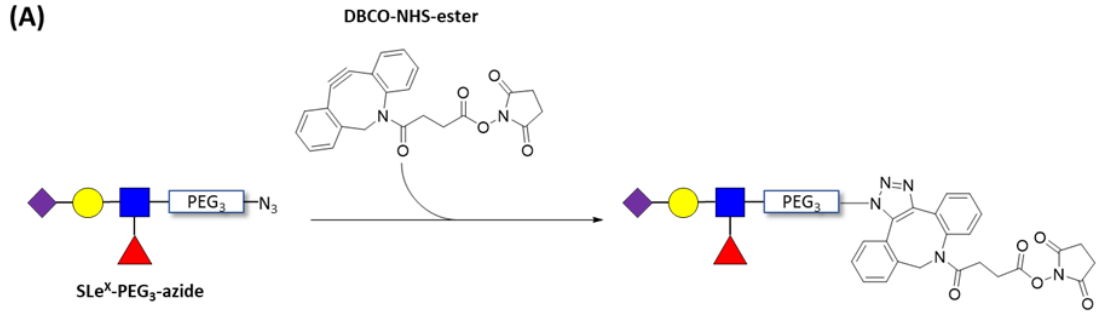


Figure 5. Identification of synthesis product after purification: (A) TLC assays for finding possible fractions. The whole reaction mixture was eluted in 70 fractions, each 1 mL in volume. 0.5 µl from each fraction was spotted on the TLC plate then the plate was stained with p-anisaldehyde. The fractions with no spot at all meant nothing and were discarded. Other fractions (38-46) were checked one by one for the identify their components. Compared with a SLe^X-PEG_3-Azide control, fraction 28 to fraction 31 were considered as pure final product. A more confidential result was tested by mass spectrometry. **(B-E)** Mass spectrometry for a confident result. All SLeX-PEG3-Azide product was in fraction 28-31. In other fractions are

unconsumed substrates and LeX-PEG3-Azide (Fucosylated LacNac-PEG3-Azide), which means the reaction efficiency could still be improved.

3.2 Modifying SLe^X-PEG_e-Azide Conjugate by Click Chemistry.

Once we could reliably synthesize our SLe^X-PEG_3-Azide conjugate, we turned our attention to anchoring the glycoconjugate on cell surfaces. N-Hydroxysuccinimide esters (NHS-esters) could react with primary amines (such as those found on lysine side chains of cell surface proteins) and form stable amide bonds in slightly alkaline conditions. Since we had an azide tail on our conjugate, we planned to add an NHS-ester to the SLe^X conjugate through copper-free click chemistry. Unlike traditional click chemistry, copper-free click chemistry does not need the catalyst of copper ion which may be toxic if we want to apply this modification on living cells. With a Dibenzocyclooctyne-NHS ester (DBCO-NHS ester), the click chemistry could proceed without any toxic catalyst (Figure 6. A). The only problem we should deal with is its solubility: DBCO-NHS-ester needs organic solvent. On the contrary, SLe^X-PEG_3-Azide dissolve in water. This could cause excess DBCO-NHS-ester to remain in the mixture after reaction. Those remaining DBCO-NHS-esters would compete with our ester linked SLe^X conjugate in the anchoring on cell surface. Thus, based on their different solubility, we set a series of experiments to find an optimal water/acetonitrile solvent ratio for the click chemistry reaction. We tested the solvent percentage of acetonitrile, from 30% to 90% for the reaction. What is shown on the TLC plate is that the click chemistry product had the same migration with SLeX-PEG3-Azide. The difference is SLeX-PEG3-Azide itself does not absorb UV light, but ester does (on account of the benzyl groups). Therefore, to distinguish whether it is the product or not, we observed the TLC plates under UV light before staining (Figure 6. B). The spots of reactions all showed UV absorbance, which proved the success of click chemistry. But none of these percentages of acetonitrile could fully consume the ester.



