ABSTRACT

Title of Dissertation:

PREPARATION AND CHARACTERIZATION OF GLYCAN MICROARRAYS USING SURFACE FUNCTIONALIZED CATANIONIC SURFACTANT VESICLES.

Neeraja Dashaputre, Doctor of Philosophy, 2014

Dissertation Directed By: Professor Philip DeShong,
Department of Chemistry and Biochemistry

Glycan microarray technology is a promising technology to screen numerous glycan-lectin interactions in a high through put manner. Currently the focus in this topic is to create microarrays that mimic a eukaryotic cell surface, in order to simulate the glycan-lectin binding in cell-cell recognition events. Previous research in our group has been focused on characterization of catanionic surfactant vesicles with surfactants sodium dodecylbenzenesulfonate (SDBS) and cetyltrimethylammonium tosylate (CTAT). Surface functionalization of surfactant vesicles was achieved by incorporating glycoconjugates in vesicle bilayers. This thesis describes the preparation, characterization, and binding studies of glycan microarrays using functionalized catanionic surfactant vesicles.

Chapter 1 summarizes the state of glycan microarray technology and introduces the proposed strengths and limitations of the surfactant vesicle technology. Chapter 2 describes the preparation and characterization of array surfaces using Hydrophobically Modified (HM) chitosan, by electrodepositing HM chitosan on patterned gold electrodes. Surface functionalized catanionic surfactant vesicles bind

selectively to HM chitosan surfaces. This chapter also describes complete characterization of the binding assay to surface deposited functionalized catanionic vesicle surfaces. Lectin binding studies conducted to monitor glucose - Con A (Conacanavalin A) binding and lactose – PNA (Peanut agglutinin) binding indicate that glycans displayed binding with their respective lectin partners. Vesicles functionalized with a complex glycan, LOS F62ΔA showed significant binding with antibody anti-GC mAb 2-1-L8 specific for LOS F62ΔA. Vesicles prepared by extracting bacterial cell membrane components can be thought of as "artificial pathogens" since they contain many of the cell surface receptors found in the pathogen itself. The binding of these ‘extract vesicles’ with both monoclonal and polyclonal antibodies, on HM chitosan surfaces demonstrated that functionalized vesicles a model for "artificial pathogens".

Lectin binding to cell surface glycans is known to be a multidentate process. Due to this, the density of the glycan on the surface plays a key role in binding affinity and selectivity. Similarly, Our surface phase studies illustrate the effect of glycan concentration on the surface on the extent of binding on GBPs.

Next we aimed to perform enzymatic transformations on glycans incorporated in vesicles. Reaction of glycosyltransferase LgtE and UDP galactose with vesicles functionalized with LOS F62ΔE as well as C_{12} glucose functionalized vesicles. This was illustrated with up to 10 fold increase in PNA binding and ~ 50% decrease in Con A binding. LgtE shows similar reaction with LOS F62ΔE vesicles deposited on HM chitosan surfaces. This effect was demonstrated with the help of an ELISA assay using LOS specific antibodies. Our experiments also illustrate the

ability of enzyme Endo A to transfer a tetrasaccharide $Man_3GlcNAc$ on glucose functionalized vesicles with up to 12 % efficiency in a single step. Finally, chapter 4 illustrates our preliminary efforts of creating glycan arrays by depositing surfactant vesicles on nitrocellulose surfaces.

PREPARATION AND CHARACTERIZATION OF GLYCAN ARRAYS USING SURFACE FUNCTIONALIZED CATANIONIC SURFACTANT VESICLES

By

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Dissertation submitted to the Faculty of the Graduate School of the
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DEDICATION

To my parents, Jaiprakash and Shubhangi Dashaputre
For teaching me that education is ones most important asset.

ACKNOWLEDGMENTS

To begin, it is with immense gratitude that I acknowledge the support and help of my advisor Prof. Philip DeShong. He has been a great mentor, guiding me in every step to complete this thesis. I will be forever grateful to him for teaching me the chemistry that I know today. I am also grateful to all of my science teachers, who helped me pave the path starting from high school to graduate school.

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I am glad that I found many friends when I moved to United States, they have made my stay here pleasant and helped me build many beautiful memories of graduate school. I will always cherish our discussion and the weekend trips we have taken

together. They have been my family away from home, supporting and encouraging me at the same time.

Last but surely not the least, I thank my husband Siddhesh, for his unconditional love and support. I am glad to have a fellow chemist with me, who understood my ups and downs in graduate school.

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List Of Abbreviations

Abs.	absorbance
ASA	hexadecenyl succinic anhydride
aq.	aqueous
C	Celsius
$CaCl_2$	calcium chloride
$CaCO_3$	calcium carbonate
$CDCl_3$	deuterated chloroform
CH_2Cl_2	methylene chloride
cmc	critical micelle concentration
Con A	concanavalin A
Conc	concentration
cps	counts per second
Cryo-TEM	cryogenic transmission electron microscopy
CTAT	cetyltrimethylammonium tosylate
DLS	dynamic light scattering
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
ELISA	enzyme-linked immunosorbent assay
Et	ethyl
EtOH	ethanol
FT	fourier transform

Fuc	fucose
FucT	fucosyl transferase
g	gram
GBP	glycan binding protein
GalT	galactosyl transferase
Glc	glucose
GlcNAc	N-Acetylglucosamine
H	proton
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HM	hydrophobically modified
HPAEC	High-Performance Anion-Exchange Chromatography
IR	infrared
K_d	dissociation constant
LOS	lipooligosaccharide
LPS	lipopolysaccharide
M	molar
μ	micro
m	medium
Man	mannose
mf	mole fraction
mg	milligram
$MgCl_2$	magnesium chloride

$MgSO_4$	magnesium sulfate
MHz	megahertz
min	minute
mL	milliliter
mM	millimolar
μM	micromolar
mmol	millimole
$MnCl_2$	manganese chloride
mp	melting point
mV	millivolt
MW	molecular weight
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NHS	N-hydroxysuccinimide
nm	nanometer
NMR	nuclear magnetic resonance
Neu5Ac	N-Acetylneuraminic acid
PEG	poly(ethylene glycol)
PNA	peanut agglutinin
R_f	retention factor
rpm	rate per minute
RT	room temperature

s	sharp
s	seconds
SDBS	sodium dodecylbenzene sulfonate
SEC	size exclusion chromatography
SialT	sialyl transferase
TEM	transmission electron microscopy
TLC	thin layer chromatography
µg	microgram
UV	ultra violet
UDP	uridine diphosphate
v/v	volume by volume
wt	weight
w/w	weight by weight

Chapter 1. Carbohydrate Microarrays using Catanionic Surfactant Vesicles as Design Motifs

1.1 Introduction

Carbohydrates play an important role in cell-to-cell communication and cell-cell recognition. The surface of a cell is decorated with a variety of cell surface receptors and the majority of these receptors are glycoprotein or glycolipid derivatives.[1] In essence, the surface of a eukaryotic cell is "sugar coated" (Figure 1.1). Glycans enable the cell to perform various cell functions such as cell adhesion, development, immune response and cell-cell recognition.[2] Pathogen-cell interactions take place with the help of the available carbohydrates on the cell surface. For example, lipooligosaccharide (LOS) of *Neisseria gonorrhoeae* is implicated critical for the adhesion and invasion of host epithelial cells.[3] Bacterial and viral infections and cancer cell metastasis are known to take place with the involvement of such complex glycans.[4] However, the exact mechanism of function of these molecules is still not clear. While human cells utilize nine types of surface glycan architectures, bacterial cell walls are known to display over a hundred different glycan structures that enhance the complexity of the surface.[5] Since the glycome of a cell affects cellular functioning and recognition, there is interest in developing microarray techniques that determine the binding between surface glycans and glycan binding proteins (GBPs).[6] Hence, there is an interest in determining the precise arrangement and distribution of these carbohydrates on the cell surface, in order to know the specifications of binding between carbohydrates and GBPs. Moreover, different types of cells are known to posses different kinds and quantities of carbohydrates on the cell surface, thus adding to the difficulty of characterization of this binding event. Additionally problematic for the

elucidation of structure-function relationships, is the intrinsic conformational flexibility of carbohydrate molecules, making crystallization extremely difficult.

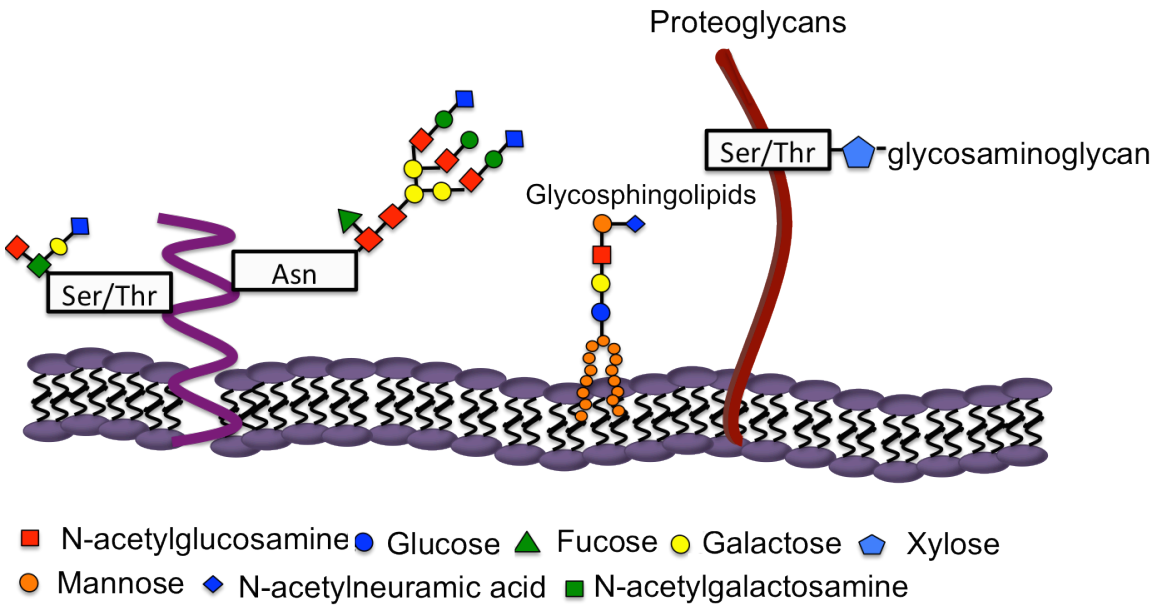

Figure 1.1. Glycans observed on the human cell surface.

1.2 Glycan Microarrays

Microarrays are devices with functionalized surfaces that allow the detection of biolological components such as nucleic acids, proteins, antibodies, inhibitors, enzymes and pathogens by an appropriate optical method, typically fluorescence. Microarray technology is an invaluable technique to detect the interaction of biological entities; accordingly, protein and DNA microarrays have been used extensively to detect the presence of nucleic acids or proteins under physiological conditions.[7, 8] DNA microarrays make use of affinity between the base pairs of complementary single strands of DNA. They have been used to study the functions of many genes simultaneously and also to elucidate transcription changes for cells affected with genetic diseases.[9]

Protein microarrays make use of protein-protein interactions in their design, these have been extensively used to determine protein functions and potential drug targets.[10]

Glycan microarray technology, on the other hand, has not been as well developed as nucleic acid or protein microarray methods due to the unique characteristics associated with glycan-glycan or glycan-protein interactions. Individual carbohydrate binding interactions are relatively weak compared with their protein and nucleic acid counterparts, having dissociation constants in the millimolar range.[11] Accordingly, GBPs are known to exhibit multivalent binding with glycans in order to overcome this low affinity associated with the weak binding of individual carbohydrate residues.[12] This phenomenon is known as cluster effect (Figure 1.2) Multidentate interactions significantly increase the selectivity of glycan binding. Multidentate binding, however, requires that both the density of glycan and its orientation on the surface of the array be controlled precisely to ensure efficient binding.

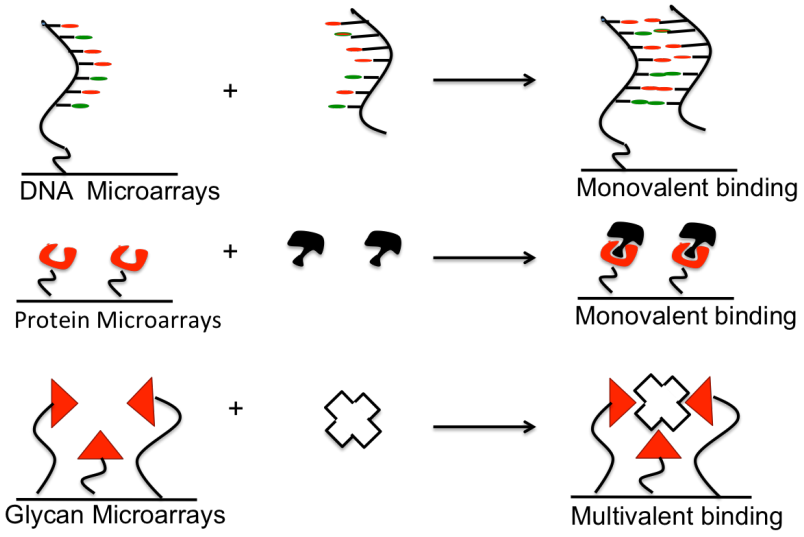

Figure 1.2. DNA and proteins predominantly exhibit monodentate binding; glycans on the other hand exhibit multivalent binding with GBPs to show cluster effect. For binding to take place, multiple glycans on the surface need to be perfectly oriented.

Thus controlling the population and distribution of carbohydrates on the surface is of a vital importance, in the design of an array to give high throughput detection.

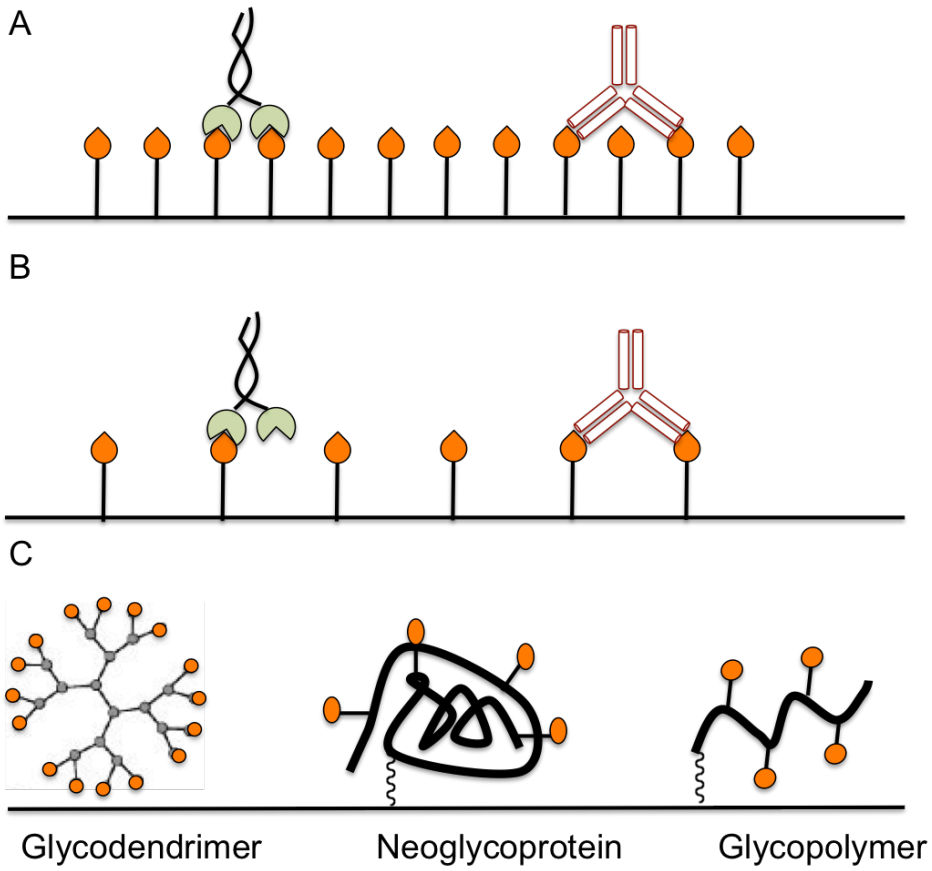

Figure 1.3. This figure depicts effect of glycan concentration on the extent of binding with GBPs. It shows binding between glycans and two different GBPs. When the glycan concentration is higher, both the GBPs bind to the surface (Figure1.3 A) At lower surface glycan density however, binding is different for these GBPs. (Figure 1.3 B) Previous attempts to control the concentration and distribution of glycans on the surface.(Figure 1.3 C) Figure adapted from reference 25.

Many groups have demonstrated the effect of glycan density and spatial arrangement on the binding to glycan arrays.[13] On the cell surface, carbohydrates often exhibit a cluster effect that increases the effective concentration and distribution of carbohydrates on the surface and plays a vital role in the binding with GBPs. (Figure 1.3 A, B) Accordingly there is greater interest in employing strategies such as

glycodendrimers, glycopolymers, and neoglycoproteins (synthetic glycoproteins) that demonstrate cluster effect (Figure 1.3 C). [14, 15]

For example, Kiessling and coworkers quantitatively established the relation between the ligand density on a synthetic polymer and extent of binding to GBPs such as concanavalin A (Con A).[16] Attempts to mimic cell surfaces have been made by preparing fluidic microarrays that allow the glycans to exhibit control of density and mobility to display multivalent interactions. For example, Zhu and coworkers observed a strong a positive correlation between extent of adhesion of E. Coli to the density of mannose on the surface of eukaryotic cells.[17] The Gildersleeve group varied the density of neoglycoproteins on the surface of an array and observed considerable difference in antibody recognition of samples.[18] Inclusion of multiple neoglycoprotein populations onto a single array allowed Gildersleeve to distinguish subpopulations of cancer patients based on differential response of their serum antibodies to neoglycoconjugates of varying densities in human serum. [18] Recent studies by the Bertozzi group have demonstrated the effect of glycan GalNAc valency and inter-ligand separation in synthetic mucin like glycopolymers on the binding of mucin like glycoconjugate in several Tn-antigen specific lectins.[19] They observed that decreasing the density of GalNAc valency on the array increased the binding avidity of mucin two-fold. However, no significant effect was seen on mucin binding at the highest GalNac valency.

1.3 Uses of Glycan Microarrays

A glycan array serves as a powerful alternative to a solution phase protein detection assay because of its ability to detect lower sample quantities and detection time.

These arrays are also highly precise because of the specific nature of glycan-protein interactions. In spite of developed synthetic methods of preparation, it is fairly difficult to obtain highly pure glycoconjugates in large quantities. A microarray overcomes this problem of lack of highly pure glycoconjugates since very small amount of pure glycan is required for detection. Carbohydrate microarrays are an efficient way to study the role that complex glycans play in the human body. For example, It is possible to decipher the glycome of a particular type of a cell by creating an array of neoglycolipids as shown by Fukui et al.[20] Information obtained from the glycan code could be further used to design drugs and trace the exact structures of respective glycoproteins.[21] Seeberger and coworkers used these microarrays to decipher the glycosamination code.[22] The sulfate pattern and repeating sequence of disaccharide of these key glycan was confirmed with the help of microarrays formed with amine functionalized heparin sugar units. Ligler et al. have demonstrated the use of microarrays to detect bacterial toxins associated with cholera, tetanus, salmonella, and listeria.[23] The respective antibodies were used in order to bind the glycan microarrays and a dose-dependent binding could be measured. Microarrays have also been used to determine the enzyme activity of carbohydrate processing enzymes and to search for new antibodies and to detect antibodies for diagnosis of specific diseases.[24]

1.4 Preparation of Glycan Microarrays

A variety of methods have been developed to immobilize glycans on surfaces and prepare glycan arrays.[25] These methods can be divided into two general categories: a) adsorption of the glycan without covalent attachment and b) covalent attachment of

glycans to the surface. Each category can be further divided into methods that attach a particular glycan region (a particular part of the glycan molecule) to the surface and the methods that rely attachment of a non specific glycan region on the surface. (Figure 1.4)

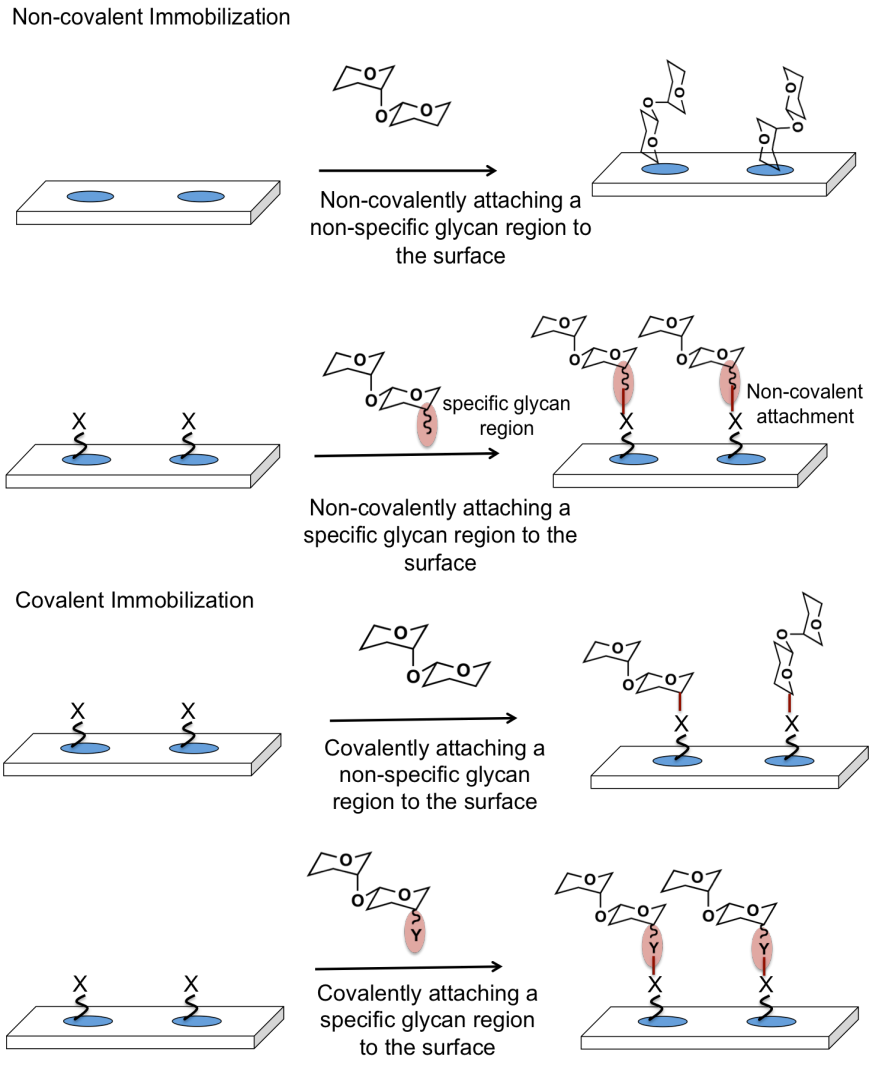

Figure 1.4. Strategies to make glycan arrays by covalent or non-covalent immobilization of carbohydrates to the surface. Figure adapted from reference 25.

1.5.1 Non- Covalent and Non Glycan - Region Specific Immobilization

In order to attach glycans to the surface non-covalently, strategies such as use of hydrogen bonding or hydrophobic interactions have been employed. Wang et al. prepared the first glycan array by simply spotting polysaccharides on nitrocellulose surfaces.[26] The process of adsorption attaches large polysaccharides attached to the surface. However, since a permanent covalent bond was not formed in this process, smaller carbohydrates were not retained on the surface and often detached in the process of washing and binding studies. This method does not ensure attachment of a specific glycan region to the surface. A second disadvantage of this method is that the density of the glycan presentation cannot be controlled during adsorption. Electrostatic forces have also served for immobilization of heparin sulfate polysaccharides on poly-L-lysine coated surfaces.[27] A major limitation of this method is that it is difficult to control the orientation of heparin sulfate on the surfaces because any of the sulfate groups on heparin can get attached on the surface. (Figure 1.5.1)

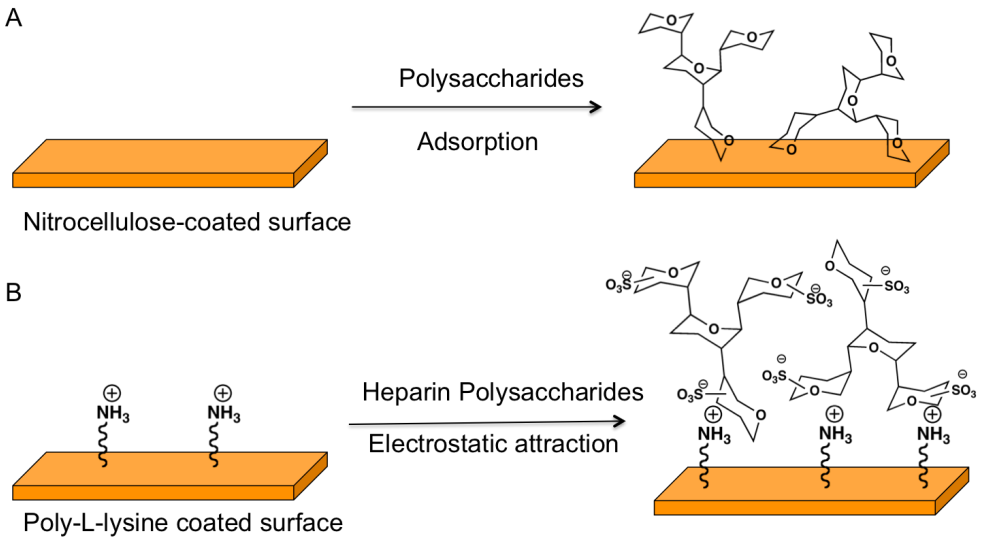

Figure 1.5.1. Non covalent-non glycan-region specific immobilization. A. Use of process of adsorption to form polysaccharide coated nitrocellulose surfaces. B. Formation of heparin sulfate microarrays using phenomenon of electrostatic attraction.

1.5.2 Non Covalent And Glycan - Region Specific Immobilization

Adsorption or electrostatic attraction is not useful to immobilize mono or short oligosaccharides because these compounds are not readily adsorbed onto surfaces. Hence many strategies were created to attach small carbohydrate derivatives to the surfaces utilizing modified glycans. For example, a method by the Wong group includes use of aluminum oxide coated glass slides.[28] In this technique perflurophosphate coated surface was deposited with polyfluorinated ($-C_8F_{17}$) derivative of mannose. The mannose derivative adsorbed to this surface using hydrophobic interactions between the two-fluorinated tails. Other strategies include use of neoglycolipids (lipid-conjugated glycans) on nitrocellulose membranes,[20, 29] or use of biotinylated glycans to bind to steptavidin coated surfaces (Kd ~ 10^{-15} M).[30-32] DNA-glycan hybrids have been used to anchor glycans to surfaces non-covalently, by labeling glycan molecules with a sequence of oligonucleotides and attaching them to surfaces labeled with the complementary DNA sequence.[33] (Figure 1.5.2)

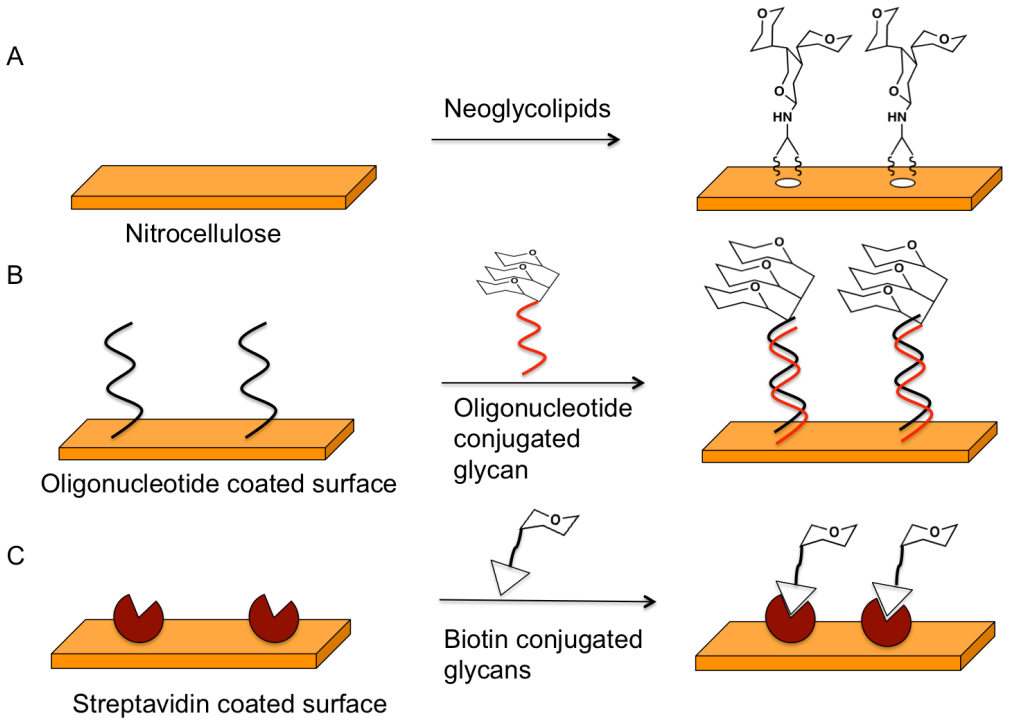

Figure 1.5.2. Non covalent glycan - region specific immobilization. Glycans attach to the surfaces with the help of non covalent forces such as hydrophobic interactions, hydrogen bonding etc. In these methods, the orientation of glycans can be controlled Figure 1.5.2 A depicts the use of neoglycolipids to create glycan arrays. Arrays created with the help oligonucleotide conjugated glycans (Figure 1.5.2 B). Biotin conjugated glycans immobilized on streptavidin-coated surfaces (Figure 1.5.2 C).

1.5.3 Covalent and Non-Specific Glycan - Region Immobilization

Methods developed in this category of glycan immobilization rely on attaching the glycans to photolabile surfaces by UV irradiation. Unmodified sugars are attached to surfaces such as azidoaryl and phthalimide coated surfaces. Azidoaryl groups on the surface get converted to highly reactive nitrene intermediates that react with the glycans to form covalent bonds.[34] Phthalimide coated surfaces yield a triplet state diradical that abstracts a hydrogen atom from a glycan molecule resulting in radical recombination to form glycan arrays.[35] Other approaches include use of boronic acid coated surfaces that react with 1,2 or 1,3 diols of glycans.[36] (Figure 1.5.3) While each of these methods results

in the attachment of the glycan to the surface via a covalent bond and thus robust linkages, these methods are not appropriate for the production of microarrays. As none of these methods can be used to control either the density of the glycan or the orientation of the glycan in presentation in the microarray.

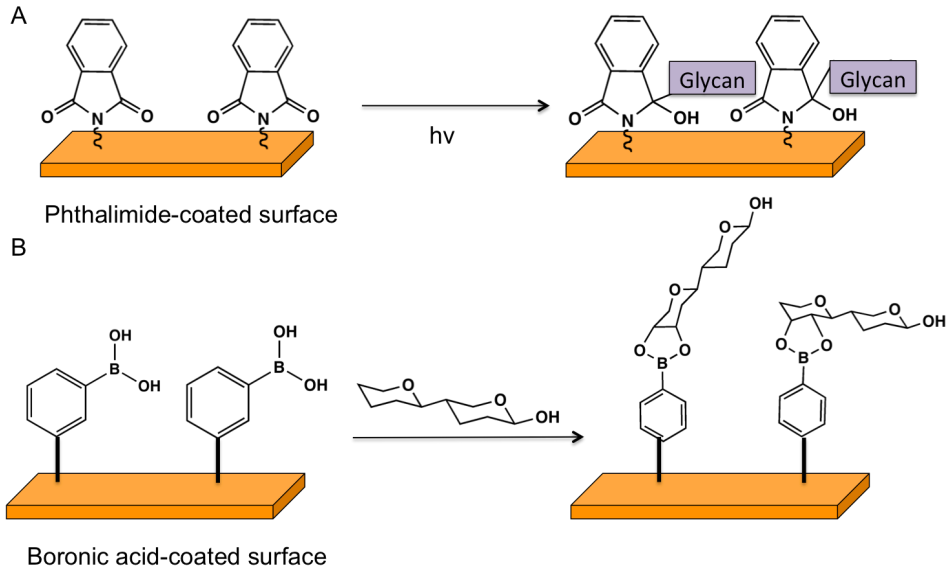

Figure 1.5.3. Covalent glycan - region specific immobilization. Glycans attach to the surface by forming a covalent bond with the surface, however the orientation of the glycans cannot be controlled as they attach to the surface.

