Part I: Nanoparticle-based designation of multivalent carbohydrate-lectin interactions. Part II: Development of new promoters for stereoselective green glycosylation

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**PART I: NANOPARTICLE-BASED DESIGNATION OF
MULTIVALENT CARBOHYDRATE-LECTIN INTERACTIONS**

**PART II: DEVELOPMENT OF NEW PROMOTERS FOR
STEREOSELECTIVE GREEN GLYCOSYLATION**

GORITYALA BALAKISHAN

SCHOOL OF PHYSICAL & MATHEMATICAL SCIENCES

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A thesis submitted to the Nanyang Technological University
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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	i
TABLE OF CONTENTS	ii
ABSTRACT	iv
INDEX OF ABBREVIATIONS	viii
CHAPTER I: <i>Nanoparticle-based designation of multivalent carbohydrate-lectin interactions</i>	
- INTRODUCTION	1
- RESULTS AND DISCUSSION	45
- CONCLUSION	92
- EXPERIMENTAL	93
- REFERENCES	108
CHAPTER II: <i>Development of new promoters for stereoselective green glycosylation</i>	
<i>Part A: Green glycosylation promoted by reusable biomass carbonaceous solid acid:</i>	
<i>An easy access to β-stereoselective terpene galactosides</i>	
- INTRODUCTION	115
- RESULTS AND DISCUSSION	118
- CONCLUSION	125
- EXPERIMENTAL	126

REFERENCES	133
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Part B: ZnCl₂/Alumina impregnation catalyzed Ferrier rearrangement: An expedient synthesis of pseudoglycosides

- INTRODUCTION	137
- RESULTS AND DISCUSSION	138
- CONCLUSION	144
- EXPERIMENTAL	145
- REFERENCES	154
LIST OF PUBLICATIONS	157

ABSTRACT

Chapter I: Nanoparticle-based designation of multivalent carbohydrate-lectin interactions

We have designed a novel bio-sensor system to explore multivalent interactions between carbohydrates and lectins. Goldnanoparticles decorated with carbohydrates resemble to natural cell systems and exert multivalent interactions on specific lectins. Design of this bio-sensor relies on two characteristic features of carbohydrates and goldnanoparticles; (a) carbohydrate diols can interact with boronic acids with high affinity through reversible ester formation (b) when a fluorescent dye tagged boronic acid couples with encapsulated goldnanoparticles, its fluorescence quenches. We have synthesized some carbohydrate-monomodified goldnanoparticles and fluorescein boronic acid ligand. When these carbohydrate- monomodified goldnanoparticles bind with fluorescein boronic acid ligand, fluorescence intensity of resulting complex drastically decreases. Upon the addition of a lectin to this system, fluorescence intensity again increases. Each carbohydrate is specific to particular lectin and based on this phenomenon the incoming lectin molecule replaces fluorescein boronic acid ligand from fluorescein-boronic acid-carbohydrate encapsulated goldnanoparticle complex to release fluorescein boronic acid ligand which in turn increases the fluorescence intensity. This system acts as “Turn-on/Turn-off” system. Based on this system we have extensively studied various carbohydrate-lectin interactions. This bio-sensor system is simple, efficient, cost effective and above all with high sensitivity. The limit of detection (sensitivity) of this system was found to be 4.9 nM. This system was successfully applied on cell cultures and the results showed fluorescein boronic acid-carbohydrate grafted goldnanoparticle system could be used as fluorescent probe.

Chapter II: Development of new promoters for stereoselective green glycosylation

Part A: Green glycosylation promoted by reusable biomass carbonaceous solid acid: An easy access to β -stereoselective terpene galactosides

In part A of this chapter we have demonstrated the remarkable catalytic efficiency of environmentally sustainable carbonaceous solid acid as glycosylating promoter. Various unprotected and unactivated glycosyl donors, which are usually prone to self-condensation and *in-situ* anomerization while performing the glycosylation, were successfully glycosylated to afford corresponding glycosides. This catalyst was also employed for the glycosylation of glycosyl trichloroacetimidates. This carbonaceous catalyst is reusable up to 7 times while persisting the high catalytic activity.

Part B: $\text{ZnCl}_2/\text{Alumina}$ impregnation catalyzed Ferrier rearrangement: An expedient synthesis of pseudoglycosides

In part B of this chapter we have employed $\text{ZnCl}_2/\text{Al}_2\text{O}_3$ as the catalytic system to synthesize 2,3-Unsaturated-*O*-glycosides under solvent free conditions. The major advantages of this method concern exclusive anomeric selectivity, rapid reaction

times, and friendly environmental conditions. Low cost reagents and no aqueous work-up are required and the alumina could be recycled up to 3 times.