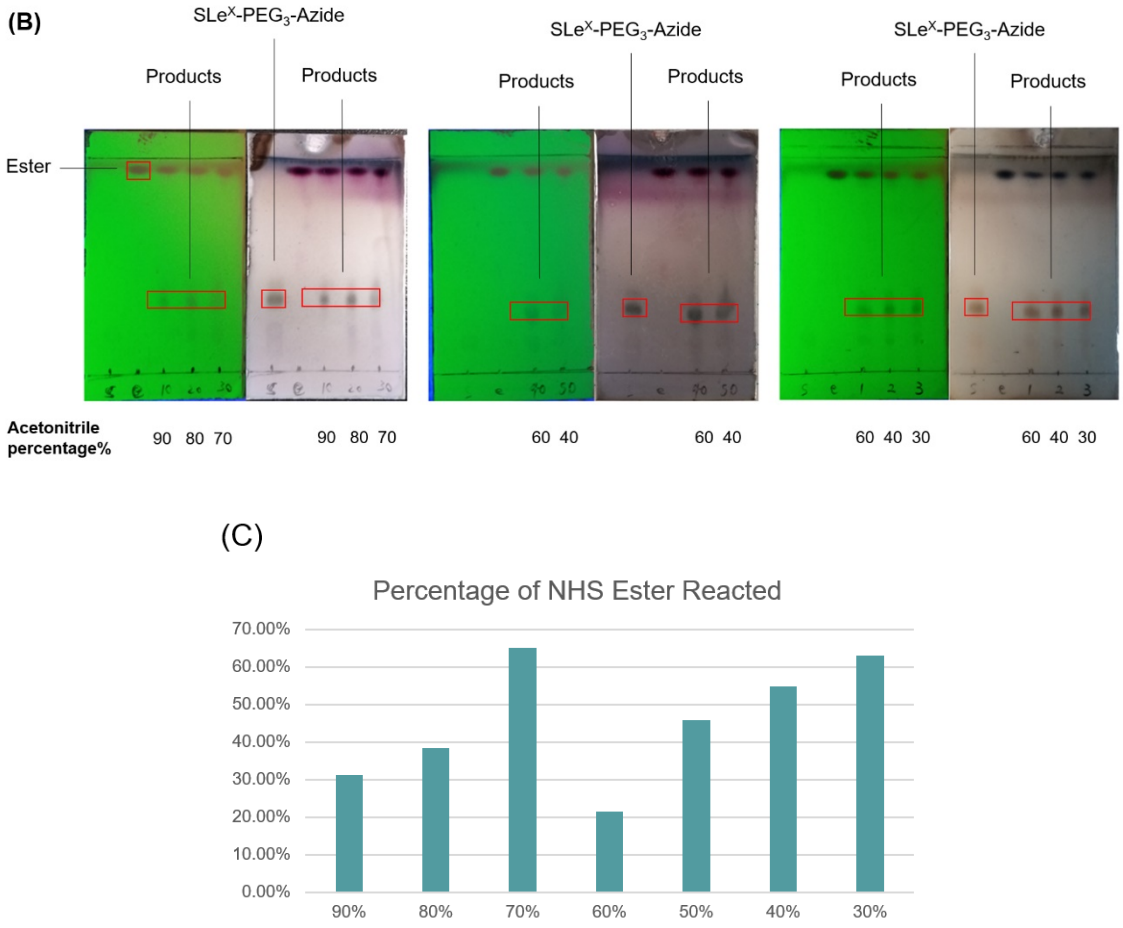


Figure 6. Click chemistry: (A) Copper-free click chemistry could process spontaneously without toxic catalyst. **(B)** TLC results of copper free click chemistry at different water percentage. The TLC plate was observed under UV light (each group left) and after staining (each group left). SLe^X-PEG$_3$-Azide has no UV absorbance so there were no spot observed under UV light. NHS-ester showed a spot on the top under UV light and the products with the same migration of SLe^X-PEG$_3$-Azide also showed UV absorbance. The shade and size of the spot could be used as a standard of the level of reaction. **(C)** Percentage of NHS-ester linked with SLe^X-PEG$_3$-Azide. Spots' intensity of the control DBCO-NHS-ester was set as 100% and spots intensity of DBCO-NHS-ester after reaction was normalized as percentage of unreacted substrate which could be used to calculate how many percentages of ester successfully linked with the azide tag. No relationship was found, and the reaction cannot go completely.

Although we found the best condition for the reaction is 40%-50% water percentage of the water/acetonitrile solvent, there were still many DBCO-NHS-ester left after the reaction (Figure 9. B, spots on the top labeled 'Ester'). With a broader investigation, we still used the DBCO group for the copper free click chemistry, but we found another alternative to the NHS ester, which consisted of a sulfo-NHS ester. It is water soluble due to the sulfate group, which makes it easy to react with cell surface protein in an aqueous environment. In addition, this water solubility also allows us to wash off the unreacted esters so that we could labeled the cell surface

with DBCO-sulfo-NHS ester first and then wash off the unbound esters, then we can process the click chemistry on them to link SLe^X-PEG_3-Azide. Based on the new plan, we tested click chemistry again with this DBCO-sulfo-NHS ester with water as the sole solvent. Similar result was observed on UV illumination and staining on a TLC plate (Figure 7). The reaction could proceed without any catalyst or organic solvent, which is compatible for treating living cells.

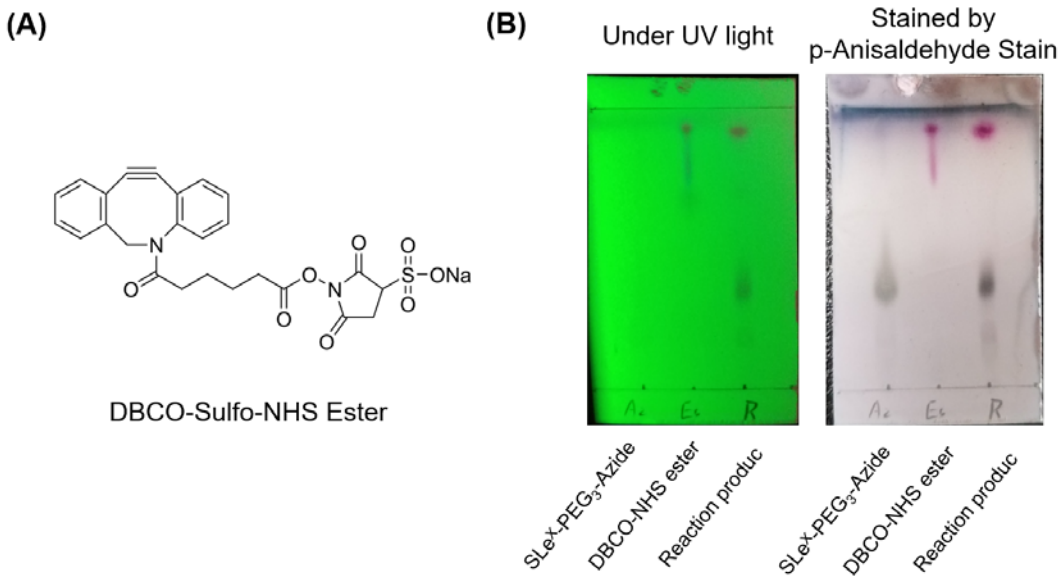


Figure 7. Click Chemistry Using DBCO-Sulfo-NHS Ester: (A) The structure of DBCO-Sulfo-NHS Ester. The sulfo group ensures water solubility and DBCO group compatible for click chemistry. (B) TLC assays was performed to test the click chemistry of DBCO-sulfo-NHS ester. Spot of product had same migration as SLe^X-PEG_3-Azide showed UV light.