1.5.4 Covalent and Specific Glycan - Region Immobilization

The most popular technique to immobilize modified glycans on the surface of an array is to employ the reaction of a modified glycan with a functionalized surface such as outlined in Figure 1.5.4 A. When applying this technique, it is important to attach a spacer group to the glycan. This linker maintains a particular distance between the surface and the glycan It has been demonstrated that this spacer is required to ensure that binding with large sized proteins can be observed.

Various functional groups such as thiols, epoxides, amines, aldehydes have served as the reactive moiety on the functionalized surfaces. Surfaces coated with a monolayer

of the reactive species are then treated with glycans labeled with complementary functional group that reacts to form a covalent bond between the surface and the glycan.

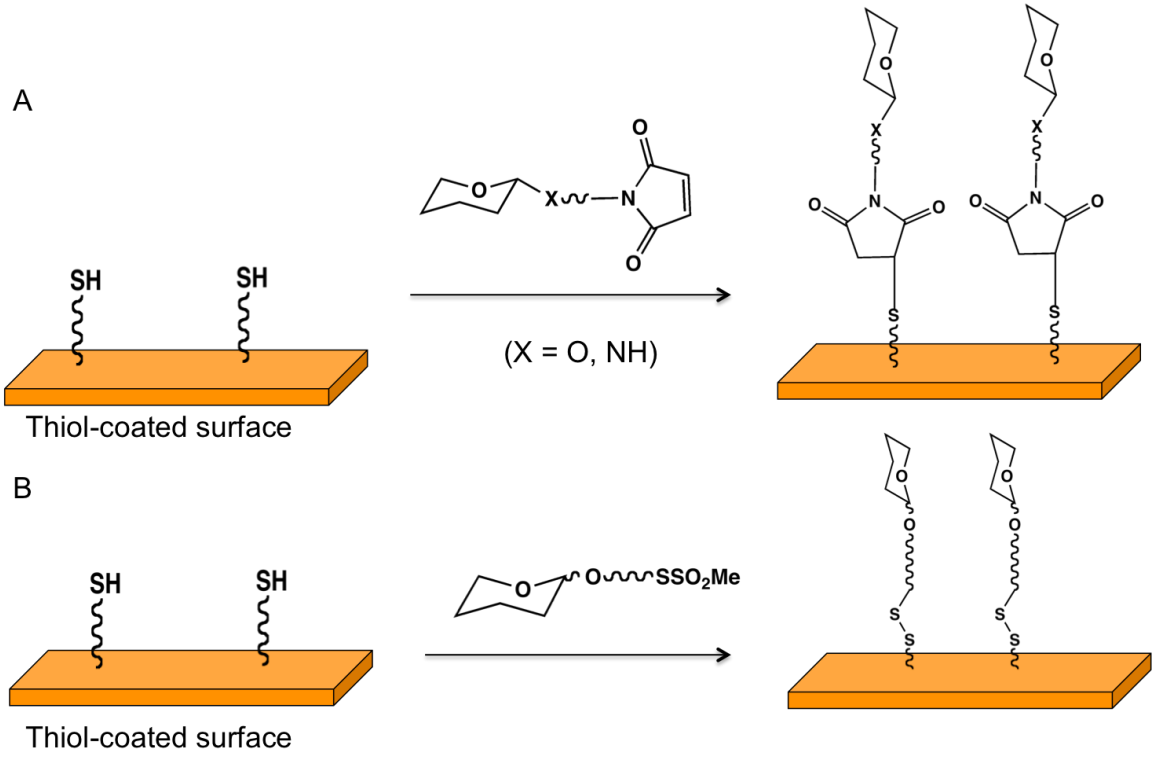

Figure 1.5.4 A. Linking glycans to surfaces using thiol-malemide and disulfide covalent bonds.

Park et al. prepared glycan microarrays via attachment of mono- and polysaccharides to the thiol-derivatized glass slides with maleimide linkers as outlined in Figure 1.5.4 [37] In this technique, tethers of appropriate length were attached to separate the sugar from the maleimide linker. Thiol surfaces can also be used to prepare arrays that link the glycans to the surface by a disulfide bond. [38,39] (Figure 1.5.4 A) In this method, thiosulfonates or thiol conjugated glycans can be used as reactants.

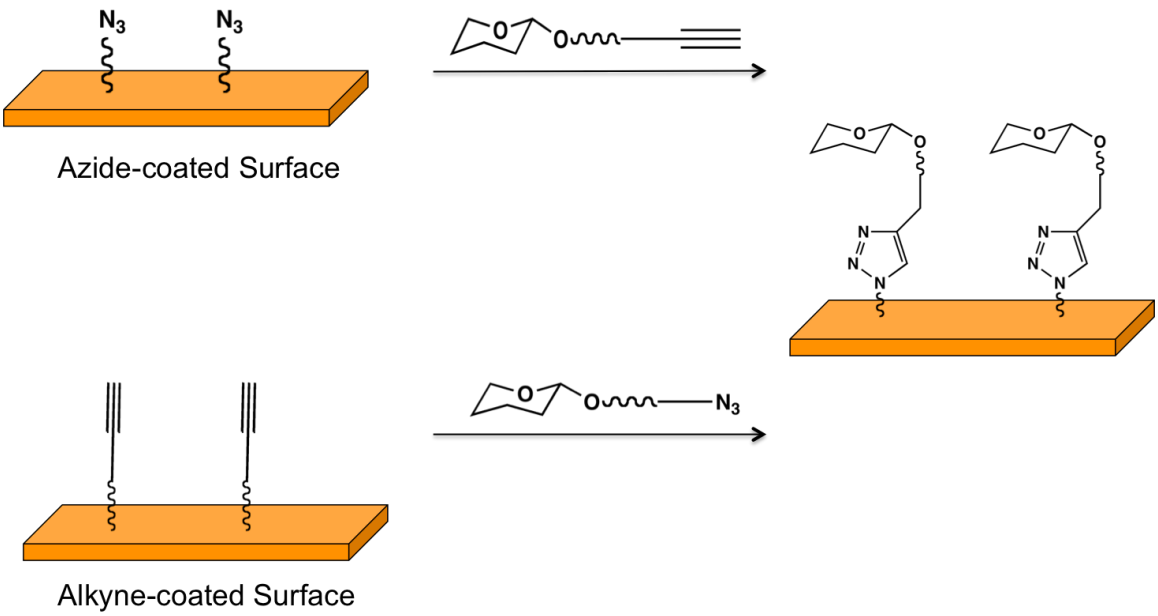

Figure 1.5.4 B. Use of Click chemistry for covalent immobilization of glycans to surfaces

Click chemistry reactions of alkyne and azides have been used extensively to prepare glycan microarrays (Figure 1.5.4 B).[40] Waldmann and coworkers have used Staudinger ligation as immobilization strategy to prepare small-molecule arrays.[41] This efficient technique makes use of azide functional group, which can undergo selective ligation with a phosphane modified glass slide.

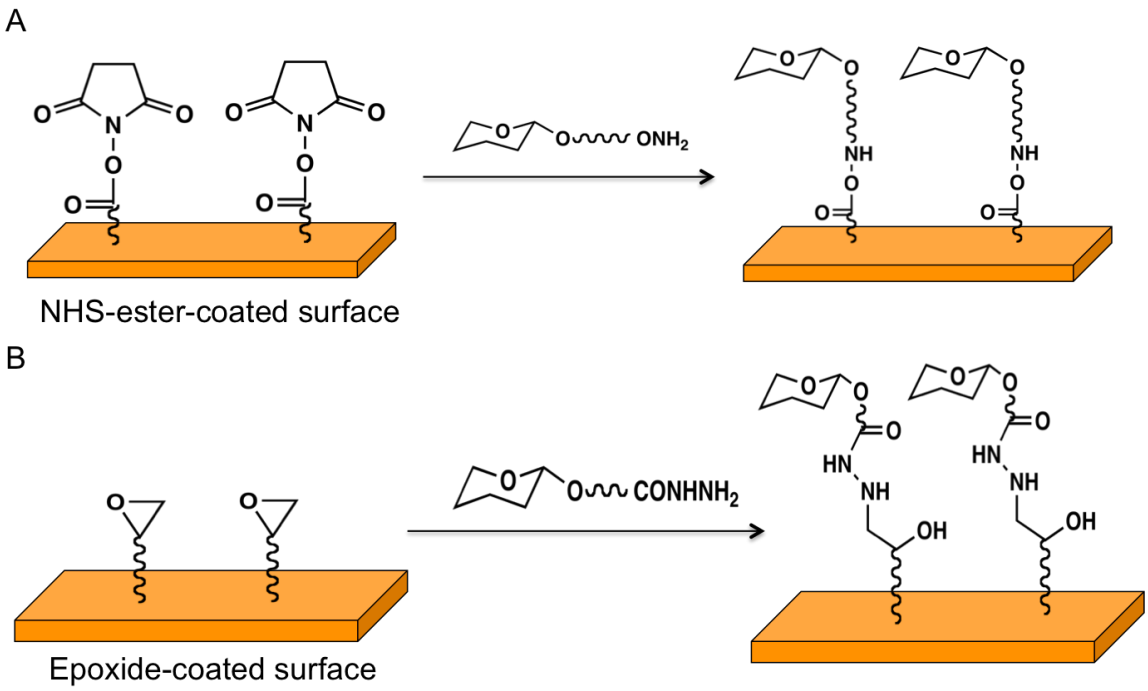

Figure 1.5.4 C. Use of ligation methods to immobilize glycans on the surface.

Ligation reactions between epoxide coated surfaces [43] and hydrazide linked glycans as well as NHS ester coated surfaces and amine linked glycans have been used to immobilize modified glycans on surfaces.

While all of these methods effectively attach glycans to surfaces, most of the methods described above cannot present the glycans in a "natural" conformation, as most of these covalent methods attach the glycans to the surface while restricting their mobility on the surface. Biomolecules attached to the surface of a cell are not anchored in a rigid manner into the membrane; they "float" in the membrane. Also, a cell's surface has a specific curvature. Creating glycan arrays on a flat surface does not present the carbohydrates as they are presented on a cell surface. It is of vital importance to create glycomic arrays mimicking a cell surface, in order to mimic glycan- lectin binding *in vivo*. As discussed

below, the production of glycan microarrays employing catanionic surfactant vesicles should be able to overcome many of the limitations with typical methodology for the production of glycan functionalized surfaces.

1.6 Surfactant Vesicles

Our research program has been focused on studying functionalized catanionic surfactant vesicles as a model for the cell surfaces of pathogenic bacteria. Catanionic surfactant vesicles are particularly useful in this regard due to the ease of their preparation, their robustness in biological media, and their cost when compared to liposomes and other self-assembled nanoscaled vesicles. Unlike liposomes, surfactant vesicles are formed from a mixture of two single-tailed surfactant molecules. It is possible to make surfactant vesicles of desired size by choosing the appropriate type of surfactant molecule and the method of preparation. Ease of preparation, stability and cost effectiveness are the characteristics that make surfactant vesicles an alternative to liposomes for a variety of applications.

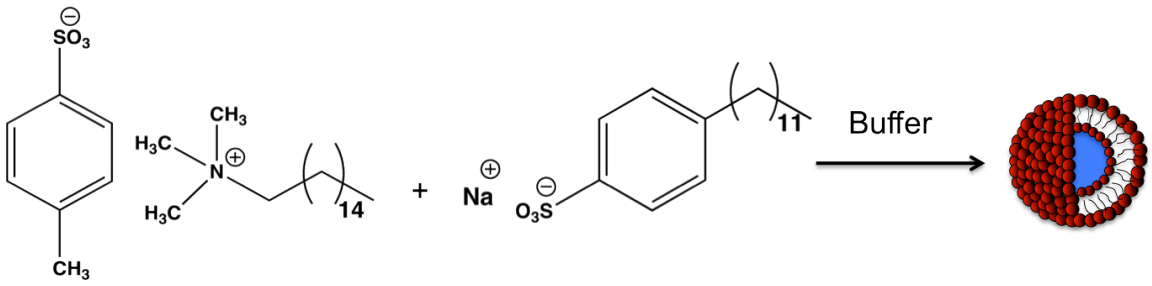

Figure 1.6. Surfactant molecules of SDBS and CTAT, when mixed in a particular ratio readily self-assemble in suspension to form surfactant vesicles

Kaler et al. first reported the formation of surfactant vesicles from the surfactants, sodium dodecylbenzesulfonate (SDBS) and cetyltrimethylammonium tosylate (CTAT).[44] (Figure 1.6) When combined in a ratio of 70:30 spontaneous formation of unilamellar

vesicles was observed. The resulting vesicles called catanionic surfactant vesicles, have an overall surface charge of the respective surfactant molecule in excess. After Kalers discovery, vesicles were looked at as ideal drug carriers since they enclose an aqueous compartment within a hydrophobic layer. Walker and coworkers were the first to decorate the outer surface of a vesicle with a biotinylated lipid.[45] Moreover, they were able to prove that these biotinylated molecules are available for binding to their streptavidin partners in order to form aggregates.

Studies have shown the ability of surfactant vesicles to incorporate charged/uncharged drug or dye molecules into the hydrophobic leaflet of the vesicle as shown in Figure 1.7 A.[46] It was observed that a molecule having a charge opposite to the charge of a vesicle encapsulates more readily into the vesicle, resulting in minimum leakage. English et al. proved that dye molecules like carboxyfluorescein could be incorporated in to the vesicles having a net positive surface charge. Anionic surfactant vesicles had a high encapsulation efficiency of cationic dyes and gave a long-term dye release profile. [46,47] Accordingly, catanionic vesicles have excellent potential as drug delivery vehicles.

1.7 Encapsulation of Glycoconjugates in Vesicles

Kaler et al. first reported that vesicles could encapsulate glucose.[44] In this instance, aqueous solutions of glucose were trapped into the lumen of the vesicles during formation of the lipid bilayer. Work by Caillet proved that loading efficiency of glucose in vesicles composed of sodium octyl sulfate (SOS) and cetyltrimethylammonium bromide (CTAB)) was only 1%.[48] However, this method results in leakage of glucose

over time presumably due to pore formation in the leaflet wall. Hence synthetic modifications were performed on the glycans in order to increase their encapsulation efficiency. Previous research in the DeShong group has been focused on incorporating various carbohydrate conjugates into surfactant vesicles and studying their properties.

It is possible to incorporate sugar linked to long aliphatic chains into vesicles presumably by insertion of the hydrophobic tail of the conjugate into the leaflet wall as indicated in Figure 1.7 B. Properties of vesicles containing glycoconjugates of glucose, maltose, maltotriose, and lactose have been studied.[49] Single-tailed glycoconjugates could be added in the preparation step of the vesicles, along with the surfactants resulting in the synthesis of vesicles in which the conjugate was embedded into the inner and outer surface of the leaflet. Single-tailed glycoconjugates have been also added after the preparation of vesicles, where the attached hydrocarbon tails simply get inserted in the bilayer of the vesicles, decorating the vesicle bilayer on the outside only. (Figure 1.7 B). The maximum quantity of glycoconjugate of glucose incorporated into the vesicles is 20 mole percent before the leaflet of the vesicle is destabilized and the vesicle ruptures.

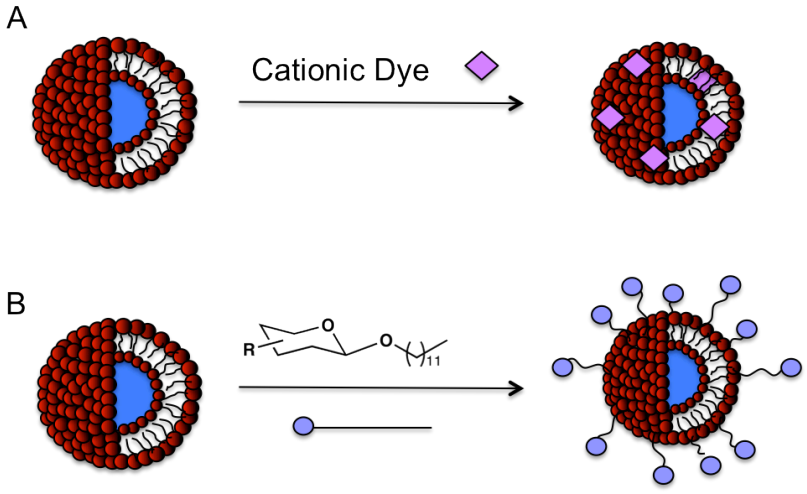

Figure 1.7 A. Incorporation of oppositely charged dye molecules in SDBS rich surfactant vesicles B. Incorporation of glycoconjugates in catanionic surfactant vesicles.

Our studies have shown that glycans functionalized with lipid tails (glycoconjugates) can be readily incorporated into vesicles. These glycoconjugates are surface expressed and are available for binding with proteins in solution.

1.8 Glycan - Lectin Binding Studies in Suspension

Thomas et al. have demonstrated that glucose functionalized catanionic vesicles to study the multidentate binding of the lectin concanavalin A (Con A).[50] (Figure 1.8) The binding of Con A to glucose functionalized vesicles was monitored by measuring aggregation. Employing catanionic vesicles in which the concentration of the glucose conjugate was varied on the surface of the vesicle, multivalent binding as a function of inter-ligand distance was studied.

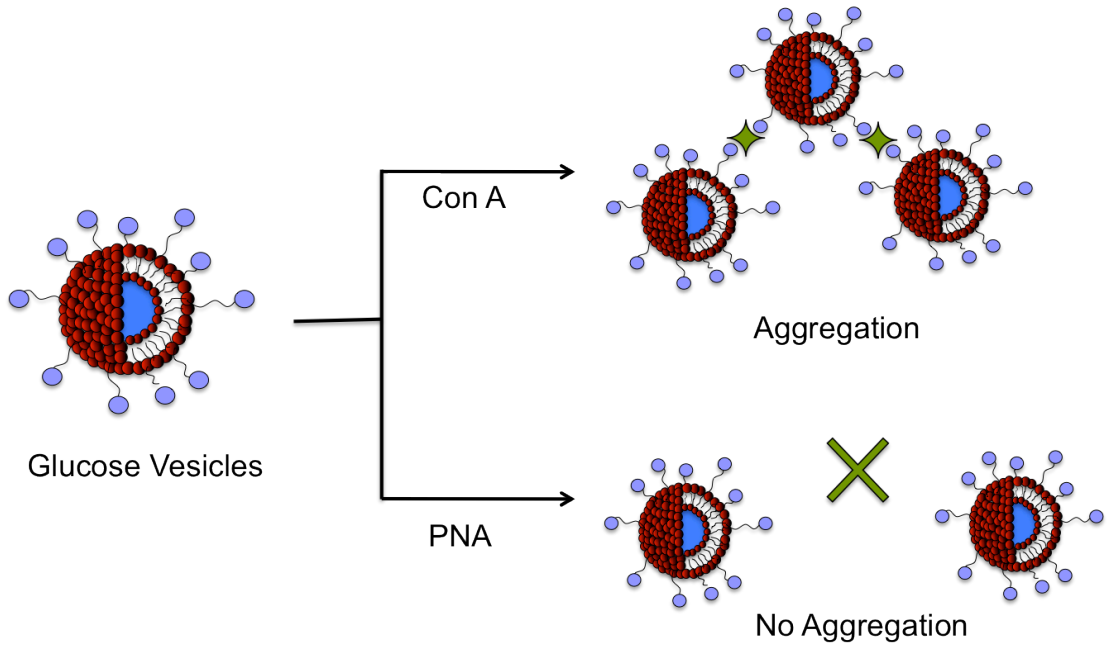

Figure 1.8. Aggregation of glucose vesicles with Con A in suspension. Glucose vesicles do not aggregate with PNA.

Assuming that the glycoconjugate formed a Poission distibution on the surface of the vesicle, it was possible to vary the surface density of the conjugate on the surface of vesicle. From the binding of Con A, Thomas determined that the distance between the two binding sites for glucose was 3.7 Å. When the O-glucoside conjugate as replaced with the N-linked analog shown in Figure 1.9, it was observed that a much lower concentration of the glucose conjugate was required to induce aggregation of the vesicles with Con A. This result with the N-linked conjugate was consistent with the formation of a cluster of conjugates thus increasing the effective concentration of glucose on the surface of the vesicle. It is hypothesized that the hydrogen bonding between the N-Linked glycoconjugates contributes to the cluster effect. (Figure 1.9)

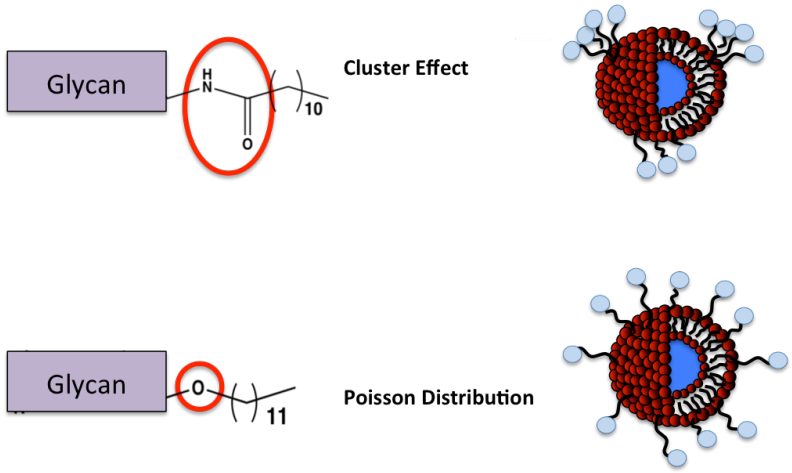

Figure 1.9 Effect of glycoconjugate structure on distribution of glycans on the surface of a vesicle.

Self-assembled monolayers (SAMs) derived from carbohydrate derivatives on gold have been shown to be useful multivalent systems for studying cell adhesion.[51] Work in our group was previously performed to create glycan modified SAMs. In order to fabricate SAMs of carbohydrates on gold, glucopyranosylamide conjugates were coupled to gold surfaces to create SAMs with different orientation of glycan molecules

on surfaces.[52] AFM and XPS studies performed on these surfaces reviled that hydroxylated glycoconjugates undergo dense packed monolayer formation on the surfaces, on the other hand, acetylated glycoconjugates were observed to form clusters on the surface.[52] The surface morphology of densely packed clusters was attributed to hydrogen bonding between functional groups of the glycoconjugates. Although preparation of SAMs provides some control over surface density and orientation of glycans on the surface, slight variations in density are not easily achieved with the help of SAMs. Surfactant vesicles provide an alternative strategy to control glycan density, applications of which will be discussed in later chapters.

1.9 Conclusions

Catanionic surfactant vesicles are excellent models to mimic cell surfaces of Gram negative bacteria because of their similar surface charge and spherical shape. These vesicles can be surface functionalized with glycoconjugates. The density and presentation of glycans on the surface of vesicles can be controlled simply. If glycan-functionalized catanionic surfactant vesicles can be deposited onto surfaces, then it should be possible to prepare arrays of glycans in which the density and presentation of glycans can be controlled for the study glycan recognition events. To achieve this goal, we first need to establish methods to deposit these vesicles on surfaces such that they maintain their integrity on deposition. Secondly, we need to prove that vesicles on surfaces undergo similar binding events with lectins when put on surfaces as in suspensions.

1.10 Research Goals

Study in the DeShong group has been focused on preparing and characterizing functionalized catanionic surfactant vesicles. Studies by Thomas, et al and Park, et al have demonstrated that vesicle bilayer can be decorated with various glycan molecules that show binding to appropriate lectins. We are able to control the concentration of glycans on the surface and the distribution of glycans on the surface is dependent on the type and the amount of carbohydrate used. Based on our solution phase studies we anticipated that preparation of carbohydrate microarrays using surfactant vesicles would provide means to construct glycan arrays with the ability to control concentration and distribution of glycans on the surface, thus addressing an important issue in this field.

The goals of my thesis study were:

1) To prepare surfaces to deposit catanionic vesicles such that they maintain their integrity on deposition.

2) To establish that, once deposited on a surface, glycans incorporated in vesicles exhibit similar binding with lectins and antibodies in solution.

3) To establish the effect of glycan concentration on the extent of binding on surfaces.

4) To perform enzymatic chemistry on vesicle surfaces in suspension and on surfaces.

Accordingly, HM chitosan surfaces were prepared for deposition of vesicles in order to prepare glycan arrays, and lectin and antibody-binding studies with deposited glycans were conducted. These results are summarized in chapter 2. Chapter 3 describes performing enzymatic reactions on glycans on vesicle surfaces to modify the glycans in

suspensions as well as surfaces. And Finally, Chapter 4 includes the description of preparation of arrays using nitrocellulose surfaces to deposit vesicles. It also discusses our efforts to print vesicles on nitrocellulose surfaces using an ink jet printer.

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Chapter 2: Preparation Of Carbohydrate Arrays Using Hydrophobic Interactions To Anchor Functionalized Vesicles On Surfaces

Some parts of this chapter are related to the submitted article
A New Type of Glycan Array Using Immobilized Catanionic Vesicles Functionalized with Glycolipids- Neeraja Dashaputre, Amanda Mahle, Vishal Javvaji, Gregory F. Payne, Srinivasa R. Raghavan, Daniel C. Stein, Philip DeShong

2.1 Introduction

We aim to use glycan functionalized catanionic surfactant vesicles to serve as motifs to build glycan microarrays. The most important requirement in the design of a microarray is the preparation of a platform to deposit functionalized vesicles. (Figure 2.2) Vesicles being soft nanomaterial, tend to deform in to bilayer films upon deposition on conventional, hard microarray surfaces such as gold or glass.

Previous research to capture intact liposomes on surfaces has focused on DNA tethering[1], electrostatic attraction[2], or protein-ligand linking schemes[3] that require functionalization of the liposome surface with linking moieties. Kool and coworkers have reported fabrication of liposome arrays by depositing DNA conjugated liposomes on phospholipid bilayers modified with complementary DNA strands. [4]

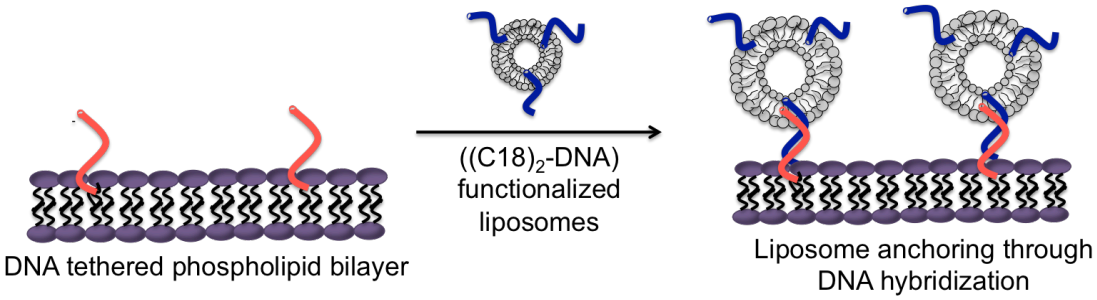


Figure 2.1. Liposome anchoring on phospholipid bilayer using DNA hybridization.[4]

Liposomes containing PEG-biotin lipids were affinity bound to NeutrAvidin® molecules immobilized onto solid supports to create liposome arrays by Vermette et al.[5] Capture of label free vesicles on surfaces has been achieved using non-covalent tethering of liposomes to lipophilic anchors on commercially available Biacore-L1 electrode.[6] Recently, Granqvist et al described their efforts to create deposited vesicle layers by depositing liposomes on thiolated PEG based surfaces.[7] Pond et al. have recently reported on the preparation of glycan microarrays by deposition of functionalized surfactant vesicles on nitrocellulose.[8]

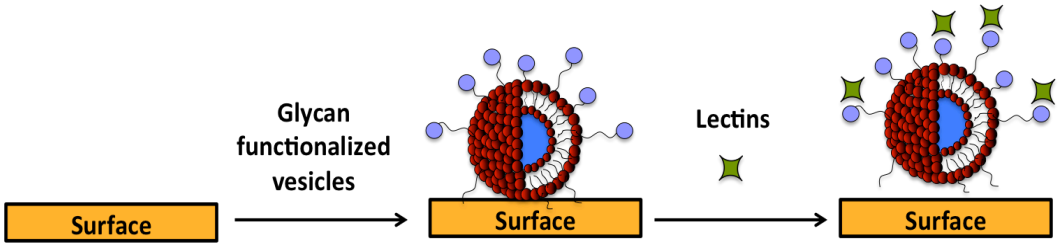


Figure 2.2. Strategy for preparation of glycan arrays using surfactant vesicles

In our group, the behavior of vesicles has been characterized and lectin binding studies have been performed in solution phase, in which the vesicles are intact and spherical.[9] It is important to keep vesicles intact upon deposition, so as to keep the morphology and aqueous compartment intact. In order to make microarrays using surfactant vesicles, the surface needs to be hydrophobic to an appropriate degree, to ensure that vesicles are prevented from rupturing upon deposition. My study was focused on preparing surfaces that will anchor catanionic vesicles in order to keep them intact on deposition.

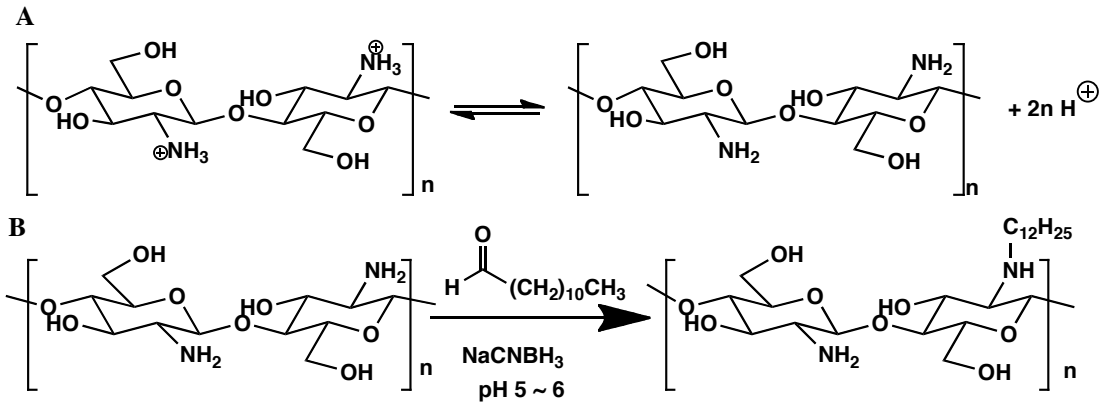
We have modified surfaces using APTES, Poly-L-lysine, alkylation reactions etc. to capture label free vesicles. Also, attempts to capture biotin labeled vesicles were made on streptavidin-coated 96 well plates. However, most of these surfaces did not anchor

vesicles on deposition. In order to confirm retention on the surfaces, vesicles labeled with dye (lissamine rhodamine) were deposited on these surfaces. This was followed by washing studies with buffer. With the surfaces listed above, the fluorescence was not retained on the surface after washing.

Surfaces modified with Hydrophobically Modified (HM) chitosan and nitrocellulose, however, retained the fluorescence after subsequent washing studies indicating that vesicles get anchored on these surfaces. This chapter discusses the work with HM chitosan surfaces to prepare glycan arrays, including modification of surfaces and binding studies for glycan – lectin and glycan-antibody binding.