INDEX OF ABBREVIATIONS

δ	chemical shift
$^{\circ}\text{C}$	degree centigrade
Bn	benzyl
br	broad
brs	broad singlet
cat.	catalytic
Con A	concanavalin A
CRD	carbohydrate Recognition Domain
cm^{-1}	inverse centimeter
d	doublet
dd	doublet of doublets
DBU	1,8-diazabicycloundec-7-ene
DMAP	4-(<i>N,N</i> -dimethylamino)pyridine
DMF	<i>N,N</i> -dimethylformamide
DMNP	dis-modified gold nanoparticles
ECorL	Erythrina Corallodendron
EDAC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EI	electron impact ionization
ELLA	enzyme linked lectin assay
equiv	equivalent
ESI	electrospray ionization
Et	ethyl
ether	diethyl ether
Fb-MMNP	fluorescein boronic acid-monomodified goldnanoparticles
FBS	fetal bovine serum

FET	field-effect transistor
Fmoc	fluorenylmethoxycarbonyl
FTIR	fourier transfer infrared spectroscopy
GalNAc	galactosamine
GlcNAc	glucosamine
h	hour (time)
Hex	hexane
HIA	inhibition of hemagglutination
HRMS	high resolution mass spectroscopy
Hz	hertz
ITM	isothermal titration microcalorimetry
IR	infrared
J	coupling constants
LOD	limit of detection
M ⁺	parent ion peak (mass spectrum)
m	multiplet
MALDI-TOF	Matrix-assisted laser desorption/ionization-Time of flight
min	minute
MMNP	mono-modified gold nanoparticles
MS	molecular sieve
N	concentration (normality)
NMR	nuclear magnetic resonance
nM	nano molar
NTFET	nanotube field effect transistor
Nu	nucleophile
PBS	phosphate buffered saline
Ph	phenyl

PET	photo induced electron
PNA	peanut agglutinin
ppm	parts per million
Py	pyridine
q	quartet
QCM	quartz crystal microbalance
rt	room temperature
RBF	round bottom flask
RPMI	roswell park memorial institute medium
s	singlet
sat	saturated
SBA	soybean agglutinin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
t	triplet
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
WGA	wheat germ agglutinin

CHAPTER 1

**Nanoparticle-based designation of multivalent
carbohydrate-lectin interactions**

1. INTRODUCTION

1.1 The importance of carbohydrates

Glycosylation is one of the most ubiquitous forms of the post-translational modification, with more than 50% of the human proteome estimated to be glycosylated.¹ Carbohydrates in the form of glycoproteins, glycolipids and glycans are the key constituents of cell membrane and extracellular matrix, playing pivotal role in cell-cell communication, cell-protein interaction and molecular recognition of antibodies and hormones.² Due to the presence of multiple hydroxyl functional groups on each monomer unit, carbohydrates are capable of forming many different combinatorial structures from a relatively small number of sugar units. Each sugar moiety could potentially carry a specific biological message, thus widening the probability of reactivity that is possible from a limited number of monomers. The chemical diversity and complexity of carbohydrates have bestowed glycans with a vast array of biological functions. Recently, sugar code and glycomics are becoming commonly used terminologies.

Over the past two decades, considerable evidence has been presented to demonstrate that carbohydrates have tremendous potential for encoding biological information in a wide variety of physiological and pathological processes.³ However, the extent, which the sugar code has been deciphered, is still very limited despite many great efforts. This embarrassment is mainly caused by lack of pure, structurally defined complex oligosaccharides and glycoconjugates and the lack of methods for molecular glycobiology study.

1.2 Interface of glycobiology and nanotechnology

Isolation of homogeneous pure polysaccharides from highly heterogeneous natural sources remains a daunting task. Synthesis of complex carbohydrates also presents a great challenge to organic chemists. On the other hand, multivalent interactions, which are characterized by simultaneous binding of multiple receptors are prevalent in biological systems, but the methods to realize multivalent interactions are very limited until functionalization of nanoscale scaffolds comes in to practice.⁴⁻⁶

Working at glycobiology, biotechnology and material science interfaces^{4,7} provides a powerful tool to understand the intricate details of biological systems at molecular level. Nowadays research on carbohydrate-modified nanoparticles has made numerous progresses, which has left a great impression on glycobiology and nanotechnology.⁴⁻⁶ Technical advances in the synthesis and structure analysis of glycosylated nanoparticles together with expanding knowledge of their interaction with pathological cell surfaces have enabled researchers to develop new biomedical instruments and to penetrate into this challenging field. For this reason there has been a tremendous effort put in to the design and synthesis of multivalent model systems⁸ that mimic natural biomolecules.