3.3 Test of Cell Labeling

After synthesis of the SLeX-PEG3-Azide and designing a method that could anchor our glycan conjugate on living cells. We moved on to conduct tests on living cells. For a cell labelling test, we chose lung cancer cell line A549 which has low intrinsic SLe^X expression. We cultured adherent cells and labeled them with DBCO-sulfo-NHS ester first then added SLeX-PEG3-Azide for click chemistry. We used Alexa Fluor® 488 anti-human Sialyl Lewis X antibody for fluorescence detection. If the anchoring works, we expect to detect fluorescence under microscopy and the fluorescence should be on the cell surface. However, the microscopy image did not match our expectation. We assumed two possible reasons for this poor result. First, there may have been some problem that happened in the course of cell surface anchoring, either the ester did not react with cell surface protein or the click chemistry did not function. Second, the antibody may have been affected due to our treatment, or it may not have been suited to our application (it is designated for use in flow cytometry). To verify that the ester-protein reaction and the click chemistry works, we applied the same treatment on BSA protein. 1% BSA protein was treated first by DBCO-sulfo-NHS ester and then SLeX-PEG3-Azide. If the labeling and click chemistry functioned well, we anticipated that there would be a small difference of migration in an SDS-page gel electrography (Figure 9). The gel images showed small but not obvious differences in the electrophoretic mobility.

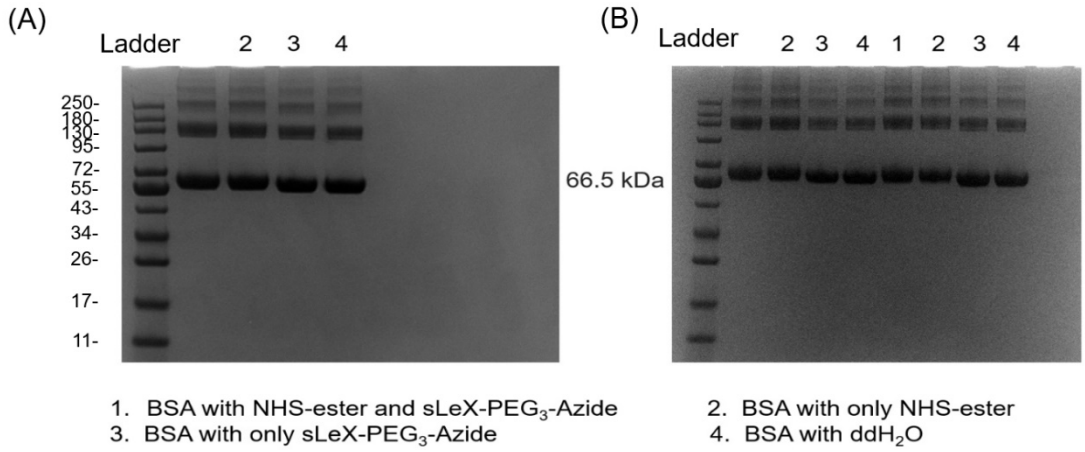


Figure 8. SDS Gel Electrography for BSA Labeling： . A tiny difference of migration showed between fully treated BSA (1.) and three control (2, 3, 4.) on an SDS page. (A) and (B) are duplicates of the experiment in to prove the slight migration is not because of 'smiling effect'.

This modest result was not sufficient evidence for successful labeling. We also tried to identify those bands using Western blot but observed no bands upon blotting with the Alexa Fluor® 488 anti-human Sialyl Lewis X antibody. We suspected the antibody (which is designated for use in flow cytometry) somehow did not work properly in this unnatural context. This particular monoclonal anti-SLe^X antibody was raised against an original cancer cell niche, and its binding was characterized against a natural glycolipid which consisted of a five-sugar glycan that includes the four-sugar SLe^X antigen. We decided to use biotinylated aleuria aurantia lectin (AAL) which binds to fucose sugar residues as an alternative to the antibody in order to identify labeled BSA through a dot blot assay. We loaded labeled BSA and three controls on the immobilon-E PVDF membrane, incubated with biotinylated AAL. If the whole modification worked the dot could be detected by the subsequent binding of HRP-Streptavidin, which could be imaged by luminescence detection of HRP-catalyzed enhanced chemiluminescence (ECL) reaction. The image result met our expectation, only BSA treated by both DBCO-NHS ester and SLe^X-PEG_3-Azide shoed a clear dark dot (Figure 10), which means our labeling approach did have the capacity to label proteins.

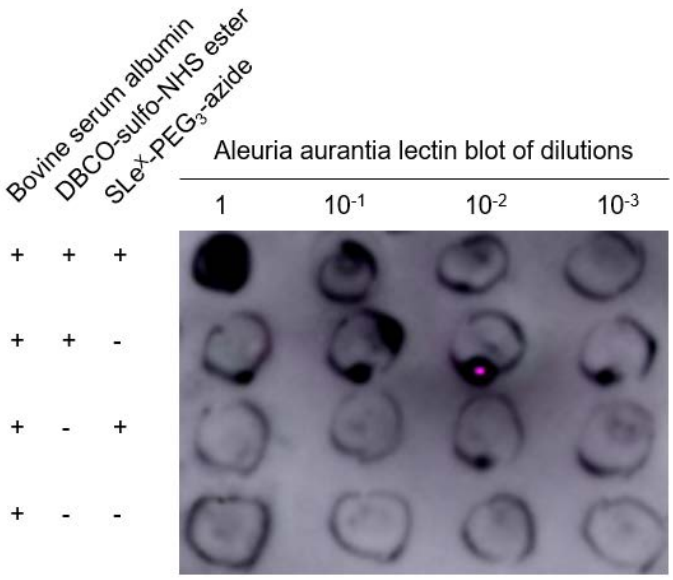


Figure 9. Dot Blot Assay for BSA Labeling: Dot blot images of ester and glycan conjugate treated sample and three control groups. Probing was done with biotinylated AAL. Only BSA with all DBCO-sulfo-NHS ester and SLeX-PEG3-Azide treatment was identified by HRP-streptavidin.