2.2 Preparation of HM Chitosan Surfaces

Work on aminopolysaccheride chitosan, by Payne and Raghavan group highlights chitosan's potential to become an appropriate platform to adsorb vesicles.[10] At low pH, amine groups on chitosan are protonated, thus making it a cationic polyelectrolyte (Figure 2.2 A). Further research by Raghavan group indicated that after modifying the chitosan molecule, by attaching aliphatic chains, converted it into an even better material for adsorbing vesicles (Figure 2.2 B).[11]



Scheme 2.1 A. Structure of chitosan B. Scheme of conversion of chitosan to HM chitosan, C_{12} hydrophobes were attached to the amine groups of chitosan via reductive amination reaction.

This new material, termed as HM (hydrophobically modified) chitosan, anchors the vesicles on itself, by inserting the aliphatic chains into the hydrophobic bilayer of a vesicle. Adding a solution of vesicles to a solution of HM chitosan results in formation of a vesicle-gel like solution, indicating that chitosan network could support the vesicles to form a hierarchical system.[12] (Figure 2.3)

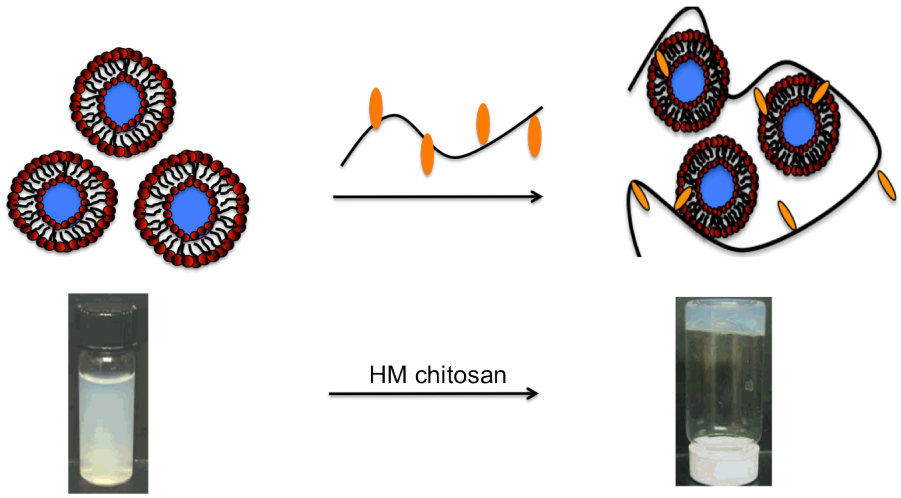


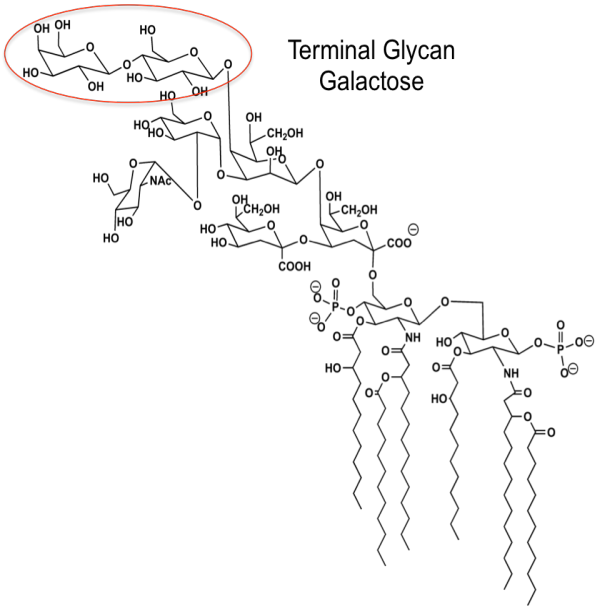
Figure 2.3. Vesicles and HM chitosan undergo a gel formation upon mixing. Gelation occurs due to hydrophobic interactions between the attached hydrophobes of HM chitosan and the hydrophobic bilayers of vesicles.

2.3 Specific Aims, Results and Discussions

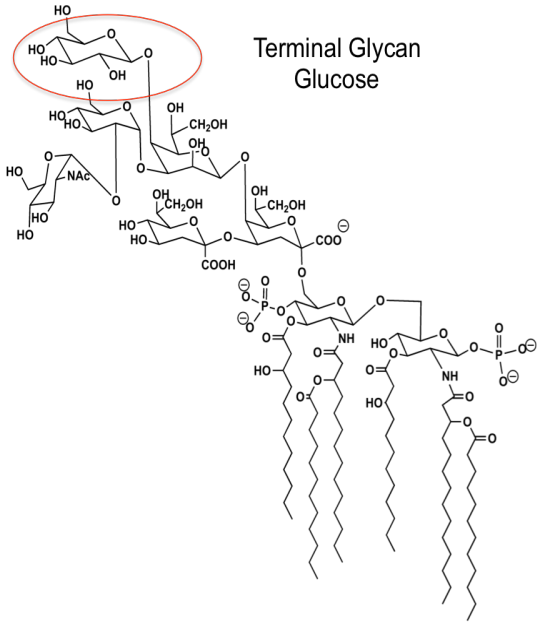
We had three goals for this study: (1) to develop an optimized procedure for the deposition of SDBS-rich surfactant vesicles onto HM chitosan surfaces, (2) to demonstrate that vesicles are deposited on the surface maintain their integrity (ligand density, distribution, etc.), and (3) to demonstrate that deposited vesicles can undergo binding with lectins from external solution.

Studies by Raghavan et al. include deposition of dye functionalized, 1 % CTAT rich surfactant vesicles on the surfaces electrodeposited with chitosan and HM chitosan. It was observed that these cationic vesicles selectively go on HM chitosan surface. [13] The authors were able prove that vesicles were intact after deposition on HM chitosan surfaces by performing a cryo-TEM of the deposited electrode. These studies were done using cationic vesicles, where as a cell, *in vivo* has a negative surface charge ~ -30 mv.[14] Thus, in order to have an accurate idea of carbohydrate-protein binding process *in vivo*, the surfactant vesicles used for the preparation of arrays need to have a zeta potential similar to that of a cell. Vesicles made with excess of the surfactant SDBS were observed to have a zeta potential ~ -55 mv (Table 2.1) Vesicles functionalized with nonionic molecules can have zeta potential close to -40 mV, depending on the type and the amount of ligand added. The structures of these molecules are depicted in figure 2.4. The systematic decrease in the zeta potential as glucose or lactose conjugate is incorporated can be attributed to the incorporation of a non-ionic surfactant into the vesicle leaflet, which results in reorganization of the leaflet components. For the same mole fraction of glycan, a lactose functionalized vesicle sample shows a higher positive-charge rendering to the membrane than glucose-functionalized vesicles. This also follows an expected

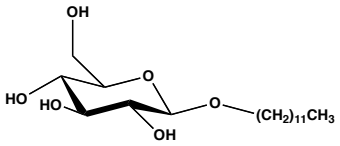
trend, as lactose being a disaccharide; it is expected to affect the rearrangement of bilayer more effectively than glucose. The much more significant decrease associated with the incorporation of the LOS F62ΔE is attributed to charged residues of the LOS (phosphate esters and carboxylates of the KDO sugars) that occupy positions in the hydrophilic outer layer of the leaflet. We have hypothesized that the zeta potentials of the catanionic vesicles are critical to high selectivity in subsequent binding studies (*vide infra*) and have chosen to maintain the zeta potential of the vesicles at >-40 mV which is comparable to the charge on the surface of a eukaryotic cell. (Figure 2.5) The SDBS-rich (anionic) vesicles utilized in this study are synthesized by combining the cationic surfactant CTAT and the anionic surfactant SDBS in a weight ratio of 30:70 (molar ratio ~ 1:2). The resulting catanionic vesicles are highly stable after formation and are unilamellar structures with a diameter 140 ± 30 nm, as measured with DLS.



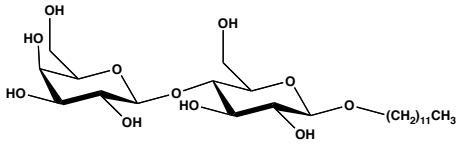
LOS *N. gonorrhoeae* F62ΔlgtA



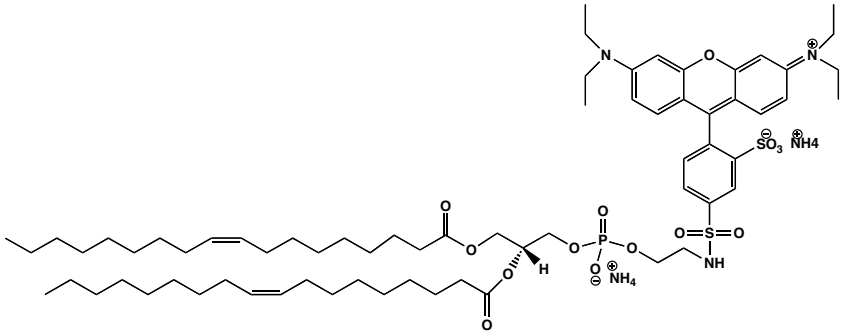
LOS *N. gonorrhoeae* F62ΔlgtE



β glucopyranosyl dodecyl ether (C_{12}-glucose)



β lactopyranosyl dodecyl ether (C_{12}-lactose)



Lissamine Rhodamine B lipid

Figure 2.4. Structures of glycoconjugates, lipooligosaccharides and dye molecules that were incorporated in vesicles.

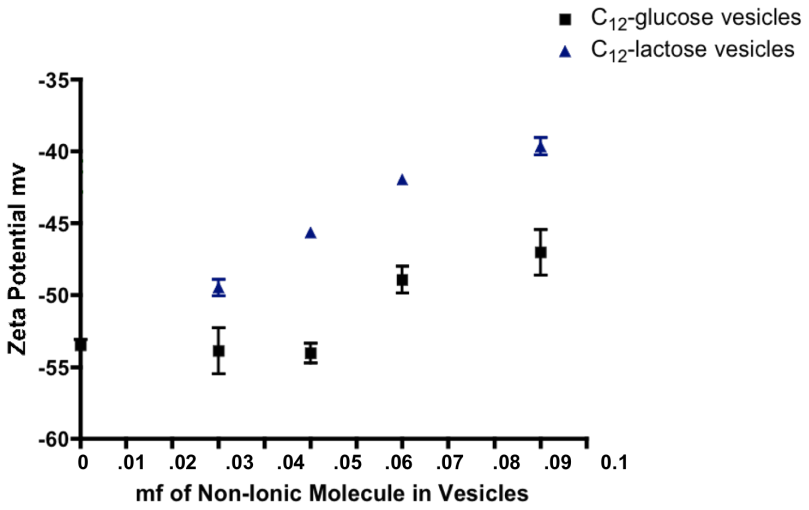


Figure 2.5. Zeta potential of functionalized with glucose or lactose functionalized SDBS rich vesicles. Non ionic molecules render a positive surface charge to the vesicle bilayer.

Sample	Mf of glycan	Zeta potential (mv)
Bare 1 % SDBS rich vesicles	-	-53.46 ± 0.66
C_{12} glucose functionalized vesicles	0.03	-53.86 ± 2.77
	0.05	-54.03 ± 1.18
	0.07	-48.9 ± 1.60
	0.1	-47.03 ± 2.73
C_{12} lactose functionalized vesicles	0.03	-49.46 ± 0.98
	0.05	-46.29 ± 0.24
	0.07	-42.17 ± 0.58
	0.1	-40.23 ± 0.42
LOS F62ΔE functionalized vesicles	0.001	-40.39 ± 0.38

Table 2.1 Zeta potential of functionalized SDBS rich vesicles

2.4 Strategy to Prepare Arrays using Surfactant Vesicles

In order to prepare surfaces for deposition of vesicles; electrodes were prepared by printing gold on silicon wafers in specific patterns. Fabrication of electrodes was carried

out on a silicon wafer by imprinting gold patterns, as described by Wu et al.[15] The dimensions of these electrodes are shown in Figure 2.7. Other alternative methods for preparation of HM chitosan surfaces were tested, which included dip coating or spin coating of HM chitosan on glass slides. However, electrodeposition of HM chitosan resulted in robust surfaces that were able to withstand the washing.

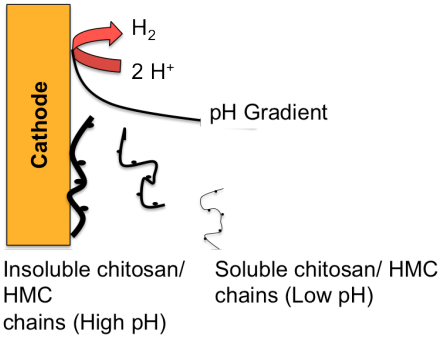


Figure 2.6. Electrodeposition of HM chitosan and chitosan on gold electrode.

HM chitosan and chitosan were then deposited on these electrodes using the process of electrodeposition. HM chitosan or chitosan derivatives have a pH-responsive character (pKa ~ 6.0) – i.e., it goes from a soluble to insoluble state as the pH is increased above 6. Accordingly, chitosan and HM chitosan films can be deposited on cathode surfaces upon application of a current due to the high local pH near this electrode. Figure 2.6 illustrates the process of electrodeposition of HM chitosan or chitosan on gold surfaces. Electrodeposition of this polyelectrolyte was performed on a gold surface, thus making a film. Previous studies by the Payne group indicated that varying the deposition time varies the thickness of this chitosan film.[15]

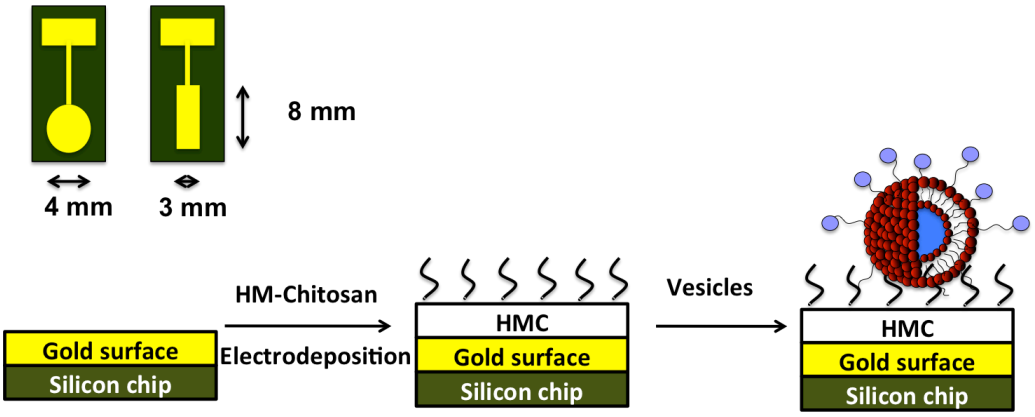
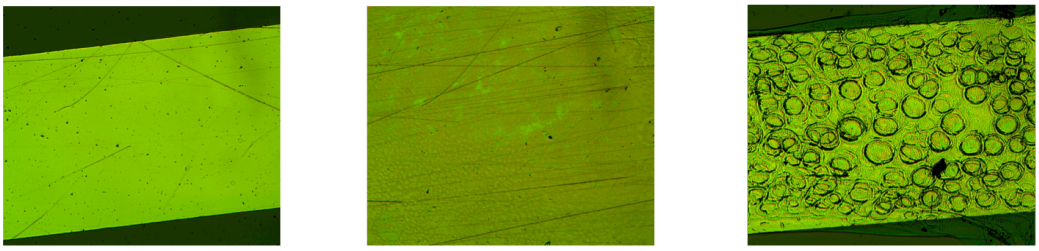


Figure 2.7 A. Strategy for the preparation of glycan microarrays using HM chitosan films for the deposition of vesicles. Top left corner depicts the electrodes used.



Bare gold electrode | Electrode deposited with HM chitosan | Electrode deposited with HM chitosan and vesicles

Figure 2.7 B. Optical microscopy images of electrodes at various stages. These pictures clearly indicate the morphology changes that occur upon deposition of vesicles.

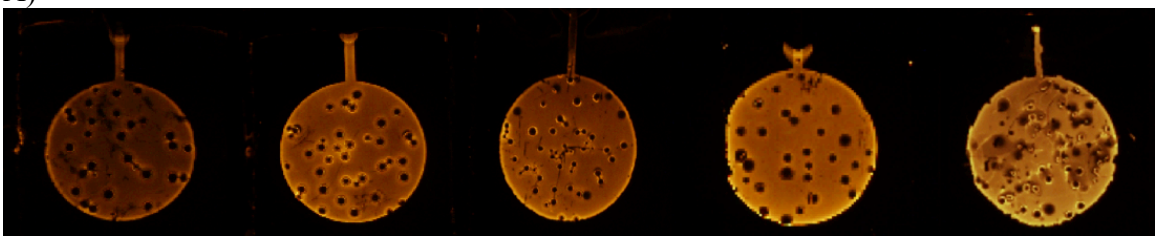
Our strategy to build glycan arrays is based on preparing HM chitosan deposited electrode surfaces to deposit glycan-functionalized vesicles. Once deposited on the surface, these glycans will serve as templates to bind proteins from external solutions. Figure 2.7 A demonstrates our strategy for preparation of glycan arrays.

2.5 Deposition of Vesicles on HM Chitosan Surfaces

Next, we wanted to check the ability of SDBS rich vesicles to adhere to HM chitosan surface. For this, the electrodes were inserted in a lissamine rhodamine dye (0.001 mf) functionalized 1 % SDBS rich vesicle solution for different intervals of time.

Fluorescence was observed after 5 min, 10 min, 20 min, 40 min and 1 h of incubation time (Figure 2.8 A). All other experimental variables, including rinsing times and image exposure time were kept constant. There is a significant increase in fluorescence between 5 to 20 min and slight between 20 and 40 min. The surface showed saturation after 40 min of incubation with surfactant vesicles (Figure 2.8 B). Hence all experiments were done with a vesicle deposition time of 40 min. Saturation of fluorescence is an indication that vesicle capture on HM chitosan proceeds by electrostatic attraction as well as surface diffusion of vesicles. As vesicles interact with free hydrophobes from the polymer, they get strongly attached to the surface. As the free hydrophobes get used up, fewer vesicles are able to bind strongly and the binding capacity of the film becomes saturated.

A)



5 min 10 min 20 min 40 min 60 min

B)

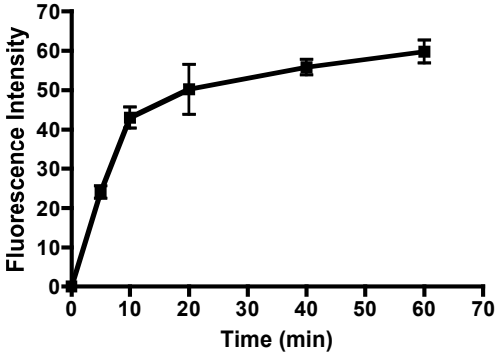


Figure 2.8 A. Fluorescence images of HM chitosan deposited electrodes with lissamine rhodamine dye labeled vesicles, deposited with varying deposition times. B. Effect of varying deposition times of vesicles on fluorescence intensity. We see saturation in fluorescence intensity after 40 min of deposition time.

2.6.1 Buffer Washing Studies

In order to verify the ability of HM chitosan surface to anchor vesicles, we deposited chitosan and HM chitosan onto gold electrodes and studied the binding of dye labeled- SDBS-rich catanionic vesicles to each surface. These surfaces were then subjected to washing with buffer solutions. In order to determine if HM chitosan and chitosan deposited on the surface, withstands the extensive washing process, fluorescently (FITC) tagged HM chitosan and chitosan was deposited on electrodes (Figure 2.9 A). These electrodes were observed under fluorescence microscope. The fluorescence on these surfaces was retained after extensive washing (Figure 2.9 B). This indicated that HM chitosan and chitosan surfaces maintain the film after extensive washing with PBS.

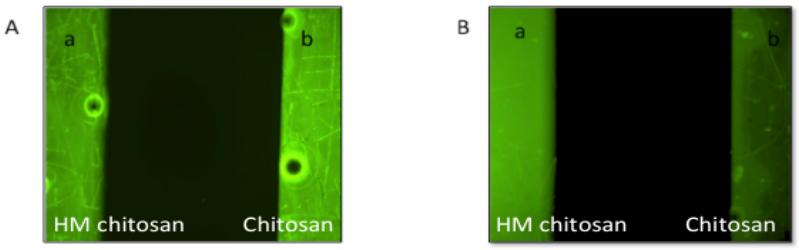


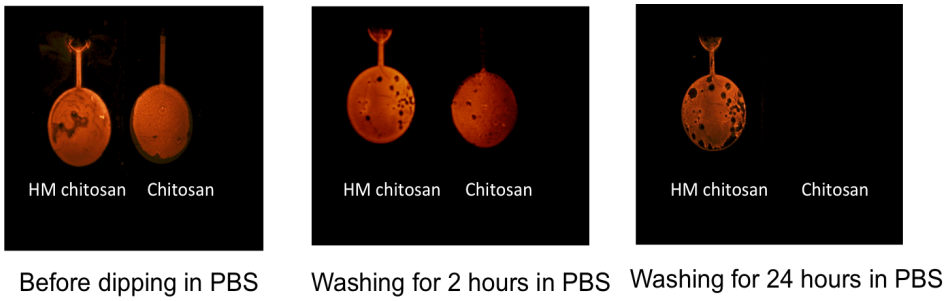
Figure 2.9 Electrodeposited fluorescein labeled HM chitosan (a) and fluorescein labeled chitosan (b) on gold electrodes A. immediately after deposition. B. After washing for six days after deposition.

2.6.2 Ability of HM Chitosan to Anchor Vesicles

Vesicles containing lissamine rhodamine dye (0.001 mf) were coated onto each of the surfaces, followed by washing of the surfaces with PBS buffer. The fluorescence of the dye on the surface was used as a measure of retention of the vesicles on surfaces. While both the chitosan and HM chitosan layers initially bound labeled vesicles, after extended exposure to buffer (20 h), the vesicles were retained only on the HM chitosan

surface (Figure 2.10 A). A control experiment in which just lissamine rhodamine dye was added on HM-chitosan surface shows negligible binding to the surface after washing with buffer. To explain this result, we propose that initially the SDBS rich catanionic vesicles go to the positively charged HM chitosan and chitosan surfaces with equal affinity. Hydrophobic chains from HM chitosan insert into the bilayers of the vesicles, thus anchoring the vesicles to the surface. Hence, after washing with buffer the HM chitosan surface retains it fluorescence. This experiment demonstrates that HM chitosan is the more effective surface for the preparation of vesicle-derived arrays. Hence subsequent experiments were conducted on HM chitosan functionalized surfaces only.

A)



B)

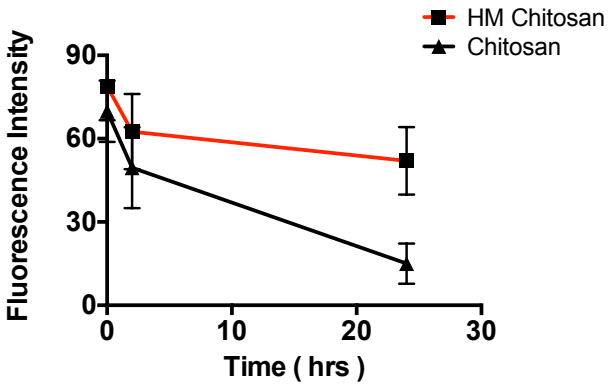


Figure 2.10 A. Images of dye labeled vesicles on deposited HM chitosan (HMC) and chitosan electrodes, B. Plot of fluorescence intensity vs. time. This plot indicates the effect of washing on fluorescence intensity of deposited lissamine rhodamine vesicles on HM chitosan and chitosan. The average intensity of fluorescence on the surface of HM chitosan decreases from 87.71 to 59.41 in 24 hours of washing, where as chitosan surface shows an intensity value decrease from 81.96 to 19.37 in 24 hours (n=5).

2.7 Integrity of Vesicles after Deposition

To verify that catanionic vesicles were intact after deposition onto the HM chitosan surface, an indirect assay was employed. SDBS-rich catanionic vesicles were deposited on a HM chitosan surface the surface was treated with PBS buffer containing 10% (v:v) Triton X100 (Figure 2.11 A). Triton X100 is known to disrupt vesicles in suspension, and the disruption of the colloidal suspension of vesicle can be monitored by DLS.

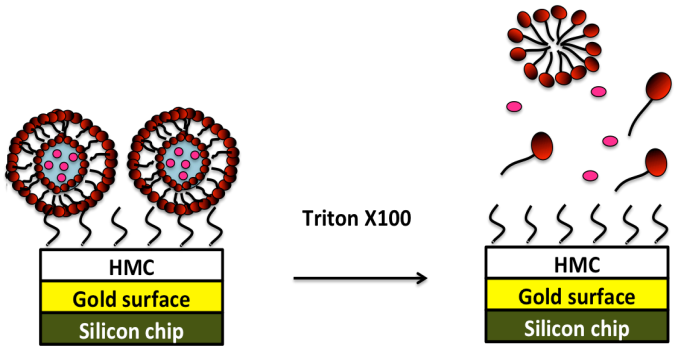
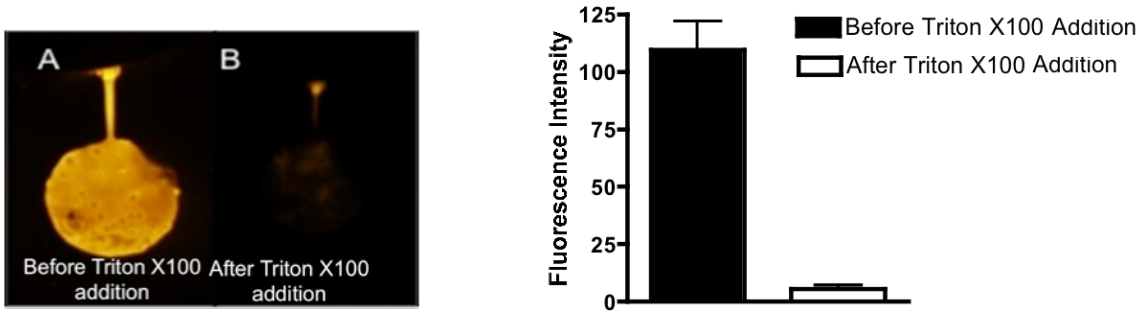


Figure 2.11. Disruption of vesicles with Triton X100. Dye functionalized vesicles lose fluorescence after treatment with Triton X100.

In addition, after disruption of the vesicle, the hydrophobic fluorescent dye is released. When vesicles deposited onto the HM chitosan surface were treated with Triton X100 in PBS buffer for 10 s, followed by washing with PBS buffer, the rhodamine fluorescence was lost from the surface (Figure 2.12 A). Since the vesicles were purified with size exclusion column, we are sure that the excess dye was removed and all dye was encapsulated in vesicles. The control experiment in which Triton X100 was not added to the vesicle-coated surface did not result in the loss of fluorescence. We interpret the loss of fluorescence to the disruption of vesicles, which in turn implies that the vesicles were intact prior to contacting Triton X100.

A) B)



2.12 A. Disappearance of fluorescence of lissamine rhodamine functionalized vesicles after treatment with Triton X 100. A shows the fluorescence of vesicles under red filter, B shows the same electrode after subjecting to 10 % triton X 100 solution for 10 s.
2.12 B. Graph of fluorescence intensity on electrodes before and after Triton X100 addition (n=5).

2.8 Lectin Binding Studies with Glycan Functionalized Vesicles

To demonstrate that vesicles deposited on the HM chitosan surface behaved analogously to their counterparts in suspension, we studied the binding of lectins to glycan-functionalized vesicles. Multivalent binding of glycans by lectins is a hallmark of glycobiology. Previous studies in our lab have demonstrated that glycoconjugates derived from carbohydrates (glucose and lactose, respectively) are readily incorporated into the bilayer leaflet of catanionic vesicles, and the resulting glycan-functionalized SDBS-rich vesicles can bind with their cognate lectin (glucose to Con A; lactose to PNA) with high selectivity under physiological conditions. [9,16]

2.9 Binding Assay

The electrode was electrodeposited with HM chitosan. This electrode was then immersed in a solution of glycan functionalized vesicles. After this step, the vesicles were immersed in FBS solution. The electrode was then rinsed with DI water and exposed to a

solution containing fluorescently tagged protein that non-covalently binds to the glycan with affinity. The electrode was then washed with FBS solution followed by a washing with water. Finally, the electrodes were observed under microscope.

To visualize the binding of glucose functionalized vesicles with Con A, vesicles functionalized with C_{12}-glucose were deposited on HM chitosan surfaces. The electrodes were then immersed in FBS solution. This step is really important to minimize the non-specific binding of FITC Con A to the surface. The electrodes were then immersed in a solution of FITC labeled Con A in FBS. Presence of ions such as Ca^{++} or Mg^{++} in solution is very important for conducting the Con A or PNA binding to corresponding glycans. Theses ions assist in formation of glycan-lectin tetramer. Typically it is achieved by adding HEPES buffer. FBS used in our experiments, contains 2 mmol Ca^{++} or Mg^{++} that assisted in binding. (Product specification, Sigma Aldrich). Hence experiments done in absence of HEPES also resulted in binding between lectins and glycans. After this, the electrodes were again washed with FBS and water, and viewed under microscope. In order to determine the Con A deposition time, we conducted studies where the Con A deposition time was varied from 10 min to 1.5 hour. Based on the fluorescence intensities of the electrodes, time of deposition of 1 hour was decided for the subsequent experiments.

A)

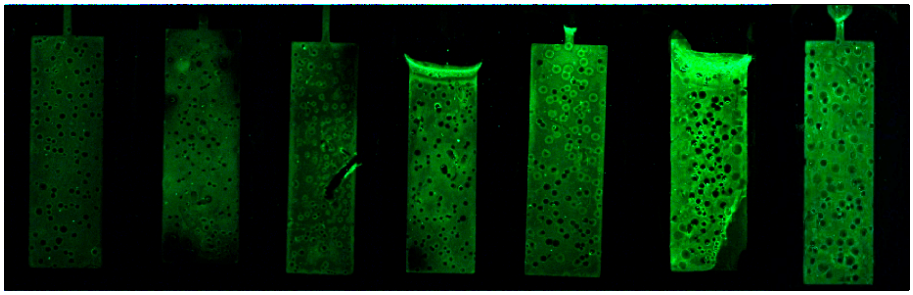


Figure 2.13 A. Images of electrodes as FITC Con A deposition time is varied.

B)

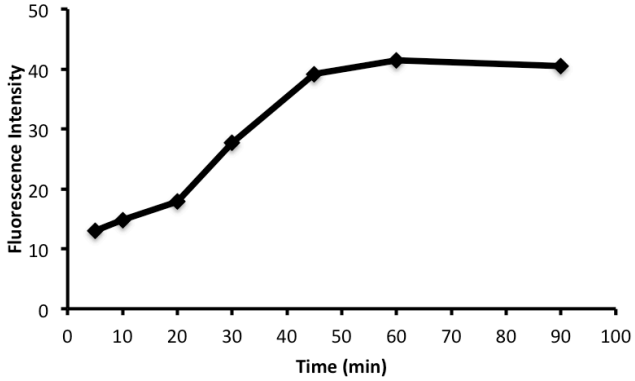


Figure 2.13.B Fluorescence intensity as a function of time of deposition of FITC labeled Con A.

2.10 Carbohydrate – Lectin Binding

Vesicles functionalized with 0.03 mf C_{12}-glucose and 0.03 mf C_{12}-lactose glycolipids were deposited onto different regions of a HM chitosan surface. When exposed to a solution of FITC-labeled Con A, the glucose region of the array preferentially bound the labeled protein, while almost no binding of the labeled PNA was detected on glucose region of the array (Figure 2.14). Conversely, treatment of the glycan arrays with FITC labeled PNA produced fluorescence on the lactose region where as FITC labeled Con A fails to bind to lactose labeled vesicles on surface (Figure 2.15). ConA specifically recognizes α-D-mannopyranosyl, β-D-glucopyranosyl, and β-D-fructofuranosyl moieties

and fails to recognize the lactosyl moieties in C_{12}-lactose functionalized vesicles. Similarly PNA recognizes β-D-lactopyranosyl and β-D-galactopyranosyl moieties and fails to recognize β-D-glucopyranosyl moieties in C_{12}-glucose functionalized vesicles. Negligible binding was observed with bare vesicles deposited on HM chitosan surfaces. These results demonstrate that functionalized vesicles coated onto HM chitosan behaved exactly as expected based on the results from colloidal suspension studies. We also confirm that glycan moieties embedded in vesicle bilayer, are available for binding with the corresponding lectins from external solution.

A)

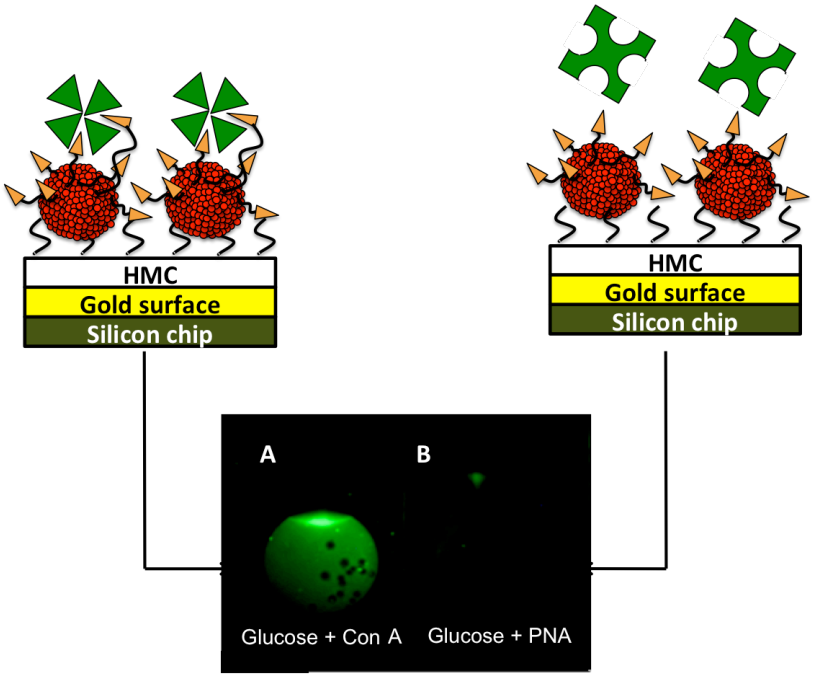


Figure 2.14 A. FITC labeled Con A binds to vesicles functionalized with C_{12}-glucose, where as FITC labeled PNA fails to bind to 0.3 C_{12}-glucose functionalized vesicles.

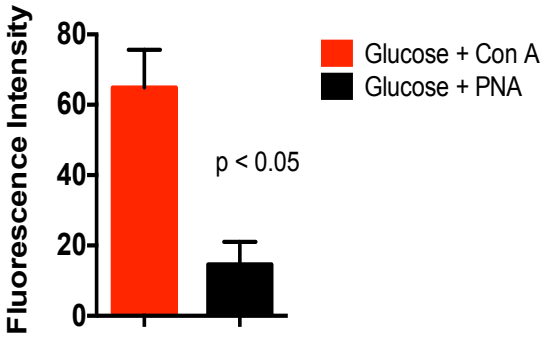


Figure 2.14 B. Fluorescence intensity on the Con A bound region (64.87 ± 18.34) is almost five times than the PNA bound region (14.63 ± 9.81), as measured with image j. The results were analyzed by a paired t test to find that the observations are significantly similar ($p < 0.05$, n=5)

A)

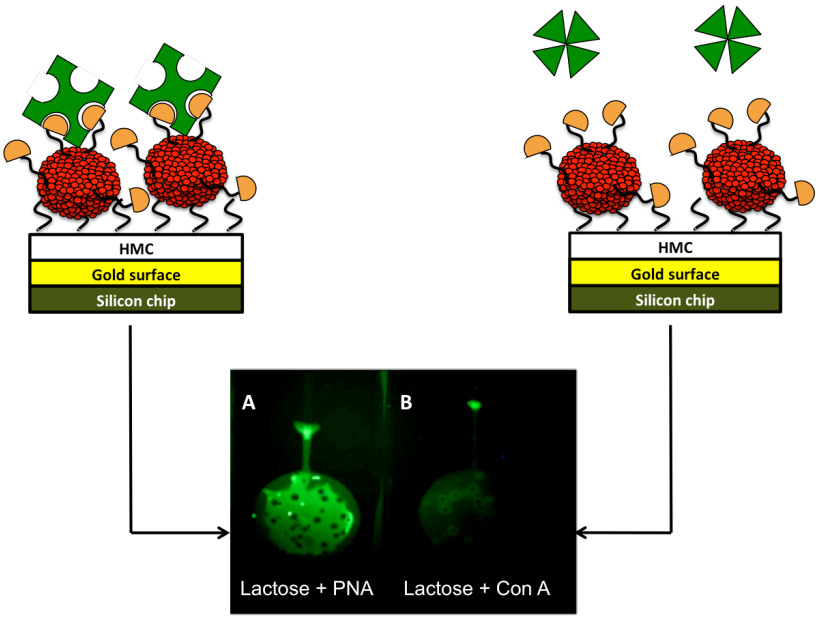


Figure 2.15 A. FITC labeled PNA binds to vesicles functionalized with C_{12}-lactose, where as FITC labeled Con A fails to bind to the lactose functionalized vesicles.

B)

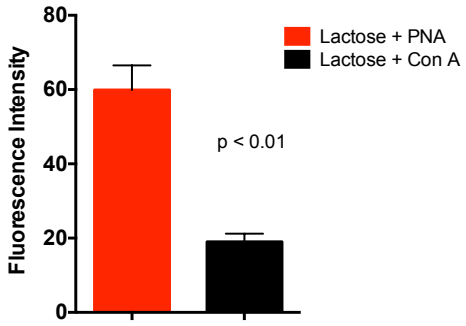
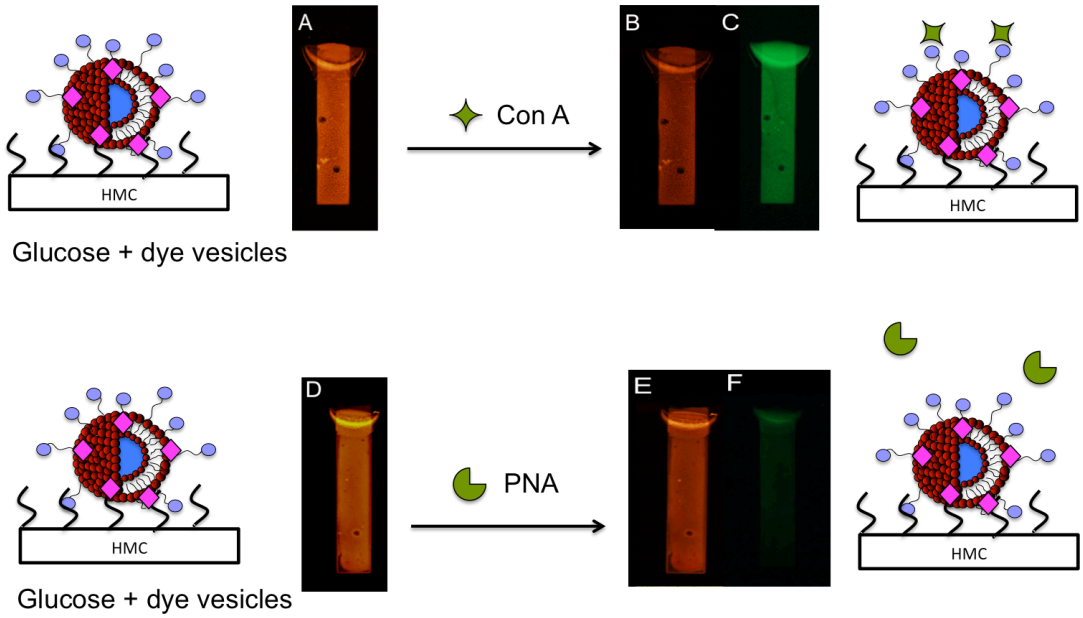


Figure 2.15 B. Fluorescence intensity is approximately 3 times higher on the PNA side (59.89 ± 11.47) relative to the Con A side (19.02 ± 3.75 gray value) as measured with image j. The results were analyzed by a paired t test to find that the observations are significantly similar ($p < 0.01$, n=5)

To further confirm the presence of deposited vesicles, vesicles were functionalized with glucose as well as lissamine rhodamine dye, thus allowing us to see them under different filters. The presence of vesicles was confirmed before (Figure 2.16 A) and after (Figure 2.16 B) the addition of FITC-Con A, by observing the fluorescence of lissamine rhodamimne dye under red filter. Figure 2.16 C indicates the binding of FITC labeled Con A to the deposited C_{12}-glucose vesicles, as observed under green filter. Repeating these experiments with peanut agglutinin (PNA) served as a complementary proof of the binding, since PNA is known to not show selective binding to glucose. Figure 2.16 D and E indicate the presence of glucose-dye vesicles, before and after exposure to FITC labeled PNA as observed under red filter. Our experiments with these vesicles show that C_{12}-glucose functionalized vesicles do not bind to FITC labeled PNA (Figure 2.16 F).

The reduction in fluorescence intensity of lissamine rhodamine dye before and after the addition of lectins (A to B and D to E) could be attributed to the extensive washing in binding assay. It is possible that washing results in loss of some vesicles from electrode surfaces. This experiment was repeated thrice to yield a intensity values as shown in the

Figure 2.17. The surface exposed to FITC Con A consistently shows higher binding (fluorescence intensity value 39.47 ± 2.95) than FITC PNA deposited electrodes (fluorescence intensity value 4.81 ± 3.95)



A: C_{12} glucose - dye labeled vesicles before FITC Con A deposition (under red filter)
B: C_{12} glucose - dye labeled vesicles after FITC Con A deposition (under red filter)
C: C_{12} glucose - dye labeled vesicles after FITC Con A deposition (under green filter)
D: C_{12} glucose - dye labeled vesicles before FITC PNA deposition (under red filter)
E: C_{12} glucose - dye labeled vesicles after FITC PNA deposition (under red filter)
F: C_{12} glucose - dye labeled vesicles after FITC PNA deposition (under green filter)

Figure 2.16. FITC labeled Con A binds to vesicles functionalized with C_{12}-glucose and lissamine rhodamine dye, FITC labeled PNA fails to bind to C_{12}-glucose functionalized vesicles. Presence of vesicles is observed under red filter.

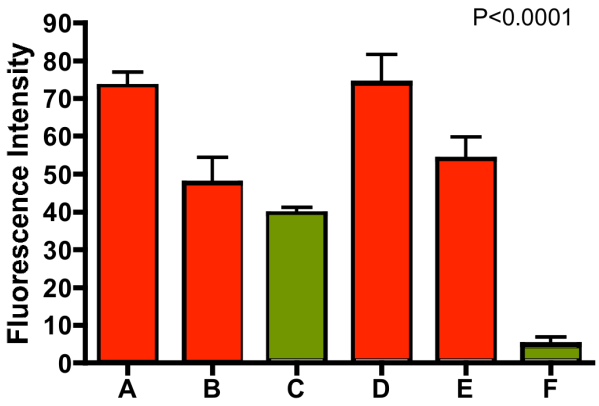


Figure 2.17. Fluorescence intensities of respective electrodes. The results were analyzed by a paired t test to find that the observations are significantly similar ($p < 0.0001$, n=3)

Studies conducted with glucose and dye functionalized vesicles confirm the integrity of glucose vesicles on the HM chitosan surface that exhibit effective binding to Con A and show negligible binding to PNA.

Our lectin binding studies clearly demonstrate that functionalized vesicles coated onto HM chitosan behaved just as expected, based on the results from colloidal suspension studies. The glycan exhibited on the vesicle bilayer are available for binding with their corresponding lectin partners in solution.

2.11 Detection of Complex Carbohydrates

Having done studies for simple carbohydrates such as glucose and lactose, we next wished to use our vesicle arrays to detect complex carbohydrates such as Lipooligosaccharide (LOS) and lipopolysaccharides (LPS) found in bacterial cell membranes.

LOS is a structural variant of LPS that terminates at the outer core region (figure 2.4). It consists of oligosaccharide component that is attached to lipid A via a Kdo (3-

deoxy-D-manno-octulosonic acid) linkage. LOS is typically synthesized by many mucosal pathogens such as *Bordetella pertussis, Haemophilus influenza, Neisseria gonorrhoeae, and Neisseria meningitidis*. In contrast to LPS, LOS can be branched with one to three oligosaccharide chains extending from the inner core heptose molecules.[17]

In order to demonstrate that our vesicle arrays can be used to detect complex carbohydrates, vesicles functionalized with LOS isolated from *N. gonorrhoeae* F62ΔlgtA were prepared and deposited onto HM chitosan electrodes. Binding of these vesicles with LOS-specific mAb 2-1-L8 conjugated with Dylite 633 was studied. With a fluorescently tagged antibody, binding could be readily detected by fluorescence microscopy. Control experiments using bare and C_{12}-glucose functionalized vesicle surfaces showed only background binding by the antibody (Figure 2.18). As represented in Figure 2.18 B, the fluorescence intensity on electrodes deposited with LOS functionalized vesicles is almost seven fold higher than the surfaces deposited with control vesicles. Binding of antibody to LOS-functionalized vesicles was very strong; indicating that arrays derived from complex glycans can serve as platforms for more sophisticated recognition strategies required for detection devices.

Figure 2.19 summarizes our attempts with binding LOS F62ΔE functionalized vesicles with polyclonal antibody tagged with cyan dye. The high-specificity binding of an antibody to the LOS vesicle suggests that the preparation of microarrays derived from the unique LOS/LPS glycans of bacterial pathogens can be used to generate arrays in which the "signatures" of pathogens can serve as the basis of diagnostic applications.

A) B)

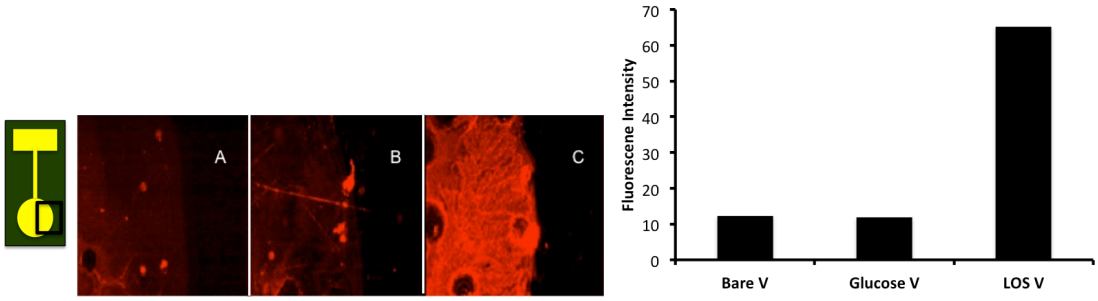


Figure 2.18 A. Detection of *N gonorrhoeae* LOS F62ΔA vesicles. Lectin binding studies of vesicles functionalized with LOS (C) with fluorescently labeled antibody goat anti GC mAb 2-1-L8 (tagged with DyLight 633) indicate LOS-antibody binding, (A, B) indicate the control experiments with bare vesicles and glucose vesicles.
Figure 2.18 B. Comparison of fluorescence intensities on the surface deposited with bare vesicles, C_{12}-glucose vesicles and LOS vesicles.

A) B)

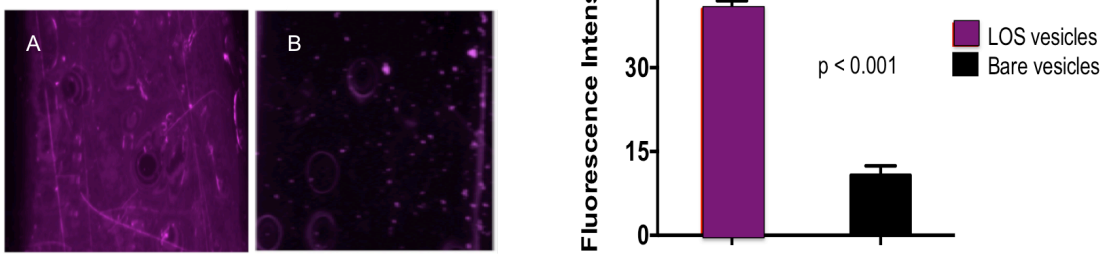


Figure 2.19 A. Cyan dye labeled goat anti-GC poly-clonal antibody was deposited on vesicles functionalized with LOS F62ΔE (A), control experiments with bare vesicles (B). These studies were repeated for four electrodes.
Figure 2.19 B. shows the comparison of fluorescence on the surface deposited with bare vesicles, and LOS F62ΔE vesicles. The results were analyzed by a paired t test to find that the observations are significantly similar ($p < 0.001$, n=4)

2.12 Detection of Artificial Pathogens using Arrays

At this point, we have demonstrated that arrays can be made with surfactant vesicles functionalized with simple mono and di-saccharides as well as complex lipooligosaccharides. A study in DeShong group done by Dr. Lenea Stocker showed that surfactant vesicles could be used to prepare artificial pathogens. Detergent-mediated

reconstitution is a common strategy used for insertion of proteins in to liposomes. A methodology developed by Dr. Lenea Stocker in the DeShong group demonstrated the way to incorporate cellular components in surfactant vesicles (Figure 2.20, manuscript under preparation)

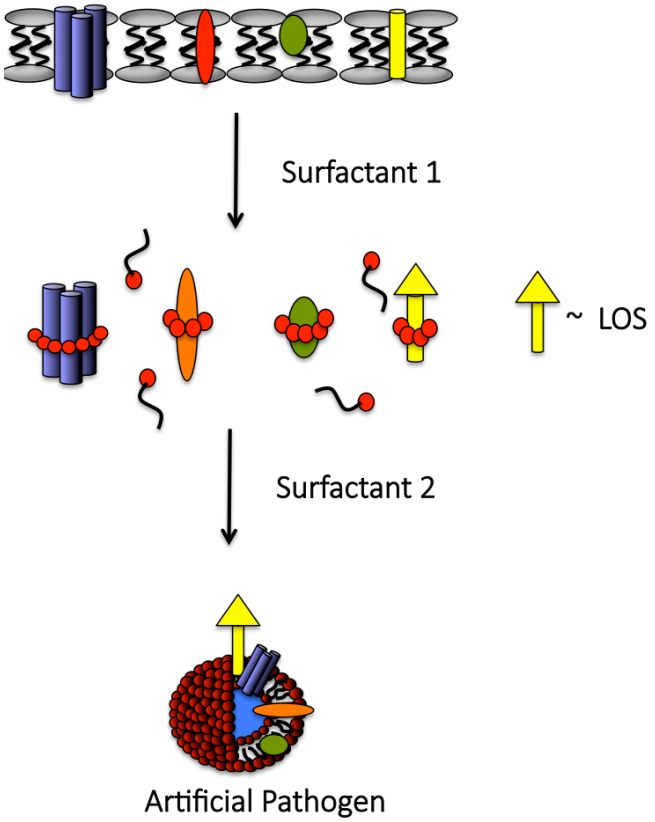


Figure 2.20. Preparation of 'Extract Vesicles'

Extract vesicles were prepared by adding SDBS solution to bacterial cell pellet and solid CTAT. After resuspension of the cell mass, the suspension was centrifuged to remove cell debris and the supernatant containing the catanionic vesicles containing cell components was purified by standard methods. In order to detect the amount of carbohydrates and proteins incorporated in the vesicles by the extraction process, carbohydrate colorimetric assay and BCA protein assay was performed.

	Protein (μg/mL)	Carbohydrate (μg/mL)
N gonorrhoeae extract vesicles	346	44

Table 2.2. Total protein and carbohydrate concentrations in vesicle extract samples determined by colorimetric BCA and carbohydrate assays. Studies conducted by Dr. Lenea Stocker (manuscript in preparation)

Western blotting of the functionalized vesicles using polyclonal antibodies provided by the Stein lab showed the presence of LOS and the two predominant membrane proteins porin (36 kD) and Opa (25-30 kD). Mass spectrometry analysis of the proteins extracted into the vesicles was performed by Ms. Avantika Dhabria in Professor Catherine Fenselau's lab. This analysis on vesicles containing gonococcal cell extracts was able to identify 157 unique gonococcal protein fragments. By comparing these fragments with a *Neisseria* DNA sequence, the majority of these fragments were associated with cell surface antigens such as porin and OPA identified by Western blot described above. Also detected were cell surface proteins for several unidentified cell surface proteins. With these "artificial pathogens" in hand, we wanted to demonstrate that vesicles functionalized with extracted proteins and surface proteins from cells can be used to make detection assays using the array strategy described above.

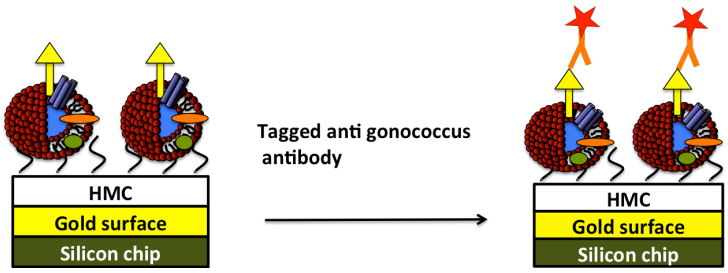


Figure 2.21 Detection of 'extract vesicles' using microarrays

In order to illustrate that neisserial LOS incorporated into the extract vesicles - "artificial pathogens" - is available for binding with external proteins we decided to configure the binding of extract vesicles with fluorescently tagged monoclonal antibody against neisserial LOS. This antibody was provided by Professor Dan Stein's lab.

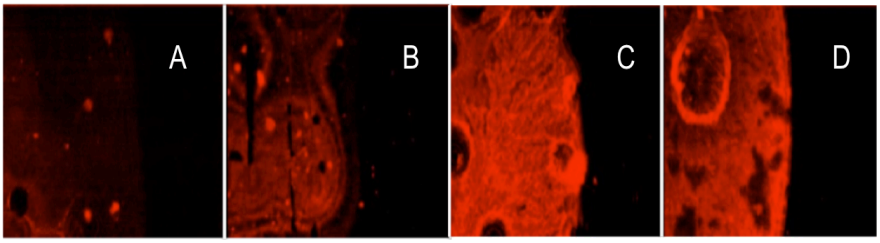


Figure 2.22 A Detection of 'extract vesicles' using microarrays
Lectin binding studies with vesicles functionalized with Extract Vesicles (D) with fluorescently labeled antibody goat anti GC mAb 2-1-L8 (tagged with DyLight 633) (A, B, C) indicate the control experiments with bare vesicles, cell extract (no vesicles) and LOS vesicles.

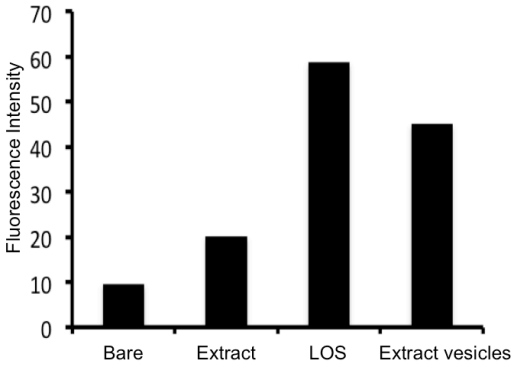
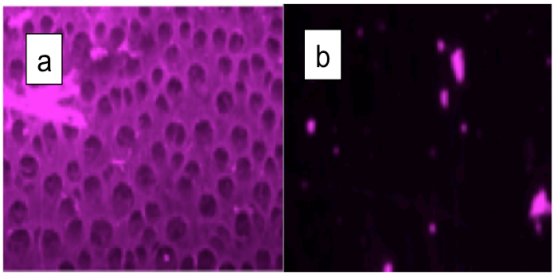


Figure 2.22 B Comparison of fluorescence intensities of fluorescently labeled antibody goat anti gonococcus on the surfaces of deposited bare vesicle, extract (no vesicles), LOS vesicles and extract vesicles

The results shown in Figure 2.22 clearly demonstrate that arrays prepared by deposition of extract vesicles show binding with goat anti GC mAb 2-1-L8 (Electrode D). Electrodes A and B show the control experiments with bare vesicles and cell extract (cell debris dissolved in SDBS solution). Electrode A shows minimum fluorescence (value 9.54) and electrode B shows comparatively higher fluorescence (value 20.14). This is could be due to parts of cell membrane in the cell debris. These cell membrane parts are expected to contain bacteria; LOS that binds to the tagged antibody resulting in fluorescence on the surface of electrode B. Electrode C, which is the positive control show highest binding (value 58.78). This is expected as this electrode is deposited with vesicles that are functionalized with purified LOS F62ΔA. Goat anti GC mAb 2-1-L8 is known to bind selectively to LOS F62ΔA, hence resulting in maximum fluorescence. Electrode D deposited with "artificial pathogens" containing LOS (as shown by Western blot, see above) gave fluorescence intensity approximately 4 times higher than the negative controls. The binding on this electrode is lower than the positive control, as the extract vesicles are not functionalized with purified LOS. The efficient binding of the goat antibody to the catanionic vesicles arising from the extraction process illustrate that these "artificial pathogen" arrays can be prepared and will function much as the natural pathogen in immunological assays.

Once binding of the goat monoclonal antibody had been demonstrated, we conducted binding studies with the "artificial pathogen" vesicles binding with goat anti-GC polyclonal antibody. Again, We consistently saw higher binding (almost four times higher intensity) on the extract vesicles electrodes than the bare vesicles electrodes as shown by the images in Figure 2.23.

A)



B)

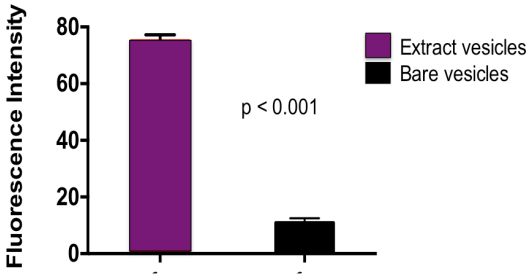


Figure 2.23 A) Cyan dye labeled goat anti-GC poly-clonal antibody was deposited on 'extract'-vesicles (A), control experiments with bare vesicles (B).
Figure 2.23 B) Comparison of fluorescence on the surface deposited with bare vesicles, and extract vesicles. These studies were repeated for four electrodes. The results were analyzed by a paired t test to find that the observations are significantly similar
($p < 0.001$, n=4)

2.13 Effect of Glycan Concentration on Binding

As described in Chapter 1.3, glycans on a cell surface often display a cluster effect which results in an increase in the effective concentration and distribution of carbohydrates on the cell surface and plays a vital role in the binding with GBPs.[18] Attempts to mimic this "natural" display of glycans on cells has been performed by preparing surfaces with "fluidic" character that allows the glycans to exhibit the mobility to provide multivalent interactions. [19-21] However, even these "fluidic" array methods are known to have limitations in achieving the "natural" environment that one finds on a cell surface.

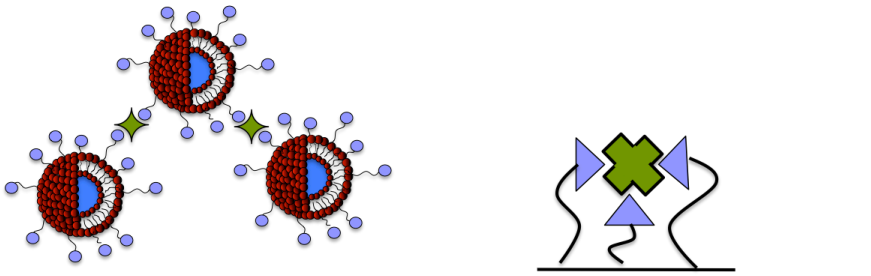


Figure 2.24. Glycans display multivalent binding to bind with glycan binding proteins (GBPs). When on cell surface, these glycans demonstrate cluster effect to bind to GBPs.

Our previous studies on lectin aggregation/binding in suspension had shown that the density of the glycan on the surface of the vesicle was important in the aggregation induced by lectins, and these studies were an excellent mimic for the "natural" environment of glycans on a cell surface. The reported distance between the two binding sites on the Con A lectin is proposed to be 2.5-6.5 nm from X-ray crystallography and FRET studies.[22] Thomas et al. demonstrated that glucose-functionalized catanionic vesicles could be used to explore the binding of Con A.[16] Based on these aggregations studies, it was found that O-linked glycoconjugate provided a Poisson distribution on the surface of a vesicle. Where as the binding curve obtained from N-linked glycoconjugate fits the binding to a cluster of conjugates. It is hypothesized that the hydrogen bonding between the N-Linked glycoconjugates contributes to the cluster effect. Based on these models, the average distance between the binding sites of Con A was found to be 3.6-4.3 nm.[16]

We questioned whether the density of ligands on surface-bound vesicles would show the analogous behavior on the array surface as their suspension counterparts. In order to investigate this proposal, binding studies between Con A and glucose-functionalized vesicles bound to a HM chitosan surface were conducted. HM Chitosan coated electrodes deposited with vesicles functionalized with varying glucose concentrations ranging from 0 mf of glucose to 0.1 mf of glucose as previously described. The concentration of glucose incorporated in vesicles was determined with colorimetric assay using phenol and sulfuric acid colorimetric assay. The amount of glucose incorporated in vesicles can be correlated to the amount of glucose added during the preparation step. (Figure 2.25) Binding studies of the glucose vesicle suspensions were

conducted with FITC-labelled Con A (Figure 2.26 A) as described above. The fluorescence intensity on the electrode surfaces was measured using Image J. Plot of fluorescence intensity against the mole fraction of glucose incorporated in vesicles follows a hyperbolic trend (Figure 2.26 B). The vesicles incorporated with higher amount of glucose displayed greater fluorescence, higher binding, with FITC Con A. The saturation of the fluorescence intensity in Figure 2.26B is presumably the result of steric crowding on the surface of the vesicle. There is a limit to the number of Con A molecules that can attach to the surface. These studies indicated that binding of concanavalin A with glucose-functionalized vesicles on the surface of the array shows a dependence on the amount of glucose incorporated in vesicles, just as was observed in the solution studies.

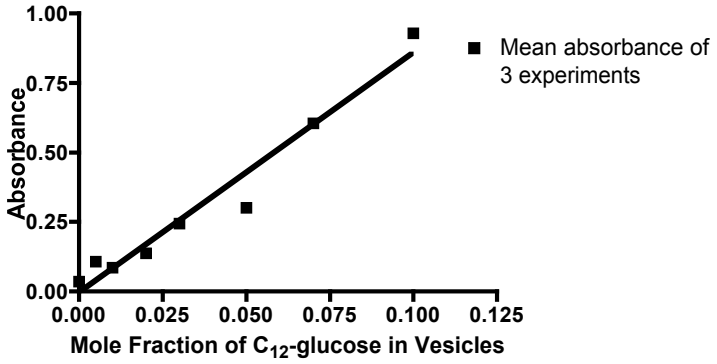


Figure 2.25 Amount of C_{12}-glucose incorporated in vesicles as detected with phenol sulfuric acid colorimetric assay.

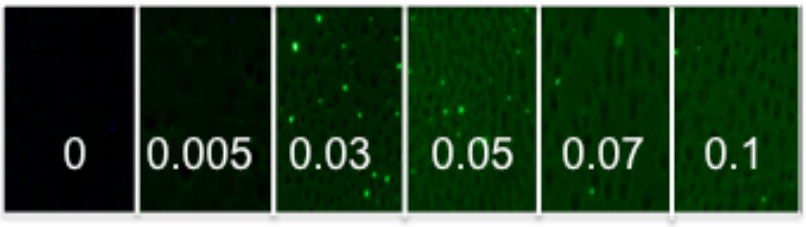


Figure 2.26 A. Images of electrodes deposited with glucose functionalized vesicles, functionalized with varying amount of glucose (Mf of C_{12}-glucose added is indicated on images).