The dot blot assay suggested to us that the biotinylated AAL and streptavidin could be also used in place of an antibody for other tests, for example, Western blot. We applied western blot assay on NHS-ester and SLe^X-PEG_3-Azide treated A549 cells' extracts (Appendix 4). The preliminary results suggest cell labeling approach may work and some further work is need for optimization.

We also tried to find whether the concentration of the ester could influence the result. We set a series of concentration from 0.25 mM to 2.5 mM of the DBCO-NHS ester treatment. We expected to see the intensity increased as the concentration went higher and maintained at a point, but the data did not show a clear relationship between ester concentration and the level of labeling.

4. Discussion

4.1 An optimized of SLe^X-PEG_3-Azide Chemo-Enzymatic Synthesis

In the project we designed a chemo-enzymatic synthesis pathway for our SLe^X conjugate of interest. Compared to chemical synthesis, enzymatic synthesis is an easier way for a relative complex structure. Enzymatic synthesis could be achieved without the need for protecting and deprotecting strategies or synthetic catalysts typical of conventional organic chemical synthesis, using only bacterial glycotransferases which could simply obtain by laboratory expression. Also, this enzymatic synthesis pathway could be extended to a larger field. It is very possible that we can synthesize all Lewis antigens in a short time with an abundant yield for different

experiments.

Although our enzymatic synthesis is practical, it can still be improved. The mass spectroscopy results in 3.1 indicated that some starting materials and intermediates were not fully converted into the desired SLeX-PEG3-azide product, we hypothesized that some key bottlenecks hindered the enzymatic synthesis. One of these bottlenecks may be due to the fact that the glycosyltransferase enzymes that catalyze transfer of sugar units from nucleotide-sugars are generally feedback inhibited by the free nucleotides that are released from the transfer reaction (UDP, CMP, and GDP for galactosyltransferases, sialyltransferases, and fucosyltransferases, respectively). We devised a recycling strategy to save expensive sugar donors and reduce the negative feedback of free nucleotide side products to increase the reaction efficiency. Our plan is to recycle the free nucleotides (UDP, CMP, and GDP) released from the reaction and convert them back into the sugar donor substrates (UDP-gal, CMP-Sia and GDP-Fuc) so that we could run the synthesis with lower amounts of initial sugar donor substrates when using same amount of the sugar acceptor (GlcNAc-PEG3-azide). The recycling strategy would also increase the extent of reaction to reduce side products like Le^X-PEG3-Azide. Recycling would also be carried out by enzymatic reactions which could work at similar conditions as the glycosyltransferases. Based on these principles, we designed a more complex synthesis pathway (Figure 12).

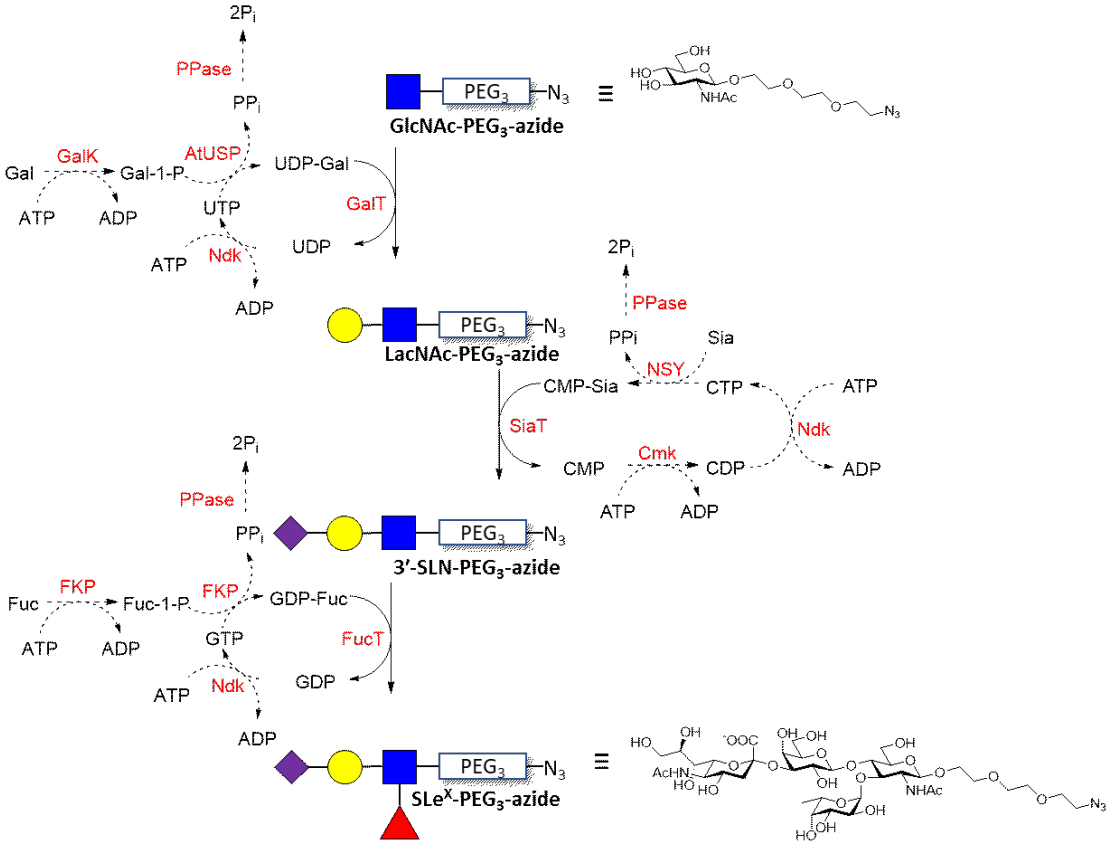


Figure 10. Recycling Strategy of Sugar Donors: With the side recycling reactions, the synthesis could start with nucleotide and sugar rather than expensive sugar donors. The recycling could also reduce the negative feedback of nucleotides.