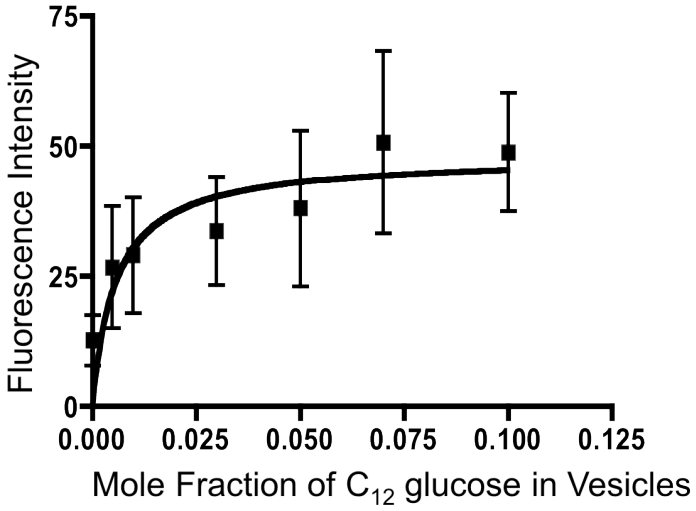


Figure 2.26 B. Plot of fluorescence intensity on FITC labeled Con A deposited on HM chitosan surfaces for different electrodes deposited with vesicles samples having varying amounts of C_{12}-glucose.

We have conducted this study multiple times and have observed that the absolute values of the fluorescence vary from sample to sample. Qualitatively, the results are consistent from sample to sample, it is only the absolute values of the fluorescence that fluctuate. This observation is a known limitation in the field of microarray preparations, arising with quantitatively uneven deposition of glycans on the surface. In order to overcome this problem, we need to create surfaces with ability to reproducibly deposit glycan-functionalized vesicles. Due to uneven deposition of HM chitosan during the electrodeposition process, the subsequent deposition of vesicles onto the HM chitosan layer is uneven for each experiment. Thus in order to prepare microarrays that can quantitatively correlate the fluorescence intensity to the glycan concentration on the surface, we need to make sure that electrodeposition of HM chitosan has reproducible morphology. This problem is currently under investigation in the lab and results relating to the study will be reported in due course.

A second concern with the microarray approach is that the study above makes an assumption that the number of vesicles residing on the surface of HM chitosan surface is equivalent for each electrode. We believe that is a valid assumption since each electrode is treated equivalently in the procedure of binding assay. However, to address this potential roadblock, the fluorescent intensity for a single glycan concentration was determined while exposing the surface to varying concentrations of Con A. We deposited a sample of glucose-functionalized vesicles on multiple electrodes and conducted binding studies with varying concentrations of FITC labeled Con A. This study was repeated with multiple glucose concentrations to obtain a set of binding curves as shown in Figure 2.27.

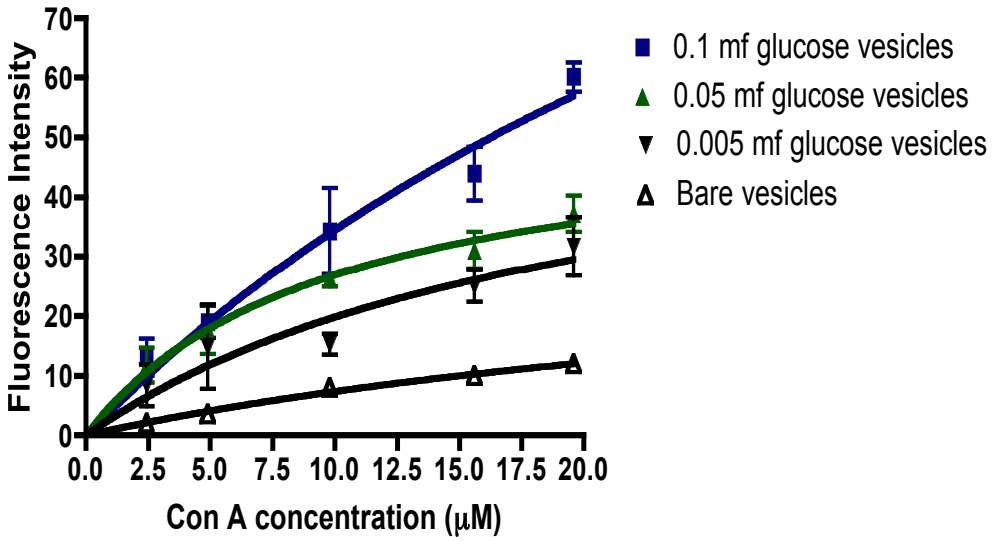


Figure 2.27. Plot of fluorescence intensity on FITC labeled Con A deposited on HM chitosan surfaces for different electrodes deposited with varying amount of FITC labeled Con A for each glycan concentrations.

The results from these studies qualitatively indicate that a higher glucose concentration on the surface, leads to higher binding with FITC labeled Con A. As displayed above, 0.1 mf glucose vesicles show saturation with FITC Con A at fluorescence intensity much higher than surfaces deposited with 0.05 and 0.005 mf glucose vesicles. The trend in the curves indicated that the surface saturated at higher Con A concentrations, independent of the glucose concentration on the surface. This shows that glycans on the surface involve in multidentate binding.

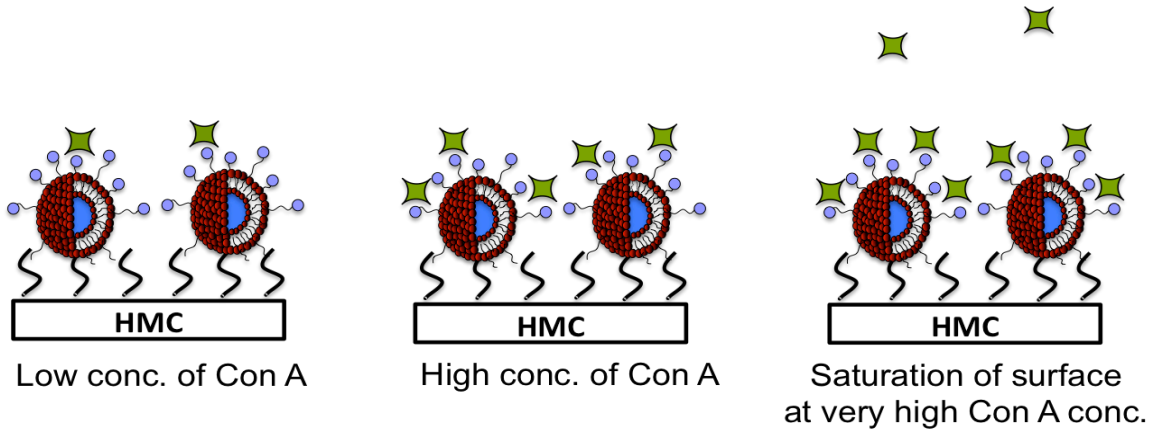


Figure 2.28 At high Con A concentrations, the surface of microarray shows saturation for Con A binding.

However, though the results are qualitatively sound, quantitatively the surface deposited with 0.05 mf glucose-functionalized vesicles only shows negligible increase in fluorescence intensity as compared to surfaces deposited with 0.005 mf glucose vesicles. (average 33 vs 25). We often observe high background fluorescence due to non-specific binding of Con A binding to the HM chitosan surface.

*2.14 Conclusion*s

In this chapter, we have demonstrated that functionalized surfactant vesicles can be utilized for the preparation of carbohydrate arrays on hydrophobically modified chitosan (HM chitosan) surfaces. A HM chitosan surface is a better motif for deposition of vesicles than chitosan itself due to its ability to insert a hydrophobic alkyl chain into the vesicle leaflet. Once deposited, the glycan-functionalized vesicles appear to maintain their integrity as indicted by triton washing studies. Binding studies with lectins and antibodies indicated that the glycans on the surface of the vesicle are available to their respective binding partners. This methodology provides a proof of concept that the surfactant vesicle technology can be employed for the production of complex glycan-based microarray systems for diagnostic and therapeutic applications. Extension of this methodology for detection of cell extract vesicles, prepared by methodology developed in our group demonstrates the possibility of creating microarrays with ease of preparation.

2.15 Experimental

2.15.1 Imaging

Fluorescence images were obtained using a Leica fluorescence stereomicroscope (MZFLIII) equipped with a digital camera (Spot 32, Diagnostic instruments). The fluorescence of lissamine rhodamine dye was observed employing an excitation wavelength of 560 nm (bandwidth of 40 nm), having an emission filter of 610 nm. To observe the fluorescence of FITC, an excitation wavelength of 480 nm (bandwidth of 40 nm) and an emission filter of 510 nm was employed.

2.15.2 NMR, IR, and UV-Vis Spectroscopy

^{1}H NMR spectra were recorded on a Bruker DRX-400 MHz spectrometer, with $CDCl_3$ or MeOD as a solvent. Coupling constants (*J* values) are given in hertz (Hz). Chemical shifts are reported in parts per million (ppm) relative to the solvent. Spin multiplicities are indicated by the following symbols: s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Infrared spectra were recorded on a Nicolet 5DXC-FT-IR spectrometer by dissolving the compounds in carbon tetrachloride. Band positions are given in reciprocal centimeters (cm-1) and relative intensities are listed as br (broad), s (strong), m (medium) or w (weak). Absorbance measurements were performed on a CHEM2000-UV-vis-spectrometer from Ocean Optics, Inc. Chemical shifts are reported in parts per million (ppm) relative to the solvent.

2.15.3 Dynamic Light Scattering

The radius of the catanionic vesicles was determined by dynamic light scattering (DLS) using a Photocor-FC light scattering device having a 5 mW laser (633 nm). All measurements were performed at a 90° scattering angle at room temperature. The autocorrelation function was measured and analyzed to determine the hydrodynamic radius and polydispersity index, assuming a Gaussian distribution of vesicle size.

2.15.4 Zeta Potential Measurements

Zeta potential of vesicle samples were measured using a Zetasizer ZS90 from Malvern Instruments Ltd. All samples were loaded in pre-rinsed capillary cells for zeta potential measurements and the reading were obtained at room temperature.

2.15.5 Chemicals and Reagents

The following chemicals were purchased from Sigma: cetyltrimethylammonium tosylate (CTAT), n-dodecyl-β-D-glucopyranoside (C_{12}-glucose), phosphate buffered saline (PBS), Sephadex G-100 as well as the fluorescein isothiocynate (FITC)-labeled lectins, FITC-labeled peanut agglutinin (PNA) from *Arachis hypogaea* and FITC-labeled concanavalin A (Con A) from Canavalia ensiformis (Jack Bean), Type IV. Sodium dodecylbenzenesulfonate (SDBS) was purchased from TCI-America. Lissamine rhodamine, 1,2-dioleoyl-sn-glycero-3-phosphoethanol-amine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) was purchased from Avanti Polar Lipids. A solution of PBS was prepared at pH 7.4 by dissolving a tablet in DI water using standard protocol. CTAT was purified by recrystallization from ethanol/acetone to produce white shiny crystals.

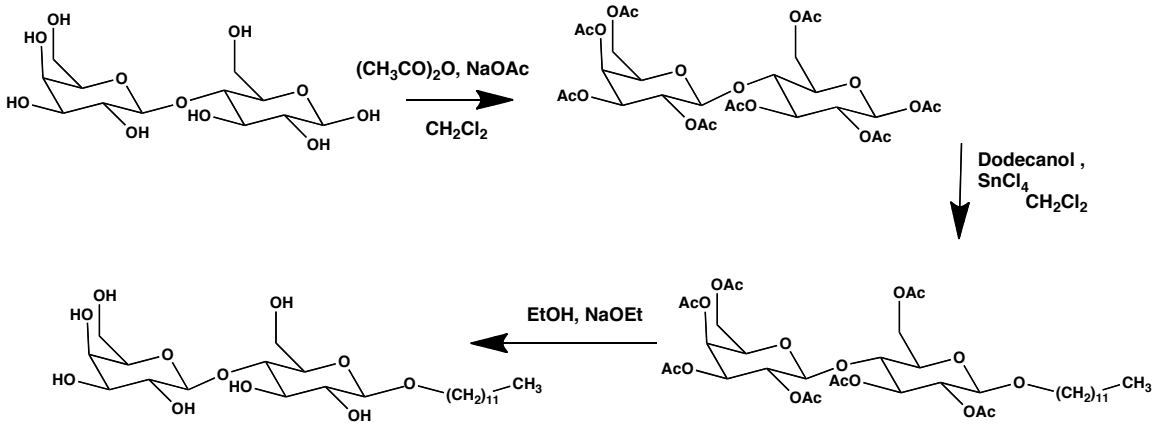
2.15.5.2 Chitosan and Hydrophobically Modified Chitosan

All reagents used were purchased from commercial suppliers and were used without further purification unless otherwise stated. Glassware used in the reaction was dried in the oven overnight. Prepared compounds were characterized by NMR and IR and melting point.

Chitosan of medium molecular weight (190-310K) and Brookfield viscosity of 286 cps was purchased from Sigma-Aldrich, which had a degree of deacetylation about 80%. It was dissolved in 0.2 M acetic acid (pH < 6.5). Hydrophobically modified (HM) chitosan was prepared by reductive amination of chitosan with dodecylaldehyde by the method reported by Desbrieres et al.[23] The degree of hydrophobic modification was 2.5 mol % based on monomer. Briefly, the method involves the following steps. Chitosan

(2.00 g) was dissolved in 100 mL water, and glacial acetic acid was added dropwise (0.2 mL with stirring). The resulting suspension was stirred for 16 h. Ethanol (100 mL) was added to this solution and stirred for 15 min. Dodecyl aldehyde (0.057 g) dissolved in 5 mL ethanol was added to the chitosan solution dropwise, while stirring the chitosan solution vigorously. After 30 min, a solution of 0.78 g of $NaCNBH_3$ dissolved in 10 mL ethanol was added thrice (2.34 g total) at intervals of 2 h. The solution was stirred for 24 h after the final addition of $NaCNBH_3$. NaOH (100 mL, 0.1 M) was added to neutralize the solution. Formation of a white precipitate was observed as NaOH was added. NaOH (15 mL of 1 M) was added drop wise to complete precipitation of HM chitosan. The resulting precipitate was washed with water until the pH of supernatant was pH 7, and dissolved in 0.2 M acetic acid. The solution was poured onto non-stick baking pans and dried for 3 days to cast films of HM chitosan. HM chitosan was dissolved in water, with drop wise addition of acetic acid to prepare 1 wt % solution (pH 5.5). Later synthesis of HM chitosan involved drying the HM chitosan directly without dissolving it in acetic acid. This resulted in a better control of pH of HM chitosan solution, thus resulting in better, more even electrodeposition of HM chitosan. Fluorescently-labeled chitosan and HM chitosan were obtained from Payne lab. These were synthesized by reacting the polymers with NHS-fluorescein, as previously reported in the literature.

2.15.5.3 Lactose Dodecyl Ether

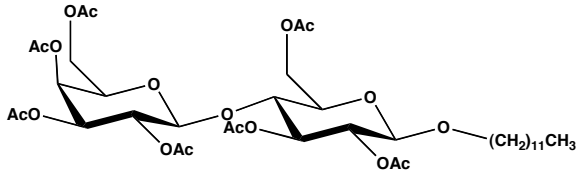


Scheme 2.2. Reaction scheme for preparation of lactose dodecyl ether

β -D-Lactose Pentaacetate

β-D-Lactose (4.50 g, 12.38 mmol, 1.0 equiv.) was added in three portions to a refluxing mixture of NaOAc (4.00 g, 49.00 mmol, 3.9 equiv.) and acetic anhydride (50.0 mL). The addition was done over a period of 30 min. The mixture was further refluxed for 3 hours. The reaction mixture was cooled to 100 ^{o}C and then added to 300 mL of ice and water mixture. This mixture was stirred for 20 h. The sticky solid formed was dissolved with 50 mL CH_2Cl_2. The organic layer was washed with satd. $NaHCO_3$ (2 X 50 mL). It was then washed with water (3 X 50 mL). The organic layer was then dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was recrystallized from acetone and diethyl ether. This resulted in 3.75 g (56%) of recrystallized product as a white powder. Spectral data were identical with that reported by Damkaci.[24]

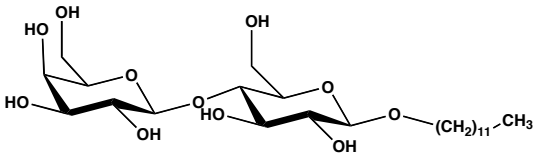
Peracetylated β Lactopyranosyl Dodecyl Ether



O-linked C_{12}- Lactose was prepared according to the method reported by Vill et al.[25]

β-peracetylated lactose (2.91 g, 4.27 mmol, 1.00 equiv.) was dissolved in 38 mL of freshly distilled CH_2Cl_2. This was followed by the addition of $SnCl_4$ (4.27 mL, 4.27 mmol, 1.00 equiv.) and dodecanol (0.25 g, 0.70 mmol, 0.70 equiv.) dissolved in anhydrous CH_2Cl_2. This mixture was stirred with 1.74 g of 4 Å molecular sieves under Ar atmosphere. The reaction was allowed to stir for 4 h. The reaction mixture was diluted with 50.0 mL CH_2Cl_2 and washed with satd. $NaHCO_3$ (2 X 50.0 mL) followed by water (2 X 75.0 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. This resulted in the formation of 1.9 g of crude O-linked C_{12} lactose as a yellow sticky solid. Purification via column chromatography (Hexanes: EtOAc, 60:40) resulted in 1.70 g (80%) of purified product as a yellow sticky solid. IR (CCl_4, cm^{-1}) 2927 (m), 2855 (w), 1758 (s), 1368 (m), 1218 (s), 1119(w); ^{1}H NMR (400 MHz, ppm, $CDCl_3$) δ 0.8 (t, *J* = 6.8, 3H), 1.2-1.4 (m, 20H), 1.97 (s, 3H), 2.06 (m, 12 H), 2.16 (d, *J* = 2.4, 6H), 3.45 (sextet, *J* = 9.6, 1H), 3.5-3.65 (m, 1H), 3.7-3.9 (m, 3H), 4.05-4.20 (m, 3H), 4.47 (dd, *J*= 12.0, 3H), 4.89 (dd, *J* = 9.6, 1H), 4.92-5.00 (m, 1H), 5.04-5.16 (m, 1H), 5.20 (t, *J* = 9.2, 1H), 5.35 (dd, *J* = 3.6, 1.2, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.2, 20.9, 21.0, 21.1, 22.9, 26.0, 29.5, 29.6, 29.8, 29.8, 32.1, 61.0, 62.3, 66.8, 69.3, 70.4, 70.9, 71.2, 71.9, 72.0, 73.1, 76.6, 76.9, 77.3, 77.6, 100.8, 101.4, 169.3, 169.8, 170.0, 170.2, 170.4.

β Lactopyranosyl Dodecyl Ether



To a solution of peracetylated O-linked C_{12} lactose (0.36 g, 1.00 equiv) in 2.00 mL EtOH was added 0.20 M solution of sodium ethoxide (18.0 mL, 8.00 equiv) and then stirred at room temperature for 24 h under Ar atmosphere. The reaction mixture was neutralized with Amberlite IR-120 resin, filtered and concentrated *in vacuo.* The product was obtained as a white solid (0.17 g, 73%) It was purified with a short silica plug using ethanol as a solvent.

NMR (400 MHz, MeOD) δ 0.9 (t, *J* = 6.8, 3H), 1.2-1.4 (m, 17 H), 1.62 (pentet, *J* = 8.0, 3 H), 1.71 (s, 1 H) 1.95 (m, 1 H), 2.45 (m, 1 H), 3.35-3.44 (m, 1H), 3.45-3.62 (m, 5H), 3.66-3.81 (dABq , 2H), 3.81-3.95 (m, 3H), 4.26-4.31 (d, *J* = 8.0, 1H), 4.35-4.39 (d, *J* = 8.0, 1H)

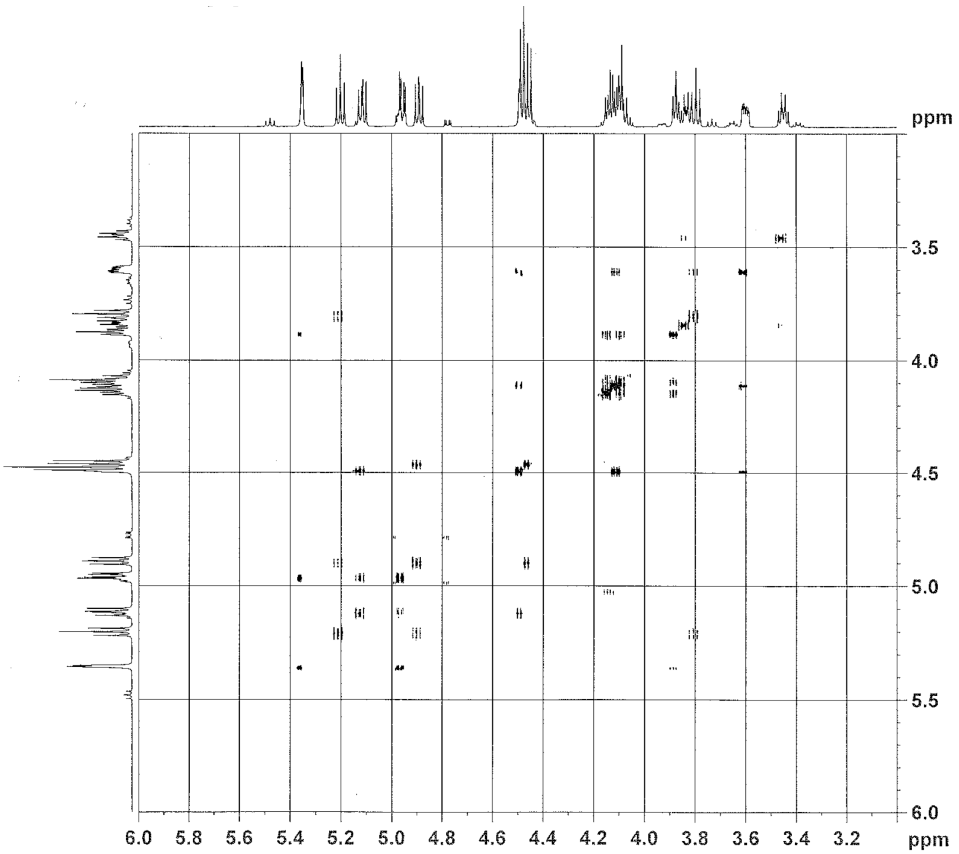
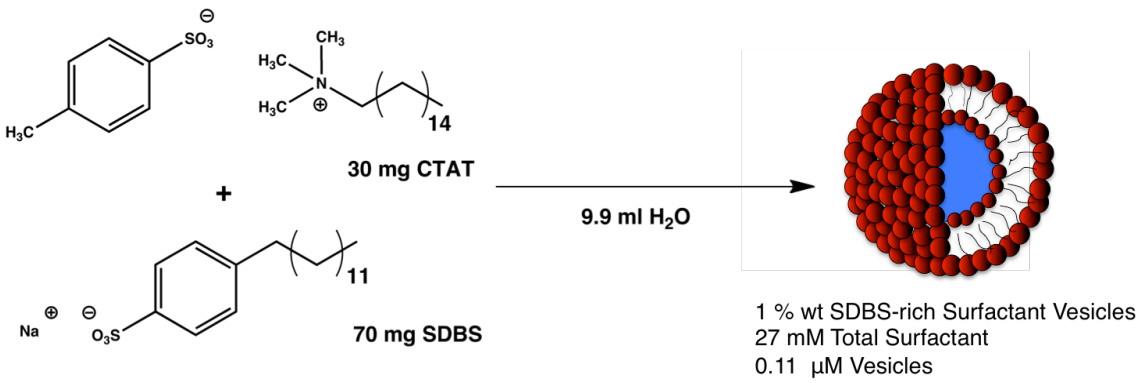


Figure 2.29 COSY (^{1}H-^{1}H) NMR spectrum of peracetylated β lactopyranosyl dodecyl ether in $CDCl_3$ on a Bruker Avance III 600 MHz.

2.15.6.1 Preparation of Vesicles

Sodium dodecylbenzenesulfonate (SDBS) was purchased from TCI America and was utilized without further purification. Cetyltrimethylammonium tosylate (CTAT) was purchased from Sigma and was recrystallized from ethanol-acetone to give a white powder. The purified solid was stored at room temperature in a desiccator containing Drierite.



Scheme 2.3 Preparation of 1% wt SDBS rich surfactant vesicles

Catanionic vesicles were prepared from SDBS and CTAT, with molar excess of SDBS. The resulting catanionic vesicles have a net negative surface charge. Anionic vesicles were prepared with 1 wt% of total surfactant by combining 70.0 mg (200 µmol) of SDBS and 30.0 mg (65.8 µmol) of CTAT. Millipore water 18 Ω (9.90 mL) was added to the mixture of surfactants, and the resulting solution was stirred for 24 h. The formation of vesicles within 1 hour of mixing was evident as the solution became turbid. Catanionic vesicles formed with this method were observed to have an average diameter of 140 ± 30 nm, as measured by dynamic light scattering (DLS).

2.15.6.2 Purification of Vesicles

Vesicles were purified with the help of size exclusion chromatography (SEC). A column (5.5 cm height; 1.5 cm diameter) was packed with Sephadex G-100 (Sigma). Vesicle solution (1mL) was added to the column and collected as the first fraction. This was followed by addition of 1 mL aliquots of water. Each 1 mL aliquot was collected in a separate vial. A total 14 fractions were collected and the vesicles were observed in fractions 3 and 4. Based on the colorimetric assay these vesicle-containing fractions were

observed to contain carbohydrate incorporated in them. Hence these two fractions were combined and used for experiments.

2.15.6.3 Glycan Functionalized Vesicles

To prepare glucose and lactose functionalized vesicles, required amount glycoconjugate was weighed in a glass vial. To this, SDBS (mole fraction 70%) and CTAT (mole fraction 30%) were combined with 9.9 mL of water. This solution was stirred for 24 hours.

2.15.6.4 Dye Functionalized Vesicles

To prepare lissamine rhodamine dye functionalized vesicles, required amount dye (0.001mf), dissolved in chloroform was pipetted in a glass vial. The solvent was removed in vacuo. To this, SDBS (mole fraction 70%) and CTAT (mole fraction 30%) were combined with 9.9 mL of water. This solution was stirred for 24 hours.

2.15.6.5 LOS Functionalized Vesicles

LOS functionalized vesicles were prepared by combining 1.00 mg of LOS in 10 mL preformed 1 % SDBS rich vesicle solution. The resulting vesicle preparation was purified as described above.

2.15.6.6 Cell Extract Vesicles

Vesicles were formed by adding 9.90 mL of an aqueous SDBS solution (0.0203 M) directly to the bacterial cell pellet and stirring for 1 h at room temperature. 30.0 mg

of solid CTAT (0.0658 mmol) was added to the suspension and stirred for 1 h at room temperature. Vesicles were centrifuged for 5 min at 5,000 rpm and the supernatant was decanted. The resulting colloidal supernatant, milky in appearance, was purified by SEC.

2.15.7 Colorimetric Assay

The colorimetric assay was used to determine the amount of glycoconjugate incorporated in the vesicles. The vesicles were passed through SEC and up to 14 fractions were collected. DLS measurements were done prior to doing the colorimetric assay. A 0.5 mL portion of each vesicle fraction was transferred to an empty vial. A 0.250 mL of 0.530 M aqueous phenol (13.3 mmol) was added, followed by addition of 1.25 mL of conc. sulfuric acid, introduced as a stream on the surface of the liquid. The samples were vortexed and allowed to sit at room temperature for 1 h to allow the color to develop. The yellow color indicated the presence of carbohydrate. Then, 0.5 mL of ethanol was added to each vial and the mixture was allowed to sit. After 10 min, the absorbance was measured at 490 nm.

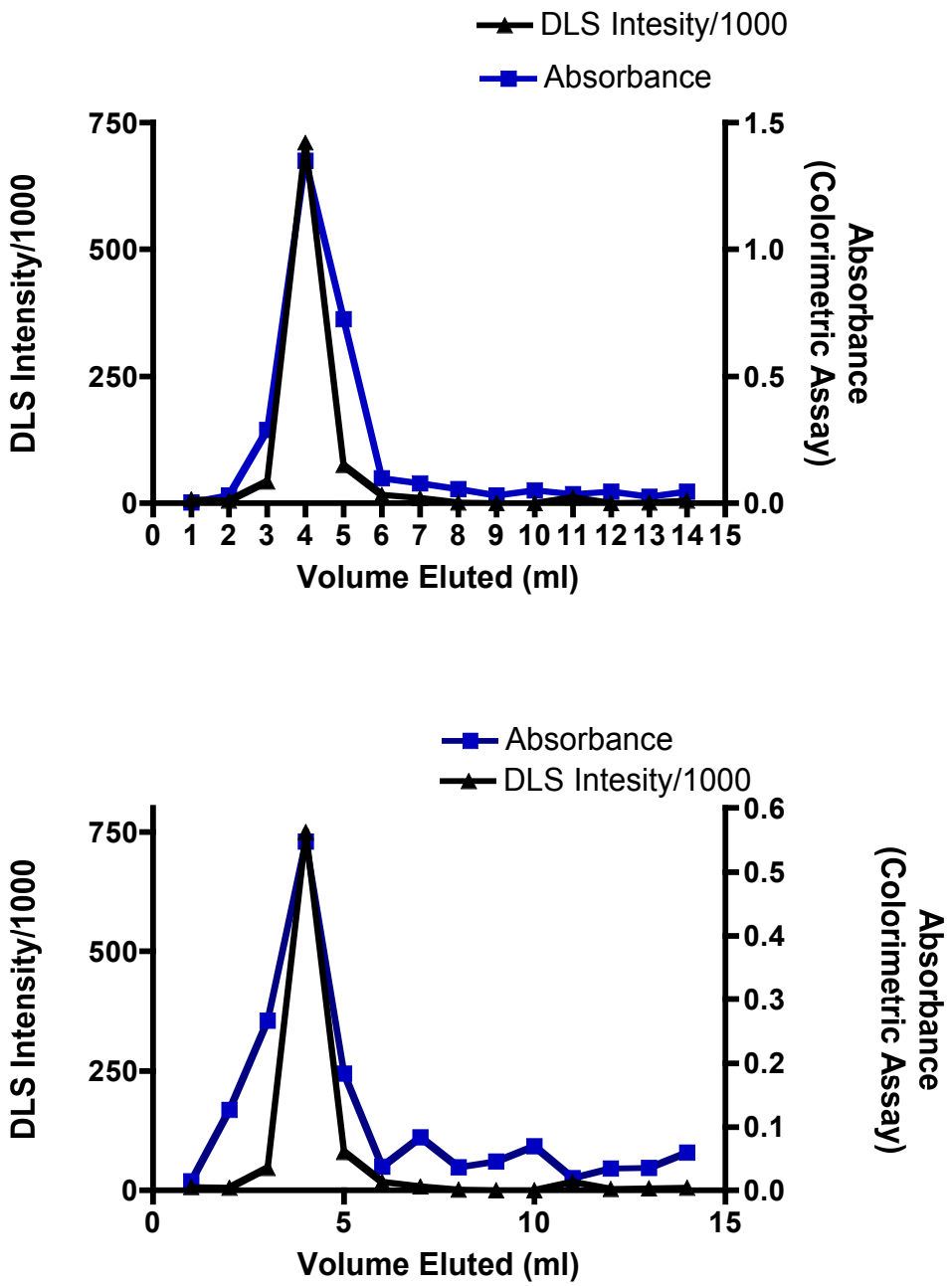


Figure 2.30. Results from SEC of C_{12} glucose-functionalized catanionic vesicles (A) and C_{12} lactose-functionalized catanionic vesicles (B) as evaluated by DLS and colorimetric assay of carbohydrate-loaded vesicles. SEC fractions were evaluated by DLS (solid black line) to identify vesicle-containing fractions. Fractions containing carbohydrates were identified with colorimetric detection at 490 nm (solid blue line).