Although this new synthesis pathway seems reasonable and we expressed all the

glycosyltransferases (Appendix 3), future work remains to be done to establish this strategy since our preliminary tests did not clearly demonstrate feasibility.

4.2 Click chemistry-A Useful Biocompatible Engineering Tool

The copper-free click chemistry plays an important role in our strategy. Previous works on *in vitro* engineering of cell surface CD15s also used NHS-ester for cell anchoring, but what they used is biotinylated NHS-ester and biotinylated sialyl Lewis X then linked them through streptavidin. Their work did inspire us, but we made our own improvement. Our modification strategy is intended to be both convenient and compatible with living cells. Our design of SLe^X-PEG_3-Azide structure and the linkage using click chemistry provides a stable linkage between SLe^X-PEG_3-Azide and DBCO-NHS-ester without introducing any heterogenous macromolecules. However, the method we developed still has some problems to solve. Like we discussed in chapter 3.3, The incomplete reaction of SLe^X-PEG_3-Azide and NHS-ester seems to limit the labeling of cell surface proteins. If we could make the click chemistry react completely, we might be able control the amount of cell surface agent we wanted to attach simply by adjusting the concentration of ester we used for treatment. Also, we believe that after establishing this technique, we could try different modifications on the azide end of SLe^X-PEG_3-Azide structure to exploit more technique based on its feature.

4.3 Future Works

In this study, we had found a method to synthesize SLe^X-PEG_3-Azide *in vitro* and we had got confident prove of the labeling on proteins. However, our attempt of labeling on living cell surface is still in preliminary stage. Further work remains before we reach our final goal of establishing an approach to modify live cells. A practical sugar donor recycling strategy could be optimized to improve the SLe^X synthesis pathway. Furthermore, factors effecting the efficiency of click chemistry should be investigated so that this step can be improved. A more practical method of testing the result of cell labeling assays needs to be established to provide evidence in which we have greater confidence, since we currently only have Western blot results. If these challenges can be met, we could start the work on the cell labeling assay for MSCs and check their engraftment in some *in vivo* experiments. We are also interested in how versatile our surface labeling method could be. Since NHS-ester could bind to any lysine side chains of surface proteins, it is also possible to label other kinds of bio-membranes. For example, exosome therapy is another advanced stem cell-based therapy. Investigation of MSCs also revealed that transplanted MSCs secrete factors are capable of cardiovascular disease recovery and some of these secrete are delivered by exosomes[47]. We would like to see our modification method could help exosomes delivery to right cellular compartments.

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Appendix

1. Expression and Purification of Recombinant Glycosyltransferases

Hp0826 enzyme used in this project is a galactosyltransferase from *H. pylori* that was previously prepared as a crude cell lysate as reported by Soroko *et al*.

After expression and purification, we obtained CST-I enzyme with the yield of 7.79 mg in an 800 mL *E. coli* culture. Following the same procedures described in chapter 2, we obtained FucT with the yield of 24.6mg in from an 800 mL E. coli culture.

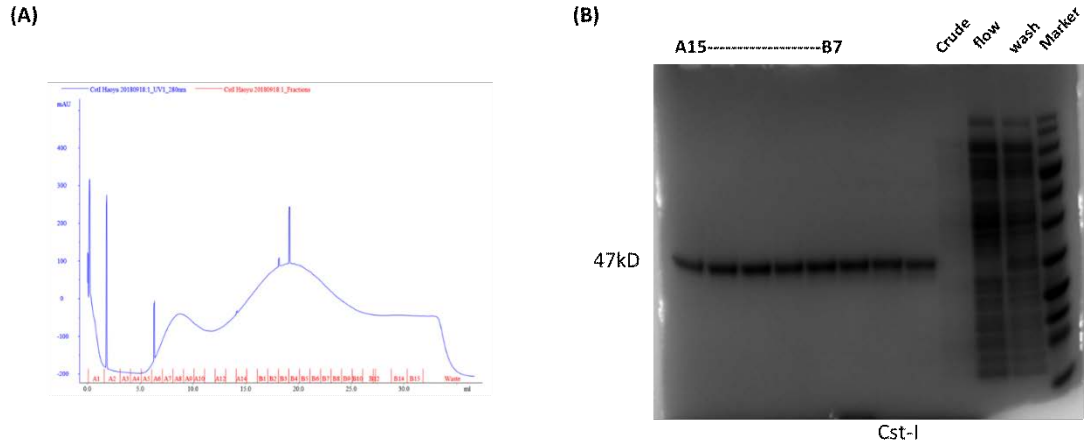


Figure A1. Purification of Cst-I: (A) FPLC curve of Cst-I purification. Fractions at the peak in the middle(A15-B7) would be the enzyme we need. These fractions were collected and teseted by SDS-page.(B) SDS-page gel electrophoresis identify Cst-I at 47kD and confirm the purity.