2.15.8 Fabrication of electrodes

Fabrication of electrodes was carried out on a silicon wafer by imprinting gold patterns, as described by Wu et al[15] Electrodes were prepared by depositing 90 Å thick chromium (Cr) and then 2000 Å thick gold (Au) films on 4 in. diameter silicon wafers already coated with patterned 1 micron thick thermal oxide film.

2.15.9 Preparation of HM Chitosan Coated Electrodes

Electrodeposition of HM chitosan and chitosan on gold electrodes. Electrodeposition was achieved by negatively biasing a specific lead, when the electrode was half immersed in an aqueous solution of HM chitosan or chitosan (1 wt % pH 5.5), respectively. A DC power supply (model 6641C, Agilent technologies) was used to pass a current equivalent to 4 μA/cm^2 for 2 min in order to deposit HM chitosan and chitosan, respectively, on the gold micropatterns.

2.15.10 Deposition of Vesicles on to HM Chitosan and Chitosan-Functionalized Electrodes

Gold electrodes that were electrodeposited with chitosan or HM chitosan were washed thoroughly with water for 15 min. The electrodes were then immersed in 300 μL vesicle sample for 40 min. These electrodes were then washed with PBS buffer for 2 hours followed by DI water for 2 min.

2.15.11 Binding Studies

Electrodes coated with vesicles incorporating glycoconjugates were treated with fetal bovine serum (FBS) for 30 min. We have conducted these studies with bovine serum albumin as well as filtered fetal bovine serum. FBS was found to be more effective than BSA in blocking the non-specific binding between lectins and the surface. Electrodes

were washed with PBS for 15 min. After this step, these electrodes were immersed in a solution of fluorescently labeled lectin (Con A or PNA) and fetal bovine serum (8.33 μM solution in FBS) for 1 h. The electrodes were washed with FBS to reduce non-specific binding of lectin with the glycoconjugate for 15 min. The electrodes were washed with PBS for 30 m prior to fluorescence imaging. For antibody binding studies, an aqueous solution of the antibody was prepared by preparing a 1:60 dilution in Millipore water.

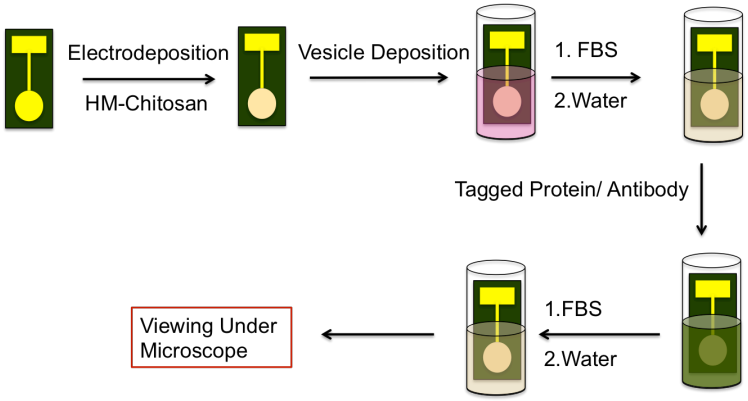


Figure 2.31. A flow chart of binding assay used for detection of carbohydrate-lectin binding

2.15.12 LOS Preparation, Purification and Analysis

Variants of *N. gonorrhoeae* expressing defined LOS structures were provided by Amanda Mahle and Daniel C. Stein of the department of Cell Biology and Molecular Genetics.

Variants of *N. gonorrhoeae* expressing defined LOS structures were maintained on gonococcal medium base (Difco) supplemented with 1% Kelloggs in a 5% CO_2 incubator at 37 °C.

Purified LOS was obtained from broth grown *N. gonorrhoeae* through the hot-phenol method followed by lyophilization. Quick preparations of gonococcal LOS were prepared from plated cultures as described by Hitchcock and Brown[26], where the sample was diluted 1:25 in lysing buffer, and boiled for 10 min immediately before loading. Approximately 0.1 μg of LOS was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 16.5% Tris-Tricine gel (from Bio-Rad) in Tris-Tricine running buffer following the protocol suggested by the manufacturer. The gel was fixed overnight in 40% ethanol-5% acetic acid, and the LOS was visualized by silver staining.

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Chapter 3. Performing Chemo-Enzymatic Transformations On Glycan Functionalized Catanionic Surfactant Vesicles

3.1 Introduction

Carbohydrates have fairly intricate molecular structures due to their structural complexity resulting out of numerous stereo centers.[1] Synthesis of complex carbohydrate structures is a well-established problem in the field of carbohydrate chemistry. Although there are multiple synthetic methodologies reported for the synthesis of complex polysaccharides, such as one-pot solution-phase synthesis[2, 3] and the solid-phase synthesis method[4, 5], there is a lack of methodologies that result in stereochemically pure glycans with acceptable yields.[6]

Biosynthesis of most abundant carbohydrates such as cellular mono, di and polysaccharides takes place with the help of enzymes such as glycosyltransferases. Glycosyltransferases are well known to catalyze the transfer of activated carbohydrate entity from a donor molecule to an acceptor molecule.[7] For example, Figure 3.1 shows the activity of β 1,4-galactosyltransferase on a donor glycan, UDP-galactose and acceptor glycan, and glucosamine. (Figure 3.1) Glycosidases on the other hand, cleave larger glycans into smaller monosaccharides. [8]

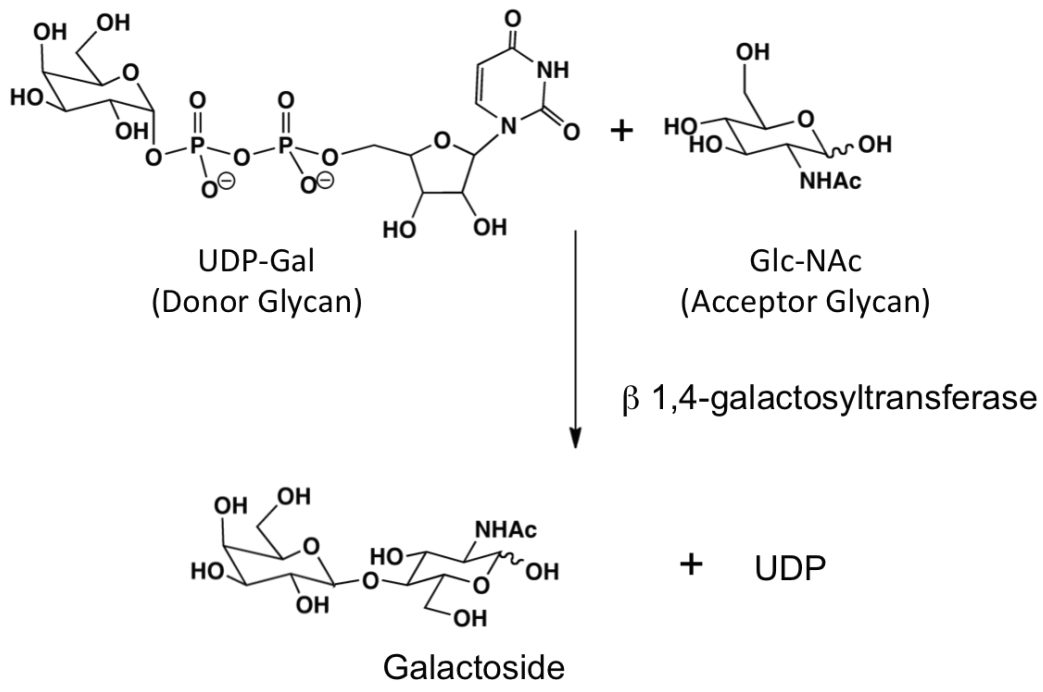


Figure 3.1 Enzyme catalyzed transformation-using β 1,4-galactosyltransferase.

Glycan synthesis using glycosidases and glycosyltransferases has an advantage over chemical synthesis methods because the enzymatic methods result in high yields of glycans with complete regio- and stereoselectivity.[9, 10] Another advantage of using enzymes is that there is no need to install and remove protecting groups as is required in traditional chemical synthesis. Thus, there is a huge interest in synthesizing glycans using chemo-enzymatic methods to create complex glycan structures.

Previous research on this topic has focused on enzymatic reactions on glycans mainly in solution phase, with considerably less focus on surface studies.[3, 11] Multiple studies performed to illustrate activity of enzymes on small molecule or protein microarrays. Activity of many glycosyl transferases was analyzed on glycan linked polystyrene microspheres by Chandrasekarn et al.[12] Glycan arrays have been previously used as platforms to create new glycan molecules using enzymatic methods. Studies by Park et al have illustrated the preparation of a complex glycan molecule,

NeuNAcR2,3LacNAc from a simple monosaccharide β-GlcNAc (Figure 3.2), using three consecutive enzymatic reactions.[13] Shin et al have demonstrated the use of glycan arrays to assay glycosyl transferases activities.[14] Although these studies are limited in scope, they clearly demonstrate that biosynthetic synthesis of glycans can be achieved on surfaces. [15]

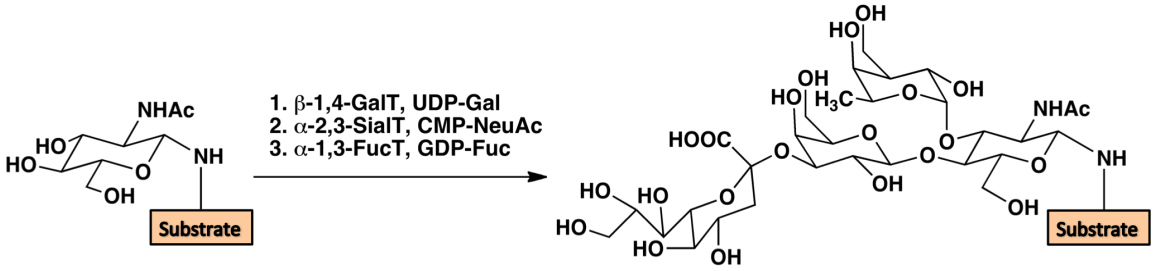


Figure 3.2 Enzymatic synthesis of NeuNAcR2,3LacNAc from β-GlcNAc, attached to a microarray.

As discussed in Chapter 2.2, functionalized SDBS-rich catanionic surfactant vesicles have a surface charge (zeta potential) that is similar to the potential of a phospholipid bilayer of a gram-negative bacterium. Hence, we hypothesized that catanionic surfactant vesicles could serve as platforms for enzymatic reactions that occur on the glycans that populate the outer membrane of bacteria. Using this reasoning, glycosyltransferases that are known to add monosaccharides to the lipid oligosaccharides on Gram-negative bacteria would be expected to add the same monosaccharide to a glycan embedded into the outer membrane of a catanionic surfactant vesicle.

Since molecules incorporated into the leaflet of vesicles have a particular orientation, only a part of the molecule protrudes out of the vesicle surface would be susceptible to the biosynthetic reaction. Previous studies performed on molecules incorporated into micelles or leaflet of liposomes have shown that a variety of chemical

transformations can be accomplished on the fragment of the molecule that extends from the vesicle bilayer. Studies by Wang et al., for example, focused on stabilization of curcumin molecule using cationic and anionic surfactant micelles and vesicles.[16] The apparent pKa value of curcumin was seen to increase in vesicles solution, thus resulting in stabilization of the molecule. The authors attributed the decrease in acidity of curcumin to arise from the inability of the base to remove the more acidic proton vinylogous acid proton (pKa 5) since it is buried inside the hydrophobic core of the leaflet. The accessible proton is the phenolic proton with a pKa of 10. (Figure 3.3)

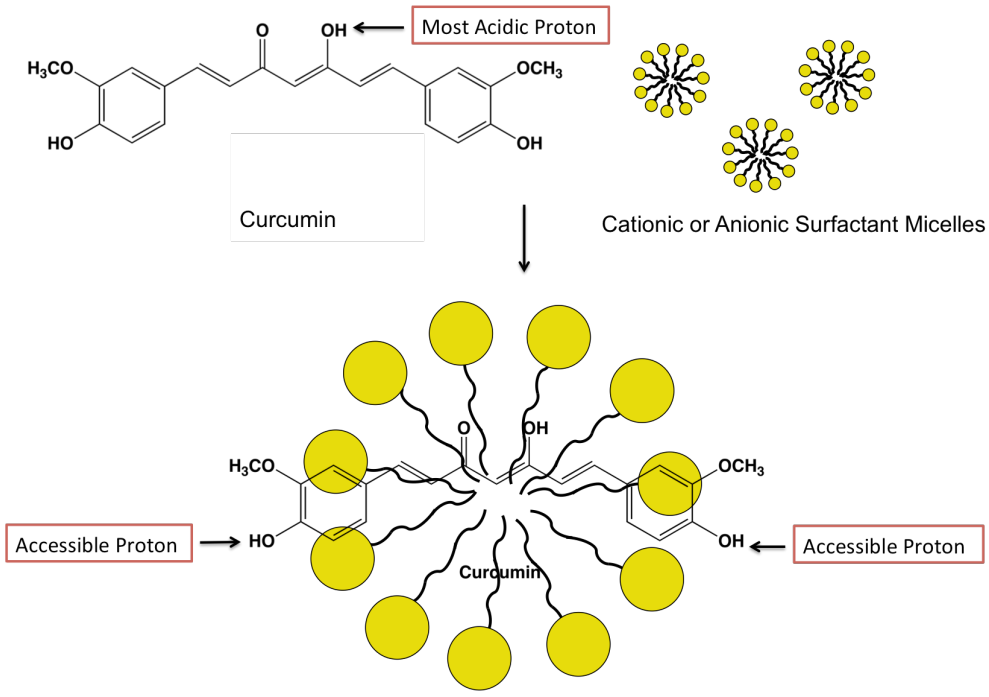


Figure 3.3 Model of curcumin embedded within a surfactant micelle, as proposed by Wang and coworkers.[16]

Based on the precedent of Wang, we hypothesized that a glycoconjugate anchored into the leaflet of catanionic vesicles would undergo glycosyltransfer with the appropriate transferase and glycosyl donor as shown in the figure 3.4.

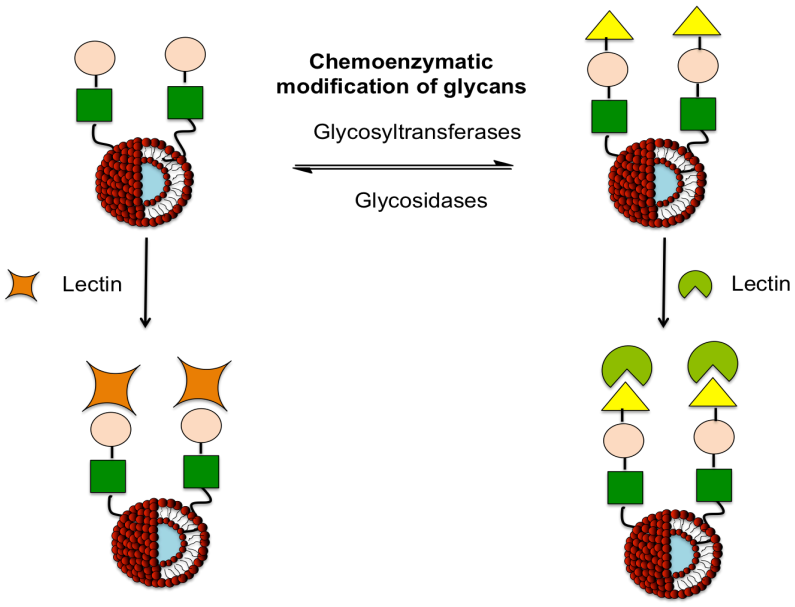


Figure 3.4 Performing chemo-enzymatic reactions on vesicle surface to modify glycans on vesicle surfaces. Changes in binding properties of functionalized vesicles occur upon performing chemo-enzymatic reactions

3.2 Specific Aims, Results and Discussions

The specific aim was to 1) demonstrate the ability of glycosyltransferases to perform glycosyl transfer of mono- and complex polysaccharide derivatives onto glycan functionalized catanionic vesicles, and 2) To perform enzymatic reactions on vesicle surfaces as they are deposited on surface of a microarray to show that glycans incorporated in vesicles are accessible for enzymatic modification when deposited on the surface.

3.3 Modification of F62ΔlgtE LOS Functionalized Vesicles using LgtE

Studies by Piekarowicz and Stein have shown that LgtE has the ability to mediate the transfer of multiple galactose moieties to whole cell LOS.[17] This observation

indicated that LgtE had promiscuous acceptor substrate specificity and could function on multiple acceptor glycans.

In collaboration with Dr. Stein's lab, we decided to determine the ability of catanionic surfactant vesicles to serve as a platform for synthesis of new glycan molecules by performing enzymatic reactions. We used flow cytometry to demonstrate LgtE activity on LOS functionalized vesicles. Upon reaction of LOS F62ΔlgtE functionalized vesicles and LgtE, we presumed that LgtE would attach UDP-Galactose to the natural substrate, LOS F62ΔlgtE. The results of this experiment, are demonstrated in figures 3.2 and 3.3**.** As the surface glycans on the vesicle surface are modified, it was hypothesized that the binding preferences and abilities of modified glycans will change accordingly. Reaction of LgtE with LOS F62ΔlgtE resulted in an approximately 52% reduction in ConA binding to LOS F62ΔlgtE LOS functionalized vesicles. Additionally, 10 fold increase in PNA binding was observed following reaction of LOS F62ΔlgtE with LgtE and UDP galactose. We concluded that reaction of LgtE with LOS functionalized catanionic surfactant vesicles results in modification of glycans. This reaction does not result in 100% conversion of surface LOS F62ΔlgtE to LOS F62ΔlgtA (terminal sugar glucose converted to galactose), as our results do not result in 100% reduction of Con A binding or 100 % increase in PNA binding. (Figure 3.5 and 3.6)

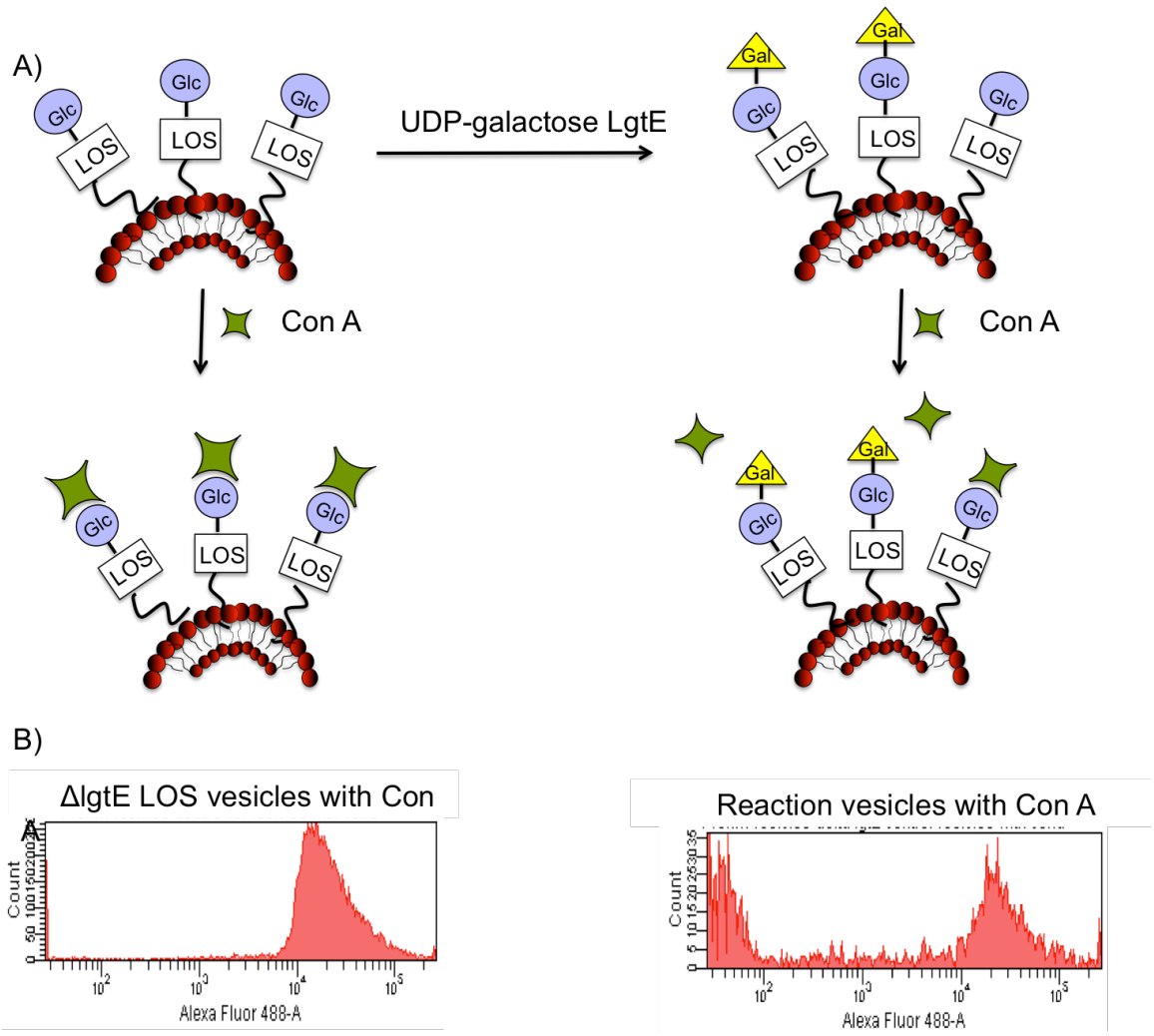


Figure 3.5 A. Schematic representations of reaction of LOS F62ΔlgtE functionalized vesicles with UDP galactose, LgtE. The change in binding with Con A is evident upon the reaction. B. Flow cytometry data of ConA incubated with LOS F62ΔlgtE functionalized vesicles before and after the reaction.

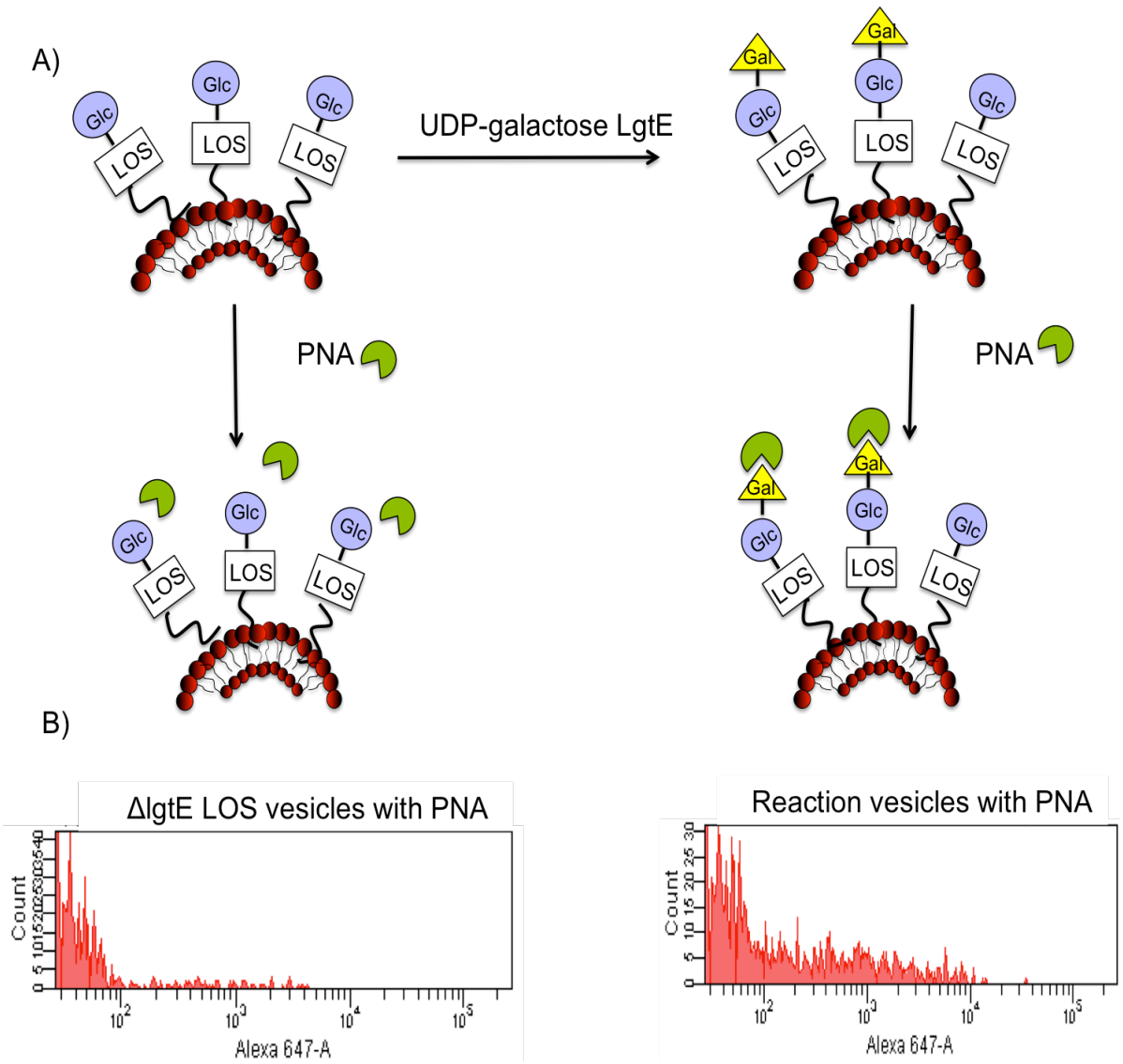


Figure 3.6 A. Schematic representations of reaction of LOS F62ΔlgtE functionalized vesicles with UDP galactose, LgtE. The change in binding with PNA is evident upon the reaction. B. Flow cytometry data of PNA incubated with LOS F62ΔlgtE functionalized vesicles before and after the reaction.

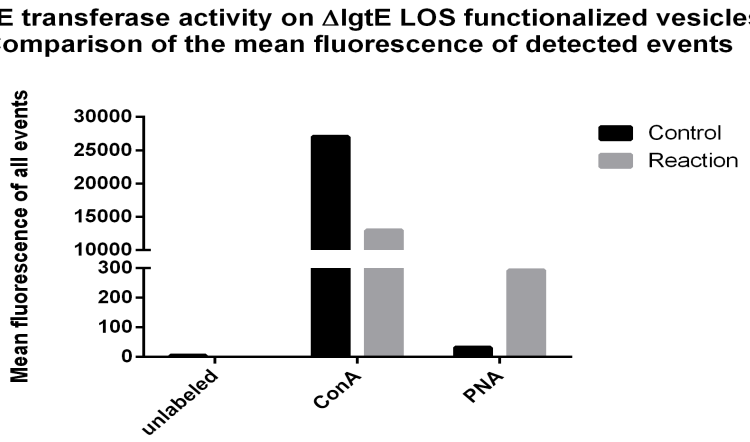


Figure 3.7. LgtE mediates the transfer of UDP-Galactose to LOS ΔlgtE functionalized vesicles. This figure shows quantification of the mean fluorescence of all events. As illustrated above, Con A binding reduces by 52 % and PNA binding increses by 10 folds upon reaction. Flow cytometry experiments done by Ms.Amanda Mahle.

3.4 Enzymatic Reactivity of LgtE on Glucose Functionalized Vesicles

In order to use glycosyltransferases to synthesize complex glycans on the surface of catanionic vesicles, there is a need to define their acceptor specificities. Methods to determine specificities of activity of glycosyltransferase have been explored that include radiochemical, spectrophotometric, immunological and chromatographic assays.

The aim of this study was to exploit the ability of LgtE to create glycans of known structure, by performing enzymatic transformations on acceptor substrates not necessarily derived from Neisserial LOS. To investigate the feasibility of exploiting LgtE to modify a diverse array of glycans, we sought to determine the activity of LgtE on C_{12}-glucose functionalized surfactant vesicles.

Having shown that LgtE can mediate the transfer of a galactose moity to its natural substrate, LOSΔE functionalized vesicles, Next, we aimed to verify if LgtE can mediate the transfer to a synthetic substrate , C_{12} glucose functionalized vesicles.

According to our experiments, LgtE is effective in transfering the galactose residue to C_{12} glucose functionalized vesicles. Flow cytometry experiments illustrate that Con A binding to glucose vesicles was reduced by approximately 38% upon reaction of C_{12}-glucose functionalized vesicles with LgtE. PNA binding to vesicles that had undergone galactosyl transfer should be increased correspondingly, and this increase in observed. There was a 10 fold in binding of PNA to vesicles after LgtE reaction. This experiment demonstrated that glycans incorporated in vesicles are capable of undergoing enzymatic transformations. (Figure 3.8 and 3.9)

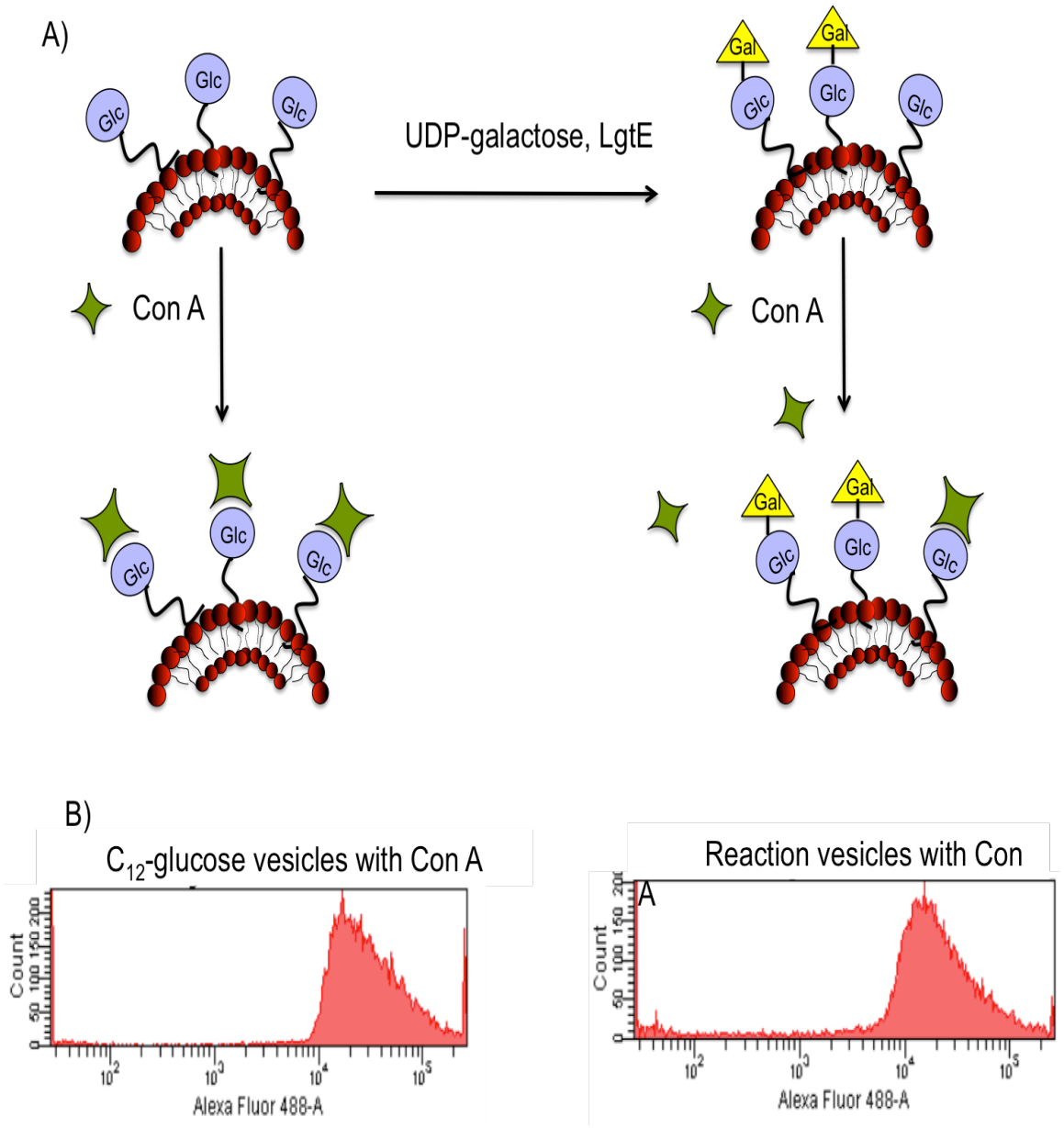


Figure 3.8 A. Schematic representations of reaction of C_{12} glucose functionalized vesicles with UDP galactose, LgtE. The change in binding with Con A is evident upon the reaction.
B. Flow cytometry data of Con A incubated with C_{12} glucose functionalized vesicles, before and after reaction with UDP galactose LgtE.

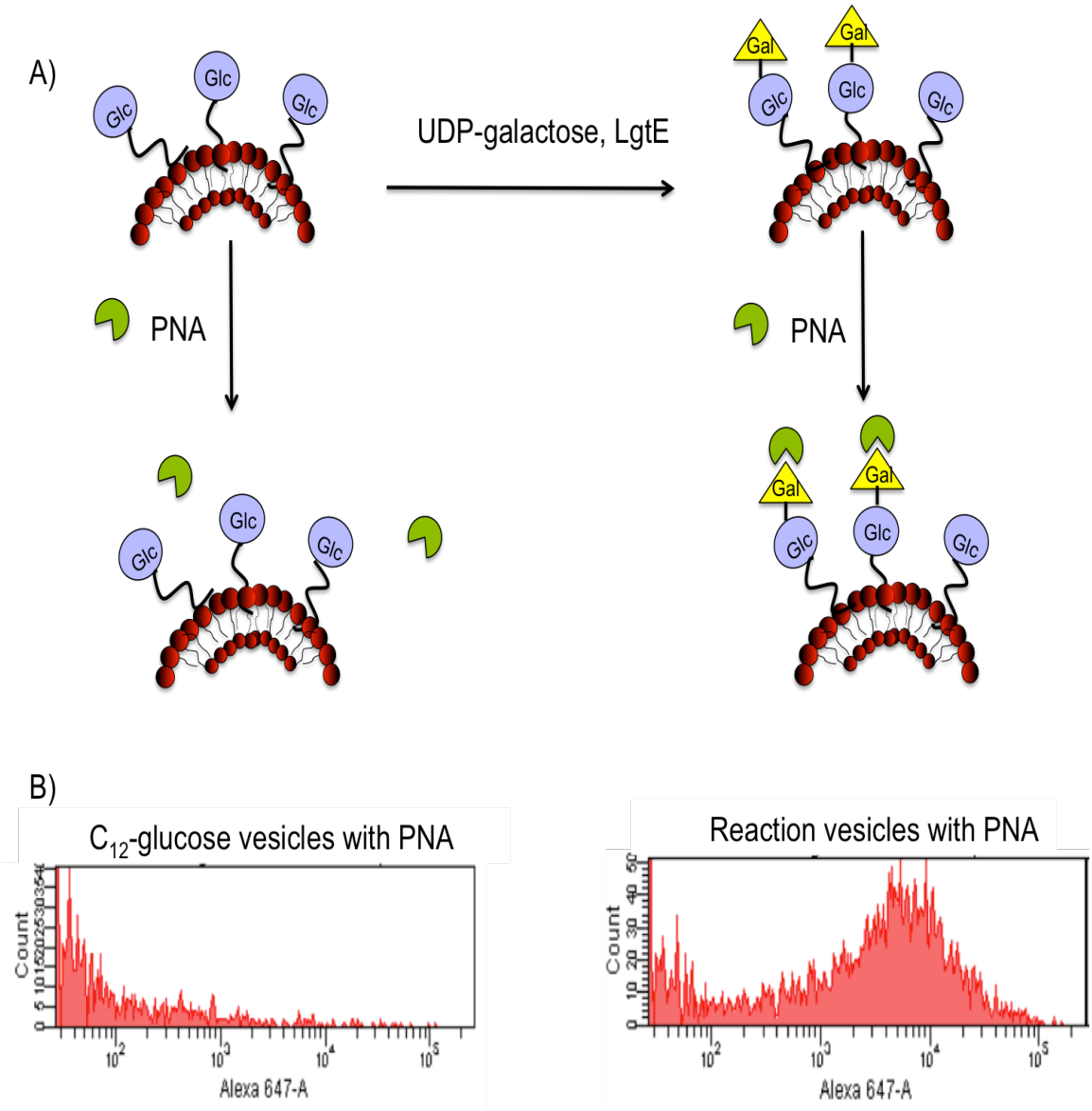


Figure 3.9 A Schematic representations of reaction of C_{12} glucose functionalized vesicles with UDP galactose, LgtE. The change in binding with PNA is evident upon the reaction. B. Flow cytometry data of PNA incubated with C_{12} glucose functionalized vesicles, before and after reaction with UDP galactose LgtE.

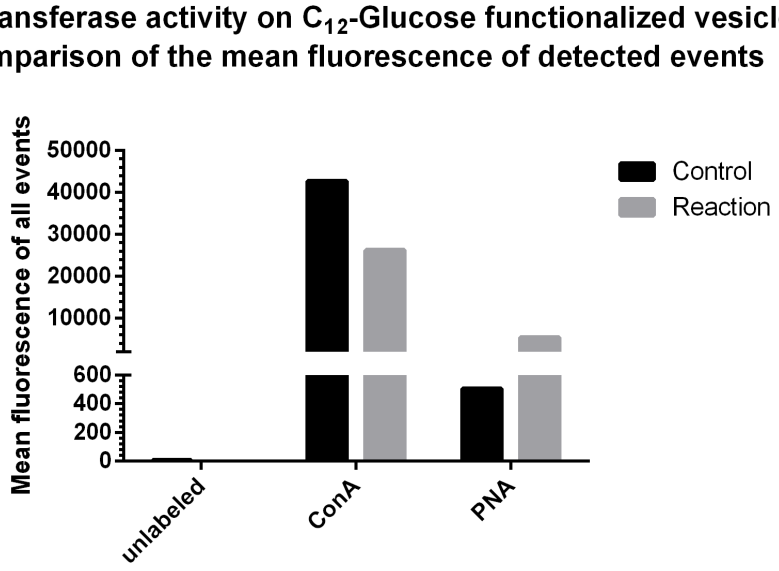


Figure 3.10**.** LgtE mediates the transfer of UDP-Galactose to C_{12} glucose functionalized vesicles. This figure shows quantification of the mean fluorescence of all events. As illustrated above, Con A binding reduces by 38% and PNA binding increses by 10 folds upon reaction. Flow cytometry experiments done by Ms.Amanda Mahle.

Our experiments with LgtE demonstrated that glycan-functionalized catanionic surfactant vesicles can be modified using glycosyltransferase. As shown by our experiments with C_{12}-glucose functionalized vesicles, biosynthetic transformations with incorporated glycans proceed even when the enzyme does not have a natural acceptor glycan incorporated in to the vesicle.

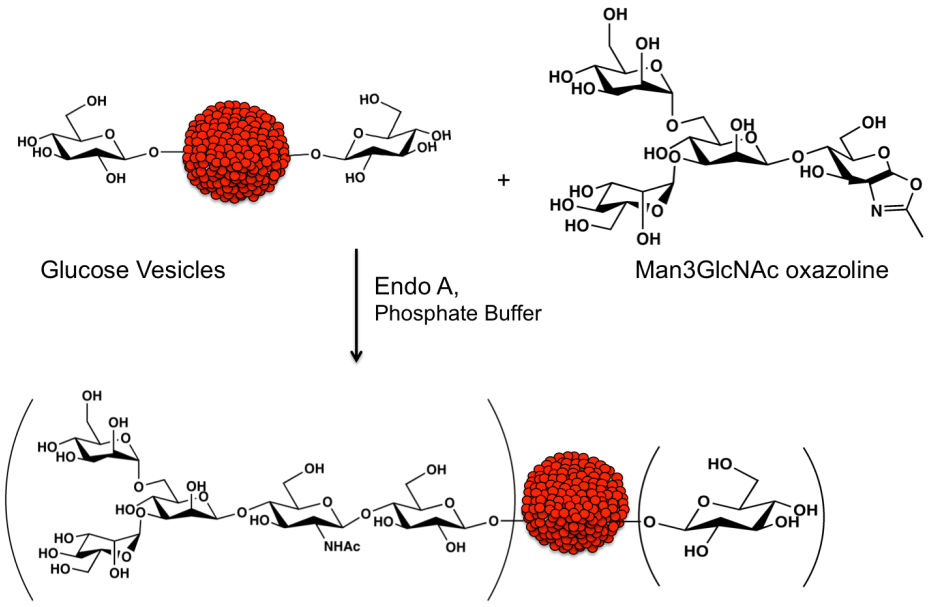
3.5 Modification of Glycans on Vesicle Surface using Endo A

Transfer of a galactose moity by LgtE to perform vesicle glycosylation, required UDP-galactose as the glycosyl donor. This method would be both expensive and tedious for synthesizing polysaccharides if only a monosaccharide is added per step.

Having shown that LgtE can mediate the transfer of monosaccharides to glycan funcitonalized vesicles, next we proposed to determine whether complex glycans could undergo enzymatic synthesis on vesicles surfaces.

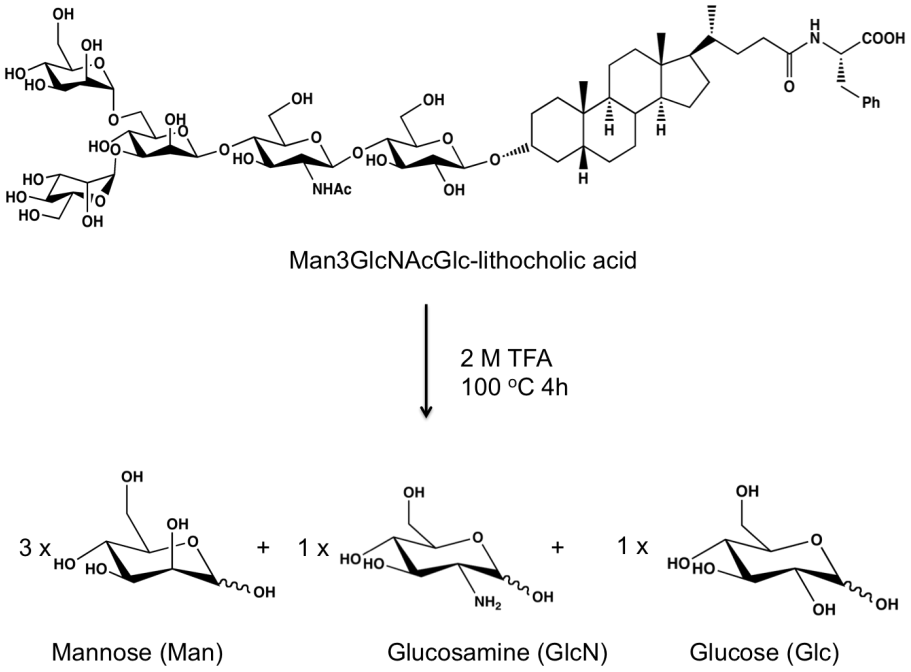
In order to create homogeneous glycoproteins and glycoforms, many enzymatic methods have been created. Transglycosylation using the endo-*β*-*N*-acetylglucosaminidase results in hydrolysis of *β*-1,4-glycosidic bond in the core *N*,*N*′-diacetylchitobiose moiety of *N*-glycoproteins. Endo-A from *Arthrobacter protophormiae* can transfer the releasing *N*-glycan to a GlcNAc-peptide acceptor resulting in synthesis of new glycopeptides.[18] The activity is particularly higher for highly mannosylated glycans. Ability of Endo A to transfer complex and intact glycopeptides in one step is unique when compared to other glycosyl transferases that typically transfer monosaccharides. This is very useful to create large glycoproteins.

As C_{12} glucose is not a natural substrate for Endo A, we first wanted to test if Endo A would mediate transfer of $Man_3GlcNAc$ oxazoline (Figure 3.11) to C_{12}-glucose *in solution*. Reaction of C_{12}-glucose with Endo A and $Man_3GlcNAc$ oxazoline resulted in 33% yield of $Man_3GlcNAcGlc\text{-}OC_{12}$. With the success of this solution phase transformation, we proposed to test the ability of Endo A to transfer $Man_3GlcNAc$ oxazoline to C_{12}-glucose functionalized vesicles.



Scheme 3.1. Generic reaction scheme of reaction between Endo A, $Man_3GlcNAc$ oxazoline and C_{12} glucose functionalized vesicles.

In order to determine the ability of Endo A of transferring large glycopeptide on C_{12} glucose functionalized vesicles, we treated C_{12} glucose functionalized vesicles with endo A and $Man_3GlcNAc$ oxazoline. The transfer of $Man_3GlcNAc$ on glucose vesicles, was analyzed by subjecting the reacted vesicle solution to High-performance anion-exchange chromatography (HPAEC).

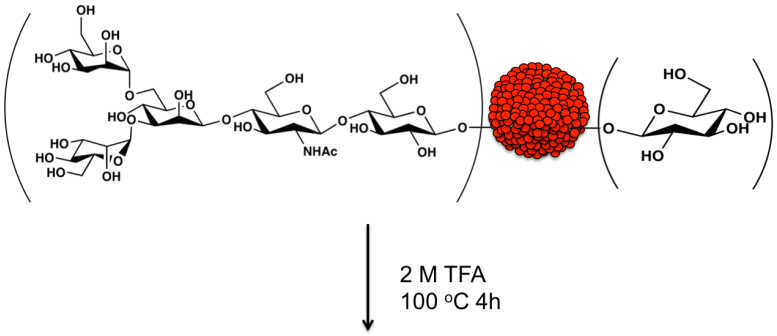


Scheme 3.2. Reaction of $Man_3GlcNAcGlc$-lithocholic acid, a control used in this analysis.

$Man_3GlcNAcGlc$-lithocholic acid was used as a control for these experiments. When $Man_3GlcNAcGlc$-lithocholic acid is subjected to hydrolysis with TFA, at 100^oC for 4 hours, it results in complete cleavage of polysaccharide linkages in the molecule. As depicted in figure 3.12, One molecule of $Man_3GlcNAcGlc$-litholic acid upon cleavage results in 3 molecules of mannose (Man), 1 molecule of glucosamine (GlcN) and a molecule of glucose (Glc).

Glucose functionalized vesicles reacted with Endo A, and oxazaline would result in similar cleavage to yield Man, GlcN, and Glc. We have to consider that not all glucose molecules on the vesicle surface would undergo transglycosilation, and hence there will be some unreacted glucose on vesicle surface.

Now if we assume that hydrolysis of this partially glycosylated vesicle results in x molecules of Man, y molecules of GlcN, and z molecules of Glc, we can calculate the yield of glycosylation can as follows



Man	+	GlcN	+	Glc	
X	+	Y	+	Z	(%)

Scheme 3.3. Calculation of glycosilation yield of Endo A activity on C_{12} glucose functionalized vesicles.

%Yield of glycosylation = (([GlcN]/[Glc] + ([Man]/3)/[Glc])/2)*100

= ((y/z) + (x/3)/z))/2

We used two concentrations of glucose vesicles for this experiment, namely 4.4 mM solution of 0.2 mf functionalized C_{12} glucose vesicles (condition 1) and 127 μM solution of 0.005 mf functionalized C_{12} glucose vesicles (condition 2) to test the effect of glucose concentrations on the glycosilation activity. The results of HPAEC analysis performed on product vesicles is as follows:

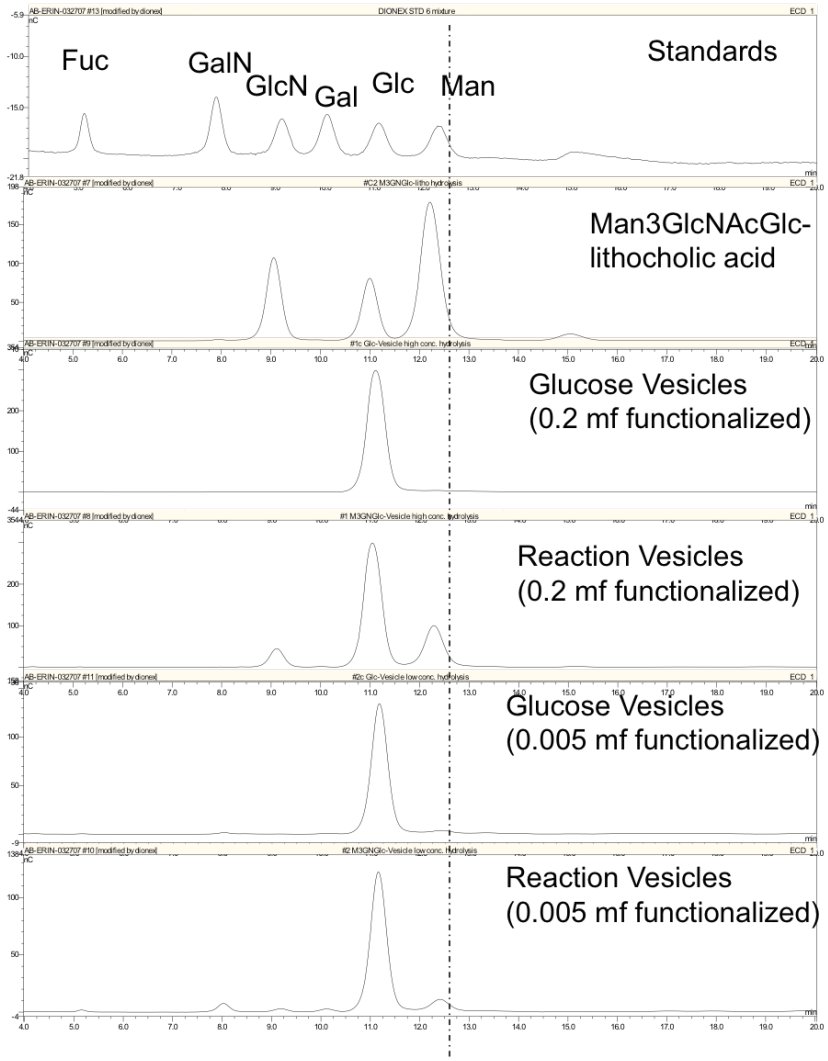


Figure 3.11 HPAEC results of vesicle glycosylation reaction with Endo A.

Sample	**Peak Area (%)**		
	GlcN	Glc	Man
Man_3-GlcNAc-Glc lithocholic acid	24.66	19.78	55.56
Reacted 0.2 mf C_{12} glucose vesicles (Condition 1)	8.07	66.63	25.30
Reacted 0.005 mf C_{12} glucose vesicles (Condition 2)	1.96	87.86	10.18

Table 3.1. Percentage of peak area of glycans obtained from HPAEC, HPAEC experiments performed by Dr. Wei Huang .

Man_3-GlcNAc-Glc-lithocholic acid shows complete cleavage under these reaction conditions as observed with HPAEC.

Yield of glycosylation for C_{12} glucose functionalized vesicles:

Condition 1: 0.2 mf C_{12} glucose functionalized vesicles

%Yield of glycosylation = (([GlcN]/[Glc] + ([Man]/3)/[Glc])/2)*100

= (((8.07/66.63) + (25.30/3)/66.63)/2)*100

= 12.4%

Condition 2: 0.005 mf C_{12} glucose functionalized vesicles

%Yield of glycosylation = (([GlcN]/[Glc] + ([Man]/3)/[Glc])/2)*100

= (((1.96/87.86) + (10.18/3)/87.86))/2)*100

= 3.04%

Based on the calculations above, 12.4% of the glucose on the surface of the vesicle was glycosylated with Man_3GlcNAc, when 0.2 mf C_{12} glucose functionalized vesicles were used for the reaction. Where as 3.04% of glucose moiety was glycosylated when 0.005 mf C_{12} glucose functionalized vesicles were treated with glycosylation conditions. Different yield of glycosylation observed for different vesicle concentration demonstrates that the reaction is dependent on concentration of glucose on vesicle surface. Since C_{12}-glucose vesicles are not a natural substrate for Endo A activity, a 12.4% yield of product formed due to tetrasaccharide transfer by Endo A serves as a proof that glycan functionalized vesicles can act as platform to performing enzymatic reactions.

3.6 Modifying Glycans on Array Surface using Enzymatic Reactions

Having shown that glycan modification can be achieved in solution phase, we wanted to examine the accessibility of our glycan arrays to perform glycan modification

on surfaces. Previous studies have focused on use of glycan microarrays to determine the extent of acceptor substrate utility of these enzymes. A number of groups have used glycan arrays built by attaching glycans to surfaces to construct assays to determine substrate specifity of various glycosyl transferases.[19-21] For example, to identify new glycosyltransferases and characterize their substrate specificities studies, Wang et al screened more than 60000 glycosyl transferases using high throughput glycan arrays. The resulting change in molecular weight due to transfer of a glycan residue was monitored using mass spectrometry.[22] This resulted in discovery of four unknown glycosyltransferases.

Having demonstrated as summarized in Chapter 2.6.2 of this thesis that glycan-functionalized catanionic vesicles deposited onto HM chitosan were sufficiently robust to serve as a platform for protein binding assays and recognition events, we wanted to determine if enzymatic synthesis could be performed on the array surface. In this scenario, we hypothesized that it would be possible to synthesize vesicles with novel glycan derivatives embedded into the leaflet of the catanionic vesicle employing enzymatic methods using glycosyltransferases. This method would allow the preparation of LOS/LPS moieties not available from pathogens. For example, treatment of a vesicle containing a LOS derivative terminating in galactose with β-galactosidase should result in cleavage of the galactose, producing a truncated LOS. Similarly, one should be able to add glycan residues to an existing LOS by treatment with the appropriate carbohydrate precursor and its corresponding glycosyltransferase.[14] While these biosynthetic transformations occur readily in SDBS-rich catanionic vesicles in suspension, we

proposed to demonstrate that enzymatic synthesis could be accomplished after deposition of the vesicle.

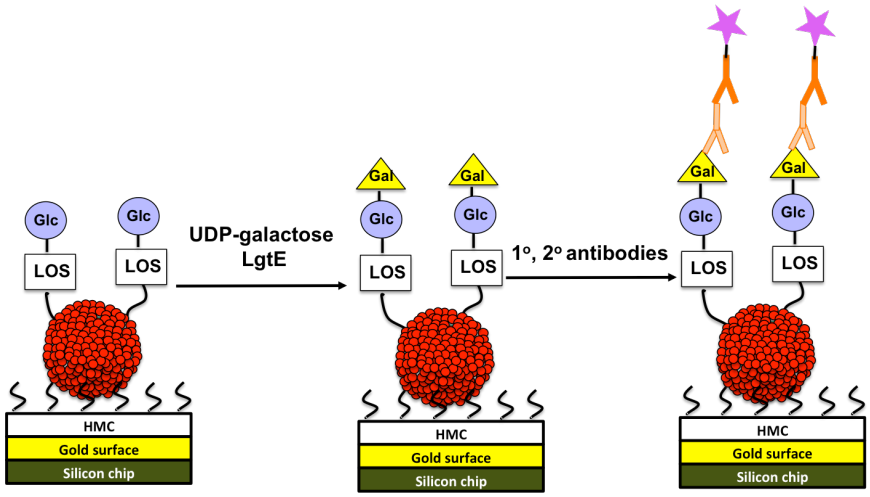


Figure 3.12. Schematic representation of performing chemo-enzymatic reactions on glycans deposited on array surfaces.

Treatment of ΔlgtE LOS-functionalized vesicle array (LOS terminates in glucose) with purified LgtE (an enzyme that catalyzes the addition of galactose onto ΔlgtE LOS) and UDP-galactose overnight at 30°C converted the ΔlgtE LOS into the ΔlgtA LOS derivative (Figure 3.13 A), confirming that the glycosyltransferase reaction had converted the ΔlgtE LOS into its ΔlgtA counterpart.

The transfer of galactose to ΔlgtE LOS to give the modified LOS was confirmed using an ELISA assay. Exposure of the functionalized vesicle arrays to a monoclonal antibody with high specifity for the galactose modified LOS (mAb 2-1-L8, provided by Dan Stein's lab) showed strong binding of the antibody in a sandwich assay (the positive control, Figure 3.13 B). F62ΔlgtE LOS-functionalized vesicles (vesicles containing an LOS identical to F62ΔlgtA LOS, with the exception that it lacks the terminal galactose) served as the negative control (Figure 3.13 A) and were not recognized by this mAb. In

each system, the presence or absence of antibody binding was detected using fluorescence.

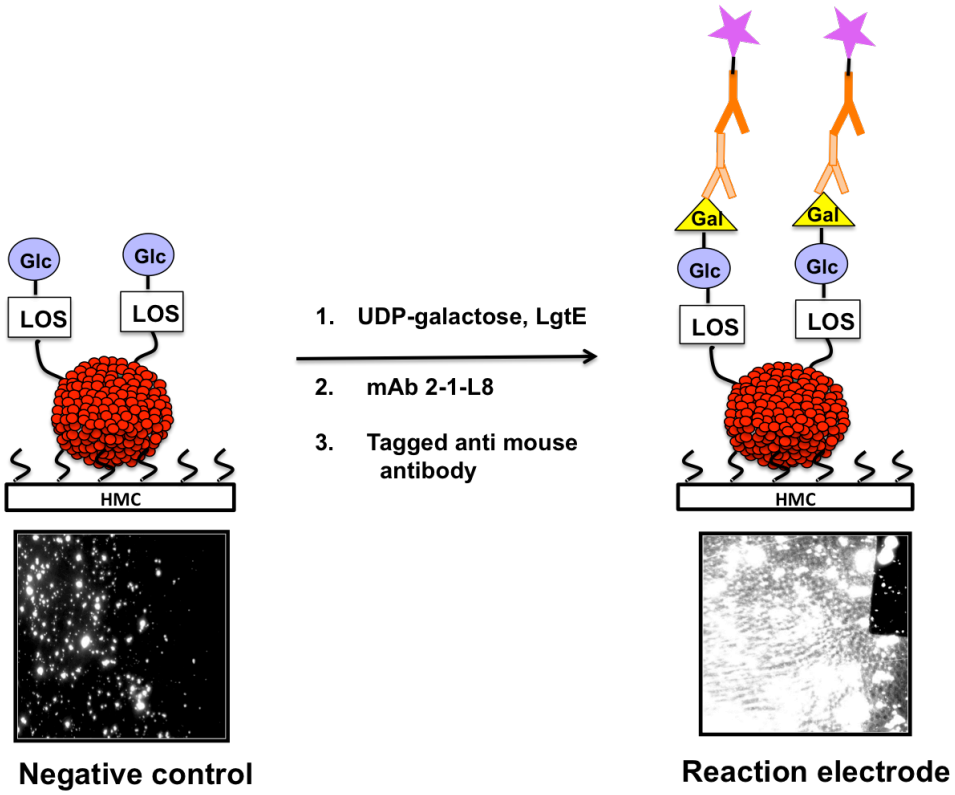


Figure 3.13 A. Catalytic modification by LgtE of functionalized vesicles, as demonstrated by an ELISA. In this assay ΔlgtA LOS functionalized vesicles were detected by binding it to primary antibody mAb 2-1-L8. The presence of this antibody was detected by a secondary antibody goat anti mouse IgG, fluorescently tagged with Alexa fluor 568. (A). ΔlgtE LOS functionalized vesicles did not exhibit binding with mAb 2-1-L8., these vesicles upon reaction with LgtE and UDP-galactose show binding with 1^{o} and 2^{o} antibodies.

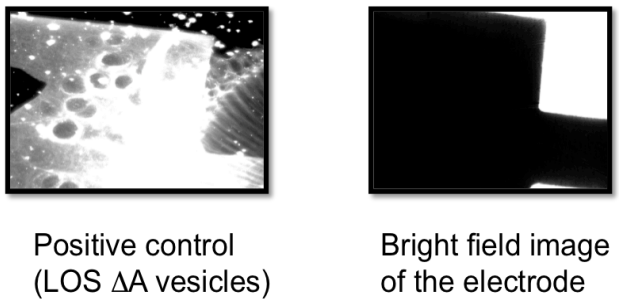


Figure 3.13 B. Positive control electrodes, deposited with LOS ΔlgtA functionalized vesicles and a bright field image of this electrode. This electrode shows the presence of mAb 2-1-L8, as observed by the binding with secondary antibody

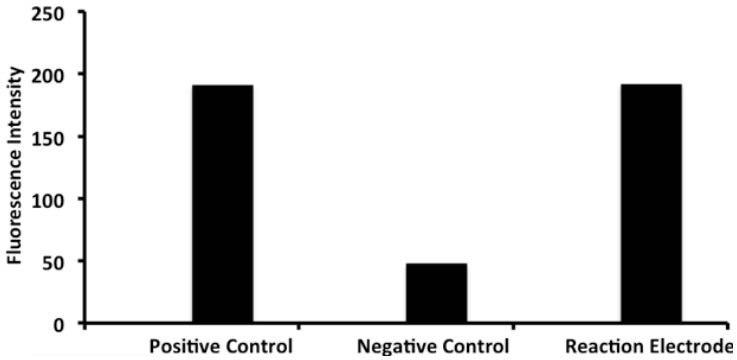


Figure 3.13 C. Graph depicting fluorescence intensity on electordes depicted in figures 3.12 A, B. Reaction electrode shows four fold increase in fluorescence intensity than the negative control upon reaction.

The ability to perform enzymatic reactions on LOS-functionalized vesicles indicated that ligands are displayed on the surface of the vesicles and that these ligands are capable of being transformation by enzymatic methods. The chemo-enzymatic reaction on the carbohydrate molecules incorporated into the vesicle leaflet opens the door for enzymatic synthesis of defined molecules and thereby the construction of diverse glycomic arrays.

3.7 Conclusions

Synthesis of complex glycans is a well established problem in the field of carbohydrate chemistry. Our efforts in solution, as well as surface phase, clearly demonstrate that we can modify glycans on the vesicle surface. This ability to selectively modify a portion of the molecule enables us to create new glycan molecules with great efficiency.

Biosynthesis of most carbohydrates takes place with the help of glycosyl transferases.

However, their use to create new glycan molecules chemo-enzymatically is still limited due to lack of a platform to perform these chemo-enzymatic reactions. Our studies with LOS vesicles show that we can modify LOSΔE to LOSΔA on the vesicle surfaces with the help of LgtE and UDP galactose in solution phase. This was illustrated with the help of change in the binding properties with the respective GBPs. Studies with glucose functionalized vesicles show that LgtE can mediate transfer of a galactose moity to glucose functionalized molecules, indicating that enzymatic chemistry could be performed on unnatural, synthetic glycans. This series of results shows that functionalized vesicles serve as an excellent platform to perform enzymatic reactions on surfaces. Reacted vesicles with modified glycan undergo binding with their appropriate GBPs or antibodies as indicated with our surface studies. Taken together, the data presented here provide a proof-of-concept that glycosyltransferases and functionalized surfactant vesicles can be used to create a complex glycan arrays for a variety of diagnostic applications.

3.8 Experimental

3.8.1 Chemicals, Reagents, Lectins, and Antibodies

All chemicals including, Sodium dodecylbenzenesulfonate, *n*-dodecyl β-D-glucopyranoside (C_{12}-glucose) were purchased from Sigma Aldrich Co. unless specified otherwise. Cetyltrimethylammonium tosylate was ordered from TCI America.