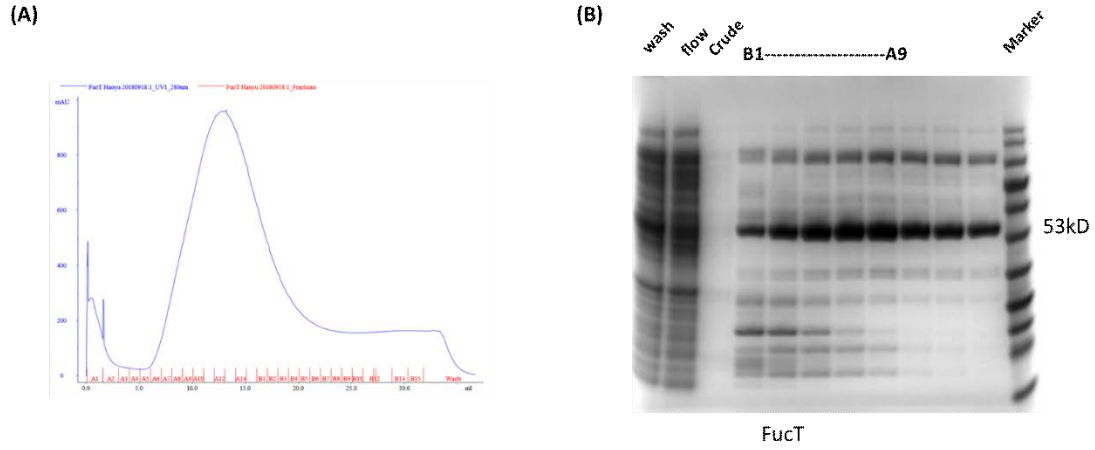


Figure A2. Purification of FucT. (A) FPLC curve of FucT purification. Fractions at the peak in the middle(B1-A9) would be the enzyme we need. These fractions were collected and tested by SDS-page.(B) SDS-page gel electrophoresis of possible fractions. FucT enzyme were at

53kD. Fractions containing the desired protein were pooled and concentrated.

2. Finding Best Condition of SLe^X-PEG_3-Azide Synthesis

We checked the result at different reaction times and reaction buffer compositions trying to find best reaction conditions. For a quick result, the new spot shown on TLC plates indicated that there are new products generated in the reaction (supplemental figure S1). We also tried to change different conditions of the reaction so that we could observe the complete consumption of substrate indicated by a disappearance of the corresponding TLC spot which is taken as a sign that the reaction proceeded to completion. With the best condition as we described in chapter 2, only each checkpoint just showed one clear spot (figure S1, D).

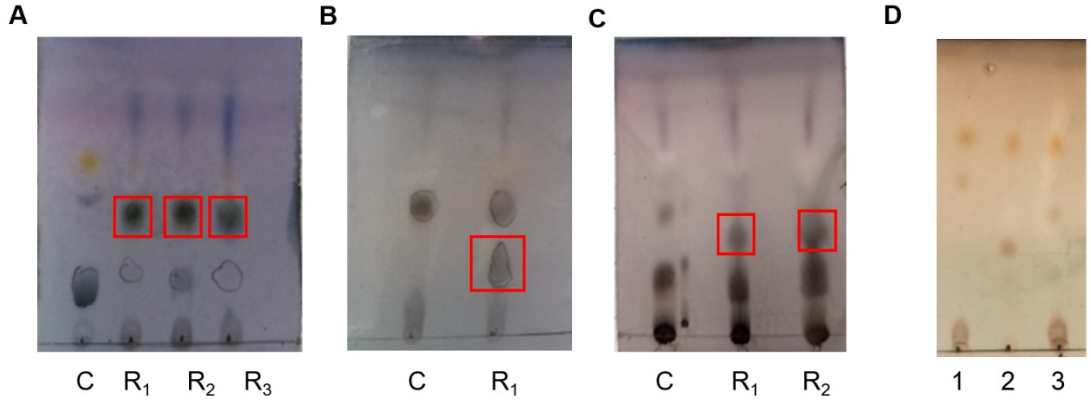


Figure A3. TLC and MS results of glycosylation: (A) TLC result of the first glycosylation step, from the left is c: no enzyme control, R_1: reaction with 0.2 mg/ml enzyme and 7mM UDP-gal; R_2: reaction with 0.2 mg/ml enzyme and 10mM UDP-gal; R_3: reaction with 0.4 mg/ml enzyme and 10mM UDP-gal. New spots was considered as product. **(B)** TLC result of the second step, from the left is C: no Cst-I enzyme control, considered as the result pf the first step; R: reaction with 0.1 mg/ml Cst-I. (C) TLC result of the third step, from the left is C: control, reaction without FucT, considered as the result of second step; R_1: reaction without $MgCl_2$; R_2: reaction with $MgCl_2$. (D) TLC result of complete sLe^X conjugate synthesis with the best condition. 1,2,3 showed the check points of each step.

3. Preparation an Optimizer of Synthesis Pathway

Since the recycling of sugar donors are also enzymatic reactions, we introduced more enzymes to our pathway.

We expressed and purified NSY in our lab (Figure 8). NSY did not have a His-tag so that we performed strong anion-exchange using Q- resin columns for purification. We obtained a yield of 9 mg of semi-pure protein from an 800 mL culture.

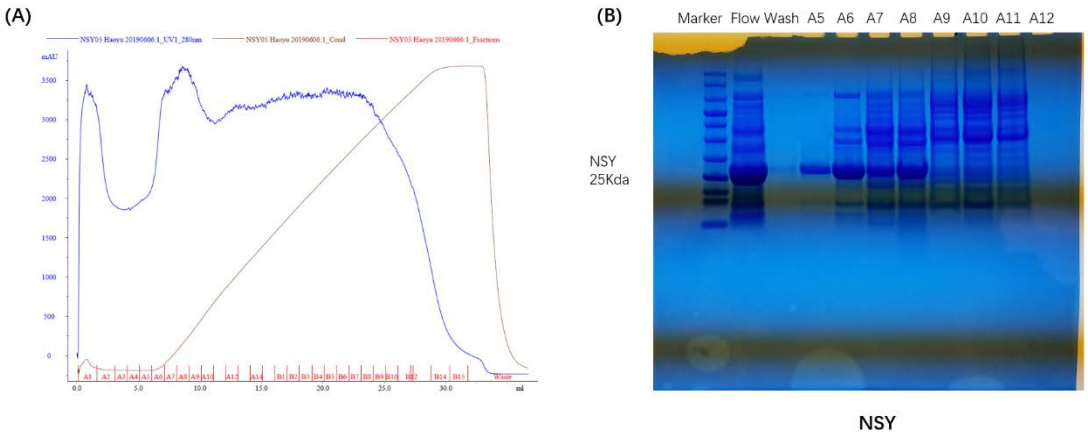


Figure A4. Purification of NSY. (A) FPLC curve of NSY purification. The curve is irregular so that we estimated that fractions from the peak (A5-A10) would be the enzyme we need. These fractions were collected and tested by SDS-page.(B) SDS-page gel electrophoresis of possible fractions. NSY enzyme were at ~25kD. Fractions containing the desired protein were pooled and concentrated.

We did not have the CMK enzyme nor a recombinant plasmid encoding it in our storage, so we had to start with gene cloning. Unfortunately, our first try with traditional gene cloning using restriction digestion and ligation failed, so we tried with Polymerase Incomplete Primer Extension (PIPE) cloning then. PIPE is a ligation-independent cloning technique that is simpler than traditional cloning and has fewer purification steps which may cause loss of DNA. We designed primers for CMK gene and the pET28 vector. We PCR amplified both DNA fragments with regions of homology at each end. The partially incomplete replication of DNA fragments during PCR leaves long complementary overhangs that allow the gene fragment and plasmid fragment to hybridize. (Figure A5). When co-transformed with both fragments the E. coli cell would complete the ligation of annealed fragments for us.

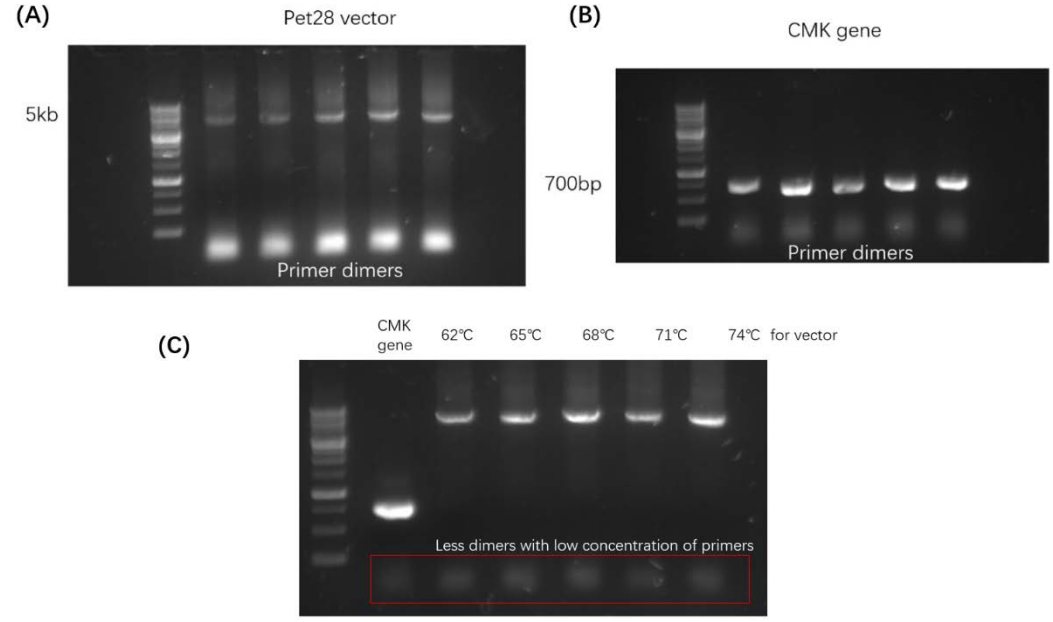


Figure A5. PCR for PIPE cloning. (A)&(B) PET28 vector and CMK gene were cloned and

PCR for amplification. Cloned vector and CMK gene were transformed into competent cells after purification. Recombined plasmid would be formed in the cell. (C) An optimization using less primer concentration to reduce dimer production.

After obtaining the recombinant plasmid, we expressed and purified the CMK enzyme in *E. coli* (Figure A6). We finally got a yield at 17.9 mg/ml enzyme from 800 ml of bacterial culture.

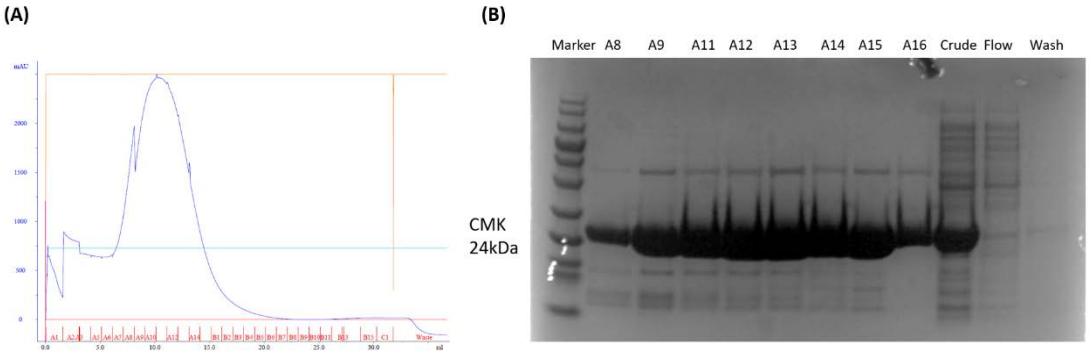


Figure A6. Purification of CMK. (A) FPLC curve of CMK purification. Fractions around the peak were collected and tested by SDS-page.(B) SDS-page gel electrophoresis of possible fractions. CMK enzyme were at ~24kD. Fractions containing the desired protein were pooled and concentrated.