MAb 2-1-L8 was graciously provided by Wendell Zollinger (Walter Reed Army Institute of Research, Washington DC). Alexa Fluor goat anti-mouse IgG (H+L), Concavalin A

Alexa Fluor® 488 conjugate, and peanut agglutinin Alexa Fluor 647 were purchased from Invitrogen Corp.

3.8.2.1 Preparation of Vesicles

Sodium dodecylbenzenesulfonate (SDBS) was purchased from TCI America and was utilized without further purification. Cetyltrimethylammonium tosylate (CTAT) was purchased from Sigma and was recrystallized from ethanol-acetone to give a white powder. The purified solid was stored at room temperature in a desiccator containing Drierite.All vesicle solutions were prepared as reported in chapter 2.15.6.1 and purified by SEC as described in chapter 2.15.6.2.

3.8.2.2 Glyan Functionalized Vesicles

C_{12}-glucose functionalized vesicles were prepared according to the method reported by Thomas et al.[23] For modification reactions with LgtE, 0.1 mf of glucose vesicles were prepared by adding 9.295 mg of glycoconjugate to preformed bare vesicles. For modification reactions with Endo A, 0.2 mf glucose vesicles (using 18.589 mg glycoconjugate) and 0.005 mf (465.0 μg glycoconjugate) glucose vesicles were prepared. All solutions were stirred for 24 hours.

3.8.2.3 LOS Functionalized Vesicles

LOS functionalized vesicles were prepared by combining 1.00 mg of LOS F62ΔE or LOS F62ΔA in 10 mL preformed 1% SDBS rich vesicle solution. This solution was stirred for 24 hours.

3.8.3 Flow Cytometry Analysis On Functionalized Surfactant Vesicles

Flow cytometry experiments were performed by Ms. Amanda Mahle from Dr. Daniel Steins lab in Department of Microbiology and Molecular Genetics.

100 μM ΔlgtE LOS or glucose terminal C_{12}-glucose functionalized vesicles were incubated in reaction buffer (20 mM HEPES pH 7.5, 1 mM $MnCl_2$, 1 μM LgtE) with or without 1 mM UDP-galactose, at $30^{O}C$ for a minimum of 2 hours. The vesicles were centrifuged at 5000 rpm for 20 min and resuspended in 100 μL 2% BSA. The solution was incubated for 1 hour at room temperature with moderate shaking. The vesicle solutions were again centrifuged at 5000 rpm for 20 min and resuspended in 500 μL 20 mM HEPES pH 7.5.The vesicles were then incubated with diluted ConA Alexa Fluor 488 conjugate (500 μg/ml solution) or PNA Alexa Fluor 647 conjugate (100 μg/ml solution) for 1 hour at room temperature with moderate shaking followed by an overnight incubation at $4^{O}C$. The vesicle-lectin complexes were subsequently washed three times with 500 μL, 20 mM HEPES pH 7.5. Labeled vesicles were subjected to FACS analysis using FACSAria (BD Biosciences) flow cytometer. Data was analyzed with FACSDiva (BD Biosciences software). Quantification of the average FSC-A (forward side-scatter) for each condition was measured as it measures of the size of the detected events.

3.8.4 Modifying Glycans on Array Surface using Enzymatic Reactions

100 μM LOS functionalized vesicles were incubated in reaction buffer (20 mM HEPES pH 7.5, 1 mM $MnCl_2$, 1 μM LgtE) with or without 1 mM UDP-galactose, at $30^{O}C$ for a minimum of 2 hours. Subsequently, the reaction mixture was spotted onto the

functionalized electrodes, and then washed 3 times with 1x PBS pH 7.2. Monoclonal antibody 2-1-L8 was then applied to the electrodes for one hour. Following a wash step, Alexa Fluor® 568 goat anti-mouse IgG (H+L) antibody (Molecular Probes®) in 20 mM HEPES pH 7.5 was applied to the electrode. A final wash step was performed and the electrodes were visualized on a Zeiss Axio Imager. Z1 fluorescent microscope (Carl Zeiss) through a EC Plan-Neofluor 10x objective in brightfield and fluorescence using filter set 43HE (excitation wavelength 550/25 nm and emission wavelength 605/70 nm; Zeiss). Photographs were taken with a mounted AxioCam MR3 (Zeiss), and the images were analyzed using AxioVision Rel. 4.8.2 software (Zeiss).

3.8.5 Modification of Glycans on Vesicle Surface Using Endo A

Enzymatic transglycosylation was carried by Dr. Wei Huang in Prof. Lei Xi Wang's group at University of Maryland, Baltimore County. These experiments were done mainly under two sets of conditions.

Condition 1: A 4.4 mM solution of 0.2 mf C_{12} glucose vesicle in a phosphate buffer (20 mM, pH 7.0, 360 mL) containing Man3GlcNAc oxazoline (12.0 mM) was incubated with Endo A (15 μg) at 30 ^{o}C for 2 hours. The mixture was subject to size exclusion chromatography with a Sephadex G100 column and eluted with a phosphate buffer. The isolated vesicle fractions were combined and concentrated. The prepared Man3GlcNAcGlc-vesicle was analyzed for its monosaccharide composition following the method described below.

Condition 2: A 127 μM solution of 0.005 mf C_{12} glucose vesicle in a phosphate buffer (20 mM, pH 7.0, 1.1 mL) containing Man3GlcNAc oxazoline (396 μM) was incubated with Endo A (15 μg) at 30 ^{o}C for 2 hours. The purification procedure was performed following the same protocol for condition 1. The saccharide analysis showed 3% of glucose moiety of the vesicle was glycosylated with Man3GlcNAc.

3.8.6 Glycan Analysis on Man3GlcNAcGlc-Vesicle

Purified Man3GlcNAcGlc-vesicle (200.0 μL) was mixed with 4 N TFA (200.0 μL) in a sealed tube and the solution was heated to 100^{o}C for 4h. The residue was cooled to RT and washed with chloroform (400.0 μL). The aqueous layer was lyophilized and re-dissolved in water, then was subject to High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detector (HPAEC-PAD) to determine the composition of released monosaccharides including mannose, glucosamine, and glucose. The transglycosylation yield was calculated based on the molar ratio of mannose/glucose or glucosamine/glucose.

3.8.7 High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

HPAEC-PAD was performed on a DIONEX DX600 chromatography system (DIONEX Corporation, Sunnyvale, CA) equipped with an electrochemical detector (ED50) and an anion exchange column (CarboPac PA10, 4 × 250 mm). The column was eluted by 18 mM NaOH to give complete separation on monosaccharide standards.

3.8.8 Enzymatic synthesis of $Man_3GlcNAcGlc\text{-}OC_{12}$

A solution of C_{12} glucose (5.0 mg, 14.4 mM) and $Man_3GlcNAc$ oxazoline (5.0 mg, 7.3 mmol) in a phosphate buffer (50 mM, pH 7.0, 200.0 μL) containing 50% DMSO was incubated with Endo A (25.0 mg) at 30 ^oC for 2 hours. The reaction residue was subject to chromatography with a silica gel column. The column was successively eluted with $CHCl_3$:MeOH:H_2O (10:10:1), $CHCl_3$:MeOH:H_2O:AcOH (10:10:2:1), and $CHCl_3$:MeOH:H_2O (5:5:2). The product fraction was combined and concentrated to give $Man_3GlcNAcGlc\text{-}OC_{12}$ as a pale yellow oil (2.50 mg, 33%). MALDI-TOF MS: calculated for $C_{44}H_{79}NO_{26}$, 1037.49; found, 1060.22 ($M+Na^+$). ^1H NMR (d_6-DMSO:D_2O 10:1) d 4.80 (s, 1H, aMan-H1), 4.60 (s, 1H, aMan'-H1), 4.45 (s, 1H, bMan'-H1), 4.34 (d, *J* = 8.0 Hz, 1H, GlcNAc-H1), 4.09 (d, *J* = 8.8, 1H, Glc-H1), 3.71-2.90 (m, 32H), 1.81 (s, 3H, Ac), 1.42-0.76 (m, 23H).

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Chapter 4: Preparation of Glycan Microarrays with Ink Jet Printed Surface Functionalized Catanionic Surfactant Vesicles

4.1 Introduction

As reported in Chapter 2, we have reported that vesicle deposited arrays could be fabricated on HM chitosan surfaces. However HM chitosan surfaces prepared by electrodeposition are quite hetergeneous in their surface morphology. We were concerned that our studies with varying vesicle-glucose concentrations might have been influenced by the uneven deposition of vesicles. HM chitosan surfaces are also not suitable for mass production, are not robust and have low shelf life.

Micro contact printing (μCP) is a promising technique to print multiple homogeneous arrays. To improve overall spot homogeneity, a technique for preparing glycan arrays utilizing microcontact printing of glycoconjugates was developed by Mirksch et al.[1] This involved a covalent immobilization of glycans on the surface using a Diels Alder reaction. Gildersleeve and coworkers have used μCP for printing neoglycolipids to prepare glycan microarrays based on epoxide opening chemistry.[2] A microcontact printing technique for printing polydiacetylene liposomes on ITO glass surfaces was developed by Shim and coworkers.[3]

Synthesizing neoglylipid arrays or the preparation of giant liposomes is a tedious process. The specific aim of the studies reported in this chapter were to combine our technique of preparing glycan arrays using surfactant vesicles and micro contact printing to prepare uniform and robust microarrays that are also easy to prepare.

Recently Pond et al. have developed a microarray by depositing catanionic surfactant vesicles on nitrocellulose paper.[4] Glucose functionalized vesicles were

deposited on nitrocellulose membranes and were detected by using a sandwich assay of biotinylated Con A and tagged NeutrAvidin molecules. (Figure 4.1)

The authors observed excessive non specific binding of NeutrAvidin to the nitrocellulose surface. The authors also did not observe a positive correlation between glycan concentration on the surface and Con A binding. We thus aimed to create an alternate assay to detect glucose- Con A binding on the vesicle surface.

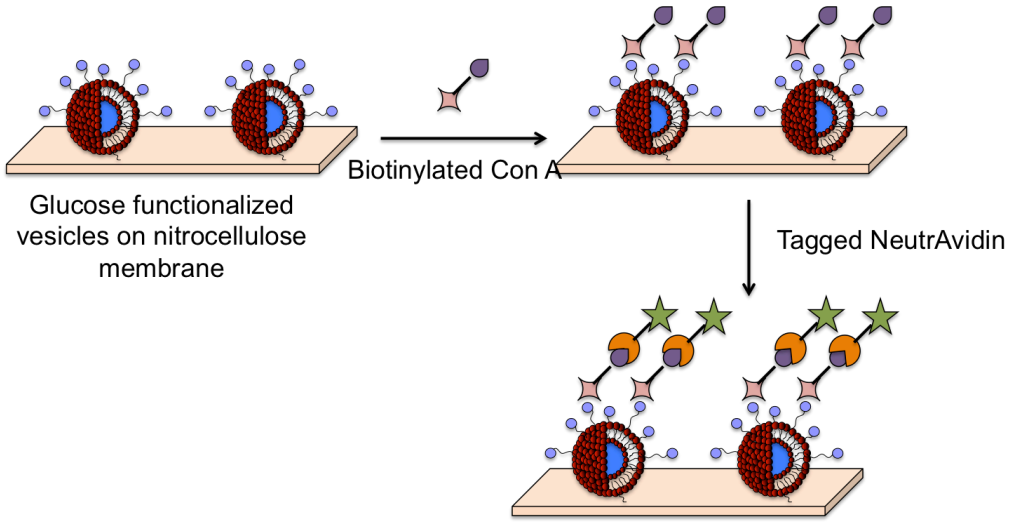

Figure 4.1 Detection assay used by Pond et Al. to detect glucose vesicle- Con A binding on nitrocellulose surfaces.[4]

4.2 Determining Substrates for Vesicle Deposition.

In order to prepare paper based microarrays, we first had to determine an appropriate material for deposition of catanionic vesicles. One can obtain papers and/or membranes with a variety of characteristics including hydrophobic or hydorphilic properties. We screened a variety of filter papers and membranes for our studies. Deposition of dye labeled vesicles was carried out on these filter papers. After the initial deposition and drying, washing studies with Triton X100 were carried out to identify

membranes that would anchor vesicles and withstand the aqueous conditions required in the anticipated binding studies.

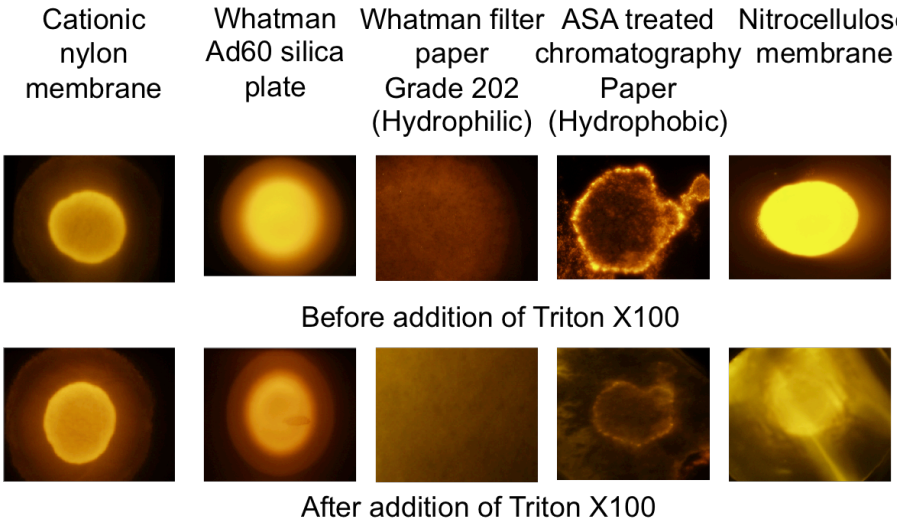

Figure 4.2 Images of surfaces following deposition of dye functionalized vesicles, before and after treatment with Triton X100.

A majority of these materials effectively anchor vesicles as indicated by the results presented in Figure 4.2. The images in Figure 4.2 show that vesicles are retained on most of the materials. But as vesicles are deposited on membranes, they may break down and the dye encapsulated in vesicles could release onto the membranes. This could result in false indication of presence of vesicles. Hence we trated the surfaces with Triton X 100 solutions. Treatment of catanionic vesicles with Triton X100 has been shown to disrupt vesicles in solution, and we assume that treatment of the vesicles deposited onto the membranes would also disrupt the vesicles and release dye. Based on this theory, dye oozes out of hydrophilic filter paper, hydrophobic filter paper, and nitrocellulose membranes upon treatment with Triton X100, indicating that vesicles can remain intact on these materials.

An indirect assay was developed to assess whether vesicles on these surfaces were intact. We observed that vesicles containing dye were able to withstand high intensity ultraviolet irradiation without bleaching of the dye. However, when high levels of Triton were added to the vesicle deposits, presumably disrupting the vesicles, rapid bleaching of the dye was observed.

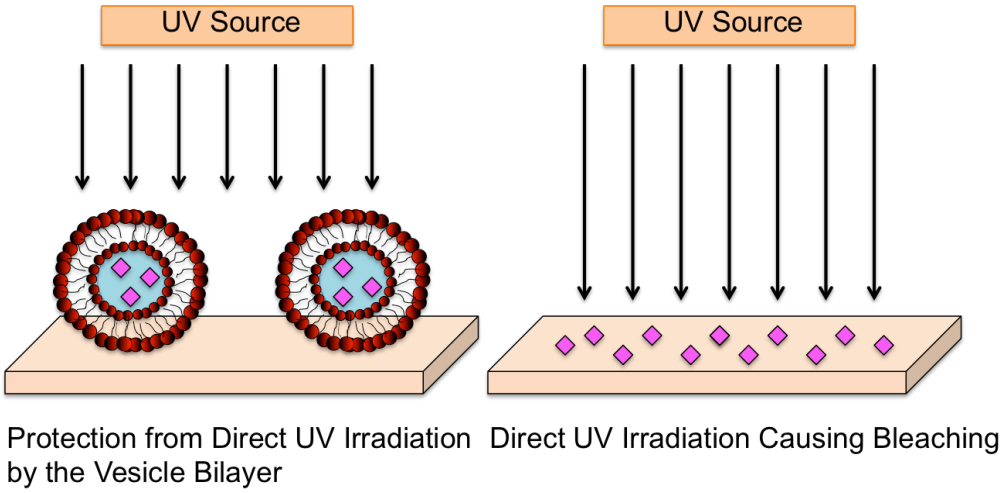

Figure 4.3 Dye encapsulated in vesicles gets protected from bleaching caused by UV irradiation. Bare dye molecules on the other hand tend to bleach much faster.

When nitrocellulose surfaces deposited with dye functionalized vesicles were exposed to UV irradiation, they retained fluorescence longer than surfaces deposited with bare dye. As dye molecules are encapsulated in vesicles, surfactant bilayer protects the dye from bleaching when exposed to UV irradiation as indicated in Figure 4.4 A. We rule out static photoquenching as a reason for this decay, as there is negligible amount of dye (0.001 mf) encapsulated in a vesicle. Further investigation is required to confirm the presence of vesicles on nitrocellulose surfaces by more direct method.

A)

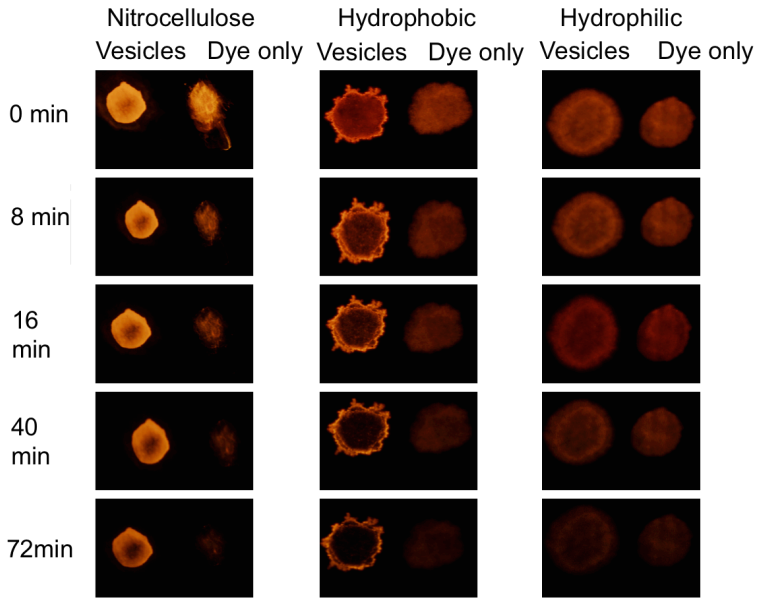

Figure 4.4 A. Images of surfaces deposited with dye functionalized vesicles, when exposed for varying time to UV radiation.

B)

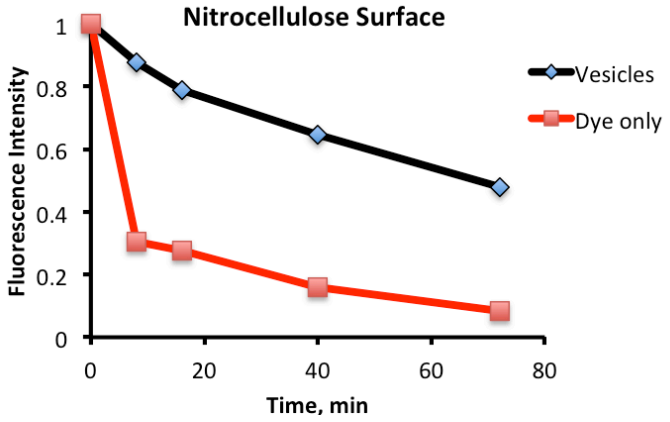

Figure 4.4 B. Vesicles deposited on nitrocellulose surfaces retain fluorescence effectively upon exposure to UV irradiation, as indicated with normalized fluorescence intensity. (n=1)

4.3 Lectin Binding Studies with Vesicles Deposited on Nitrocellulose Surfaces

Having shown that vesicles deposited on nitrocellulose surface maintain their integrity, we investigated the use of these surfaces for lectin binding studies. Preparing

microarrays on nitrocellulose membranes would allow us to build robust, uniform microarrays.

For these studies we deposited glycan- (lactose or glucose) and dye-functionalized vesicles on a nitrocellulose membrane, and conducted lectin binding studies with FITC labeled Con A, as indicated in Figure 4.5.

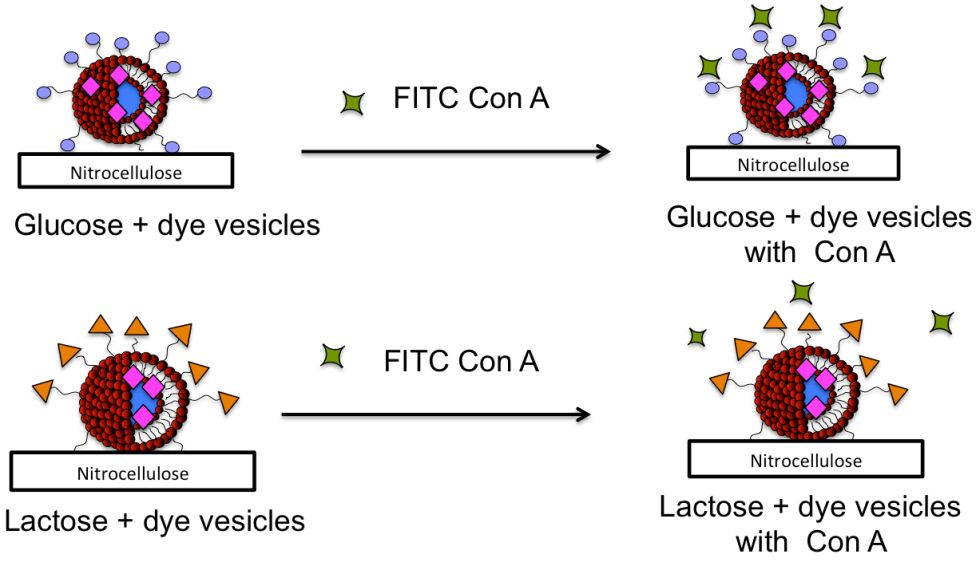

Figure 4.5. Glucose functionalized vesicles bind to FITC Con A. Lactose functionalized vesicles however should exhibit negligible binding with FITC Con A.

A)

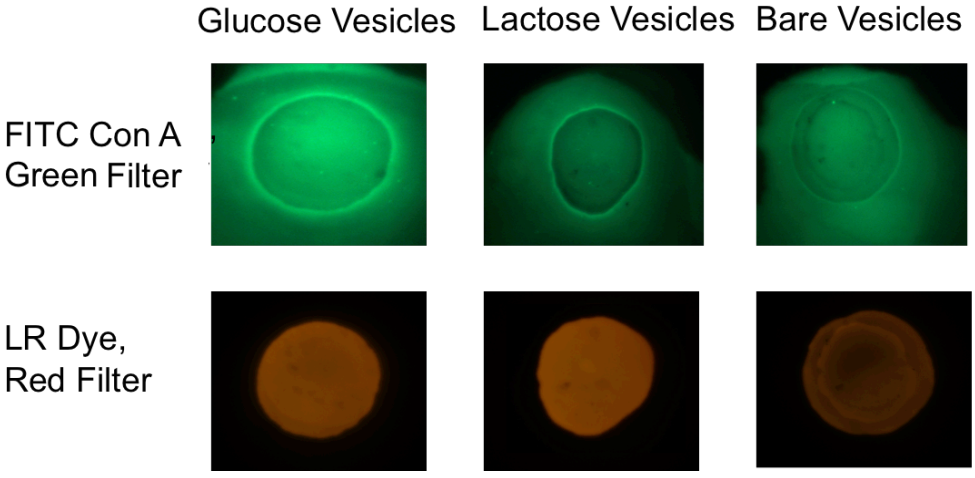

Figure 4.6 A. Surfaces deposited with glycan functionalized vesicles; show differential binding to FITC Con A.

B)

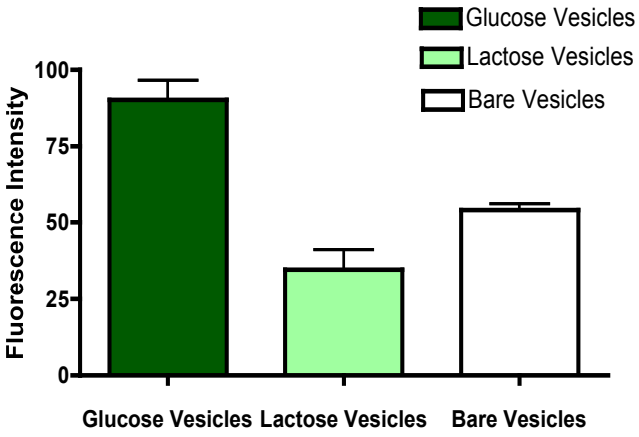

Figure 4.6 B. Fluorescence intensities observed on surfaces (6 replicates) deposited with functionalized vesicles.

As our results indicate, we observed higher binding of FITC Con A to glucose-functionalized vesicles (up to two fold higher) than lactose functionalized vesicles. We also observed a considerable non specific binding to bare vesicles. Nitrocellulose surfaces, often used extensively for proteins or nucleic acid immobilization non specifically bind to FITC Con A. We screened various blocking solutions such as bovine serum albumine (BSA, varying concentrations), caesin and fetal bovine serum (FBS) as agents to inhibit non-specific binding to the glycan surface. Based on our results, FBS is most useful in blocking the non-specific binding of Con A to the surface. Hence, lectin binding studies were conducted with FBS as a blocking agent.

Lectin binding studies with glucose- and lactose-functionalized catanionic vesicles indicated that vesicles on nitrocellulose surfaces exhibit binding properties similar to their solution phase counterparts. For example, Con A bound preferentially to glucose-functionalized membranes; while PNA bound preferentially to lactose counterparts. However, further studies need to be conducted with other glycan-lectin pairs to conform our hypothesis. Our studies to illustrate the effect of glucose concentration on Con A

binding have so far been inconclusive, indicating that we need a better control on non specific binding of the lectins to the nitrocellulose surfaces.

4.4 Ink Jet Printing Dye Functionalized Vesicles on Surfaces.

Paper-based devices are a promising approach towards building extremely low cost devices for molecular detection.[5] Ink jet printed surfaces are particularly of higher interest due to ease of fabrication and lower cost.[6] Vesicles exhibit a zeta potential of ~ -55 mv on bilayer surface, thus making them perfect candidates for ink jet printing. Our preliminary efforts to ink jet print vesicles on hydrophobic and nitrocellulose paper surfaces are summarized in Figure 4.7. We obtain a perfect spacial control, as vesicles are ink jet printed on surfaces. This technique could be used for ultra cost effective mass production of ink jet printed vesicle microarrays.

Figure 4.7 Ink jet printing of catanionic surfactant vesicles on ASA treated chromatography paper (hydrophobic filter paper) and nitrocellulose membrane surfaces.

4.5 Conclusions

Our preliminary studies have indicated that vesicle deposited- nitrocellulose surfaces can serve as an excellent replacement for HM chitosan derived vesicle arrays. Glycan functionalized vesicles are intact when deposited on nitrocellulose surfaces and display

binding to corresponding lectin binding partners. Vesicles when ink-jet printed display excellent spacial control on surface, opening doors to fabricating cost effective microarrays.

4.6 Future Studies

Studies will be performed to estimate the ideal blocking agents to minimize non specific binding of lectins to nitrocellulose surfaces. More glycan-lectin pairs should be tested for lectin binding studies using our binding assay. Studies will focus on determining the effect of glycan concentration on the surface on the extent of lectin binding. We also aim to conduct these studies on ink-jet printed vesicle surfaces. Our current studies conducted

4.7 Experimental

4.7.1 Imaging

Fluorescence images were obtained using a Leica fluorescence stereomicroscope (MZFLIII) equipped with a digital camera (Spot 32, Diagnostic instruments). The fluorescence of lissamine rhodamine dye was observed employing an excitation wavelength of 560 nm (bandwidth of 40 nm), having an emission filter of 610 nm. To observe the fluorescence of FITC, an excitation wavelength of 480 nm (bandwidth of 40 nm) and an emission filter of 510 nm was employed.

4.7.2 Chemicals and Reagents

The following chemicals were purchased from Sigma: cetyltrimethylammonium tosylate (CTAT), n-dodecyl-β-D-glucopyranoside (C_{12}-glucose), phosphate buffered saline (PBS), Sephadex G-100 as well as the fluorescein isothiocynate (FITC)-labeled concanavalin A

(Con A) from *Canavalia ensiformis* (Jack Bean), Type IV. Sodium dodecylbenzenesulfonate (SDBS) was purchased from TCI-America and was recrystallized from ethanol-acetone to give a white powder. The purified solid was stored at room temperature in a desiccator containing Drierite. Lissamine rhodamine, 1,2-dioleoyl-sn-glycero-3-phosphoethanol-amine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) was purchased from Avanti Polar Lipids. A solution of PBS was prepared at pH 7.4 by dissolving a tablet in DI water using standard protocol. Fisherbrand chromatography paper, 0.19 mm in thickness, treated with hexadecenyl succinic anhydride (ASA) was obtained from Prof. Ian White's lab. This surface is referred as hydrophobic filter paper in this manuscript. Whatman Filter paper grade 202
(referred to as hydrophilic filter paper) and Whatman AD60 silica plate was obtained from GE Healthcare Life Sciences. Cationic nylon membrane, Biodyne A was obtained from Thermo Scientific.

4.7.3 Triton X 100 Washing Studies

100 μL of 0.001 mf Lissamine rhodamine dye functionalized vesicles were deposited on various surfaces using a micropipette. These surfaces were washed with PBS buffer for 15 min. 100 ML of 10% w/w solution of Triton X 100 was deposited on these surfaces for 10 sec. Fluorescence images were taken before and after addition of Triton X100.

4.7.4 Preparation of Vesicles

4.7.4.1 Preparation of Bare Vesicles

Catanionic vesicles were prepared from SDBS and CTAT, with molar excess of SDBS according to the method previously reported by Thomas et al.[7] All vesicle solutions were purified as described in 2.15.6.2.

4.7.4.2 Glycan and Dye Functionalized Vesicles

Lissamine rhodamine dye (0.001mf) dissolved in chloroform was pipetted into a glass vial. The solvent was removed *in vacuo*. To this, SDBS (mole fraction 70%) and CTAT (mole fraction 30%) and required amount of glycans (0.05 mf, 4.647 mg C_{12} glucose or 0.05 mf, 6.801 mg C_{12} lactose) were combined with 9.9 mL of water. This solution was stirred for 24 hours.

4.7.5 Deposition of Vesicles on to Nitrocellulose Surfaces

Nitrocellulose membrane surfaces were deposited with 100 μL vesicle sample for 40 min. These electrodes were then washed with PBS buffer for 2 hours followed by DI water for 2 min.

4.7.6 Binding Studies

Surfaces coated with functionalized vesicles were treated with fetal bovine serum (FBS) for 30 min. Electrodes were washed with PBS for 15 min. After this step, these surfaces were immersed in a solution of fluorescently labeled lectin (Con A) and FBS (8.33 μM solution in FBS) for 1 h. The surfaces were washed with FBS to reduce non-specific

binding of lectin with the glycoconjugate for 15 min. The electrodes were washed with PBS for 30 m prior to fluorescence imaging.

4.7.7 Vesicle Printing

Refillable ink cartridges, purchased from Alpha D Development Inc. were filled with a suspension of lissamine rhodamine dye functionalized catanionic vesicles. An Epson Workforce 30 inkjet printer was used to print vesicles on surfaces. To prevent clogging of the print head, the vesicle solution was filtered using a 0.2 μm Millipore PTFE membrane filter prior to filling the cartridge. An open source vector graphics editor, Inkscape, was used to print arrays onto hydrophobic and nitrocellulose membranes. Five printing runs were carried out on the same paper to get a detectable print signal.

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