For the recycling of GDP-Fucose, we used the L-fucokinase and L-fucose-1-P guanylyltransferase FKP to synthesis Fuc-1-P and GDP-Fuc. We expressed FKP in the lab as well (Figure 11) and obtained a yield of 6.99 mg/ml enzyme from 800 ml of bacterial culture.

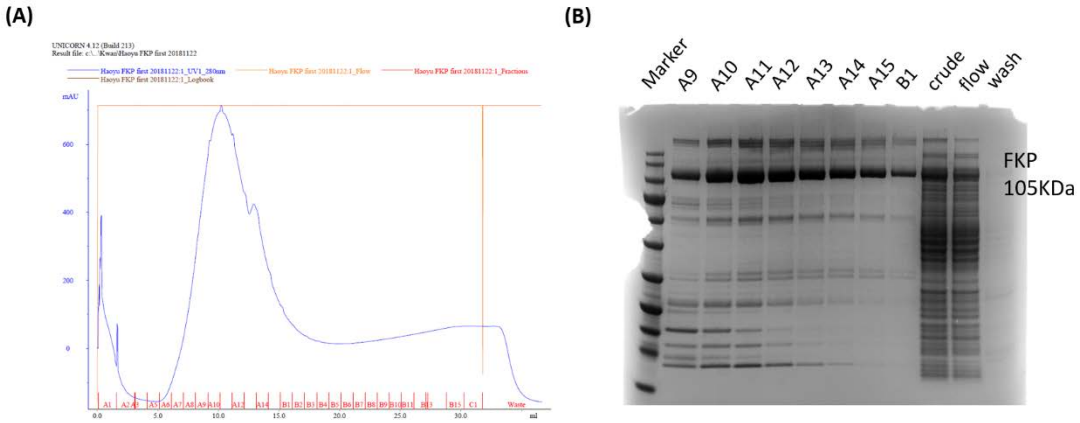
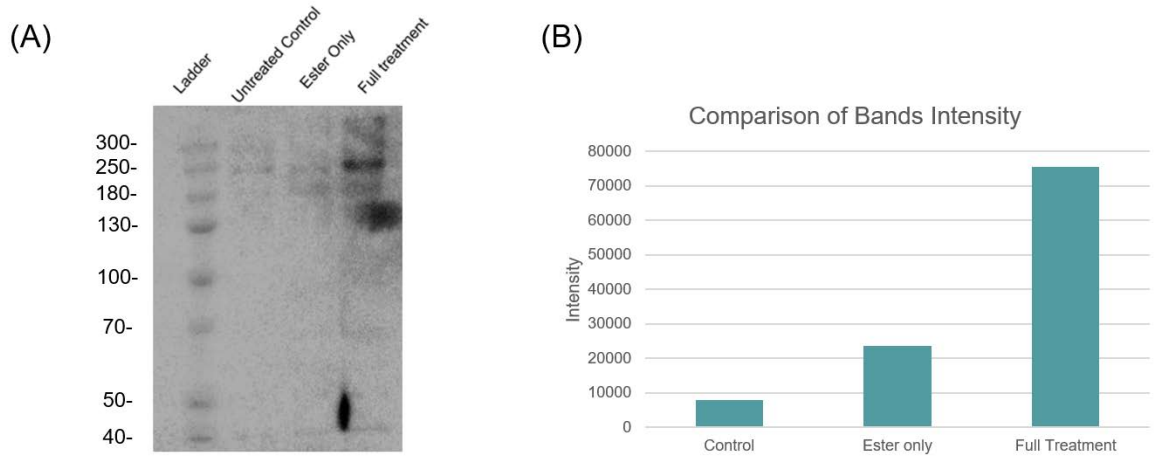


Figure A7. Purification of FKP. (A) FPLC curve of FKP purification. Fractions around the peak were collected and tested by SDS-page.(B) SDS-page gel electrophoresis of possible fractions. FKP enzyme were at ~105kD. Fractions containing the desired protein were pooled and concentrated.

4. Western Blot Assay of Cell Extracts to Test Cell surface Labeling

We designed another cell-based assay to prove our cell labeling. After our treatment on living cells, we extracted them and run a Western blot to check whether cell-surface proteins were labeled. Although the biotinylated AAL would also binds to fucose sugar residues on other surface glycans, our treatment should give a stronger signal. The Western image matched our expectation, the cell extract from cells treated with 1 mM DBCO-NHS ester and 1 mM SLe^X-PEG_3-Azide showed a higher intensity (the whole lane) than no treatment control and DBCO-NHS ester treated cells (Figure 11). This result gave us more confidence about viability of our living cell labeling approach.



FigureA8. Western Blot Assay of Treated Cell Extracts. (A) Comparison of band intensity between controls (cells with no treatment and treated only by 1mM DBCO-NHS ester) and fully treated (1mM DBCO-NHS ester and 1mM SLe^X-PEG_3-Azide) cell extracts. Treated cells showed higher intensity than the controls. **(B)** Quantification of bands intensity.