1.3 Lectin-carbohydrate interactions

1.3.1 Lectins: Structure and interactions with carbohydrates

Enzymes and immunoglobulins are the two important classes of proteins, specific to carbohydrates and widely appearing in nature. Both of these proteins interact with specific carbohydrate moieties noncovalently. ‘Lectins’ (derived from Latin word *legere*, to pick out or chose) are another class of carbohydrate binding proteins, widely

occur in plants, animals and microorganisms.⁹ Lectins neither possess catalytic activities as enzymes nor are produced due to the immune response as immunoglobulins. In 1926, Prof. James Sumner first crystallized urease enzyme (isolated from jack bean *Canavalia ensiformis*) to that he named 'Concanavalin A', most popularly known as 'Con A'. Later 1936, Sumner and Howell showed that Concanavalin A is responsible for the agglutination of erythrocytes and yeasts.¹⁰ They further demonstrated carbohydrate specificity towards lectins, by showing that hemagglutination of lectins is inhibited by sucrose ligands. Lectins are di- or polyvalent as they contain more than two carbohydrate-binding sites through which they append reversibly with mono and oligosaccharides¹¹⁻¹³ (Figure 1).

Figure 1: Cell surface lectin-carbohydrate interactions. Lectins assist numerous kinds of cells and viruses to attach to other cells via carbohydrate on their surface. (Reproduced with permission from Ref. 18; Copyright: Oxford University Press, 2004)

They not only bind to carbohydrates externally but also promote cross-linking between cells thereby causing cell agglutination. Lectins also form cross linkings between polysaccharides and glycoproteins leading to their precipitation in the solution. This unique phenomenon corroborated the discovery of human A, B and O blood groups by Karl Landsteiner in 1900.¹⁴ More precisely lectins can be defined as “cell-agglutinating, carbohydrate specific proteins”.

Ever since the lectins were discovered, understanding complex carbohydrate structural and functional aspects became much easier. For example, lectins help in investigating the physiological and pathological changes that take place on cell surface.¹⁵ Now lectins are believed to act as “recognition determinants” in an array of biological processes.^{16,17} Some of the major applications and involvement in various biological processes of lectins include¹⁸ (a) cell identification and separation (b) detection and isolation of glycoproteins and understanding structural aspects of glycoproteins (c) investigation of glycoprotein bio-synthesis (d) proper selection of lectin-resistant mutants (e) mapping of neuronal pathways (f) studies of carbohydrates that present on cell surface and in subcellular organelles (g) glycoprotein clearance from the circulatory system (h) adhesion of pathogenic agents to host cells (i) cell-cell interactions in the immune system.

It's essential to explore the structural features of lectins to understand the binding properties of lectins and carbohydrates at molecular level. As the ultra sophisticated analytical tools come into use such as X-ray crystallography, it became scientifically feasible to elucidate the structural features of lectins there by paving the way to understand the lectin-carbohydrate binding factors. High resolution X-ray crystallography of lectin complexed with its natural carbohydrate ligand has not only confirmed the amino acid sequence of protein but also the type of bonds formed

between them.^{19,20} Edelman *et al* (1972) first established 3D structure of Concanavalin A with the aid of X-ray crystallography²¹ (Figure 2). In fact Con A is the first lectin that has been elucidated through amino acid sequencing and X-ray crystallography. WGA (wheat germ) was the next lectin shown to have complexed with N-acetylneuraminic acid and N-acetylglucosamine (Wright *et al*).²² During the past few years a number of 3D structures of lectin-carbohydrate complexes have been solved.

Figure 2: Concanavalin A and Peanut agglutinin represented as ribbon diagrams. Con A exhibits β -trefoil fold. The grey spheres represent metal ions. (Reproduced with permission from Ref. 18; Copyright: Oxford University Press, 2004)

It is indeed interesting to note that even though the primary sequence of lectins differ from each other, a high degree of similarities observed with their tertiary structures. For example, one such tertiary structure commonly known as ‘lectin fold’ initially noticed in the legume lectins, which possess a characteristic 2 β -sheet oriented jelly roll (Figure 2).²³ The same kind of lectin fold can be found in galectins (galactose specific animal lectins) and pentraxin (a type of animal lectin). By late 80’s X-ray crystallography has revealed exact chemical composition on lectins and the

corresponding sugar ligands and type of bonds formed between them, that is, hydrogen bonds and hydrophobic interactions. According to Sharon “just like proteins recognize ligands, lectins also recognize carbohydrates in a distinct way”.²⁴

Lectin specificity with carbohydrates:

Sharon has classified lectins into 5 types on the basis of preferential specificity towards various monosaccharides i.e., (a) mannose (b) galactose/N-acetylgalactosamine (c) N-acetylglucosamine (d) fucose and (e) N-acetylneuraminic acid. Among above-mentioned sugars except fucose all other sugars constitute for D configuration. Majority of the eukaryotic cell surfaces consists of above stated five monosaccharides. However, human serum amyloid P component (SAP) lectin binds with a rare carbohydrate ligand 4,6-cyclic pyruvate acetal of galactose.²⁵ Despite the weak affinities between monosaccharides and lectins, as evident by low range association constants (normally in millimolar range), they are marked with exceptional selectivity to each other.¹¹ A particular lectin usually interacts with specified sugar only. For example (a) lectins that are specific for galactose ligand do not combine with glucose or mannose ligands (b) lectins specific for N-acetylglucosamine do not react with N-acetylgalactosamine similarly N-acetylgalactosamine specific lectins do not react with N-acetylglucosamine ligands. Noteworthy, lectins (such as soybean agglutinin, SBA) that combine with galactose also capable of interact with N-acetylgalactosamine with 25-50 times higher affinity. The above stated example emphasizes the need for Gal/N-GalNAc type of classification of sugars for specific lectins. However, some lectins such as peanut agglutinin (PNA) do not react with N-acetylgalactosamine.

Sometimes lectins combine with monosaccharides that possess similar structural features in the space. For example, wheat germ agglutinin (WGA) combines both N-acetylglucosamine and N-acetylneuraminic acid due to the topographical similarities at C-2 position (acetamide group) and C-5 position (hydroxyl group) of N-GluAc to those of C-5 position and C-4 position of N-acetylneuraminic acid respectively. Similar observations were found with mannose (binds with animal lectin e.g rat MBP's) and fucose due to the structural resemblance of monosaccharides. Most of the sugars bind with lectins irrespective of their anomeric specificity (either α or β). However, in some cases anomeric selectivity of sugars plays a vital role in the lectin-sugar interactions. Structural features on the glycoside also determine the binding capacity of the glycoside and lectin. Hydrophobic aromatic moieties on the glycoside cause strong interaction with lectin than aliphatic moieties. In some cases, this hydrophobic functionality effect has large influence on sugar lectin interactions. For instance, methyl- α -glycosides usually will have greater interactions with lectins over corresponding β -glycoside. However, if the β - anomer equipped p-nitrophenyl moiety, it exhibits greater interaction with lectin than corresponding α -anomer.²⁶

The association constant for lectin-oligosaccharide complex is 1000 fold higher than lectin-monosaccharide complex. This clearly shows the strong binding between oligosaccharides and lectins. In fact oligosaccharides act as natural ligands of lectins. The high affinity of oligosaccharides to lectins could be attributed to their shape and size, as each monosaccharide unit in oligosaccharide can freely rotate around glycosidic linkage that leads to 'conformational heterogeneity'.

Based on the structural features, lectins can be classified into 3 groups (a) simple (b) mosaic (or multidomain) (c) macromolecular assemblies. In this introduction, only

simple lectins were described as all the experiments were performed using them. Simple lectins consist of all known plant lectins and galectins (animal lectins specific to galactose). They usually weigh about 40 kDa. Based on the structural properties simple lectins can be further grouped into 7 sub groups.

A) Legume: Till now more than 100 legume-lectins have been isolated from plant seeds and all of them were thoroughly characterized.²⁷⁻²⁹ Concanavalin A from jack bean belongs to this sub-group, which is the first lectin to be isolated. Other well-characterized lectins in this sub group include: peanut agglutinin (PNA), soybean agglutinin (SBA) and Erythrina Corallodendron (ECoRL). Legume lectins contain Ca^{2+} and Mn^{2+} ions per sub unit, which are essential for carbohydrate binding.³⁰ Apart from carbohydrate binding site, most of the legume lectins possess a hydrophobic site, which is useful for binding with nonpolar moieties such as adenine and indole acetic acid. Each sub-unit of legume lectin consists of single polypeptide chain of 250 amino acids. In these amino acids about 20% of them are invariant and 20% of them similar. Such conserved amino acids mainly involve in hydrogen bonding and hydrophobic interactions with the sugar ligands at the lectin-binding site and most of the amino acid residues coordinate with the Ca^{2+} and Mn^{2+} ions. A typical dome shaped subunit of legume consists of 2 antiparallel β -sheets, one of the sheet comprises six strands (flat sheet) and another one seven strands (concave sheet). These strands of sheets constitute for a jelly rolls also known as lectin fold.²³ Seven-strand concave sheet usually bears shallow carbohydrate and metal ion binding site. This concave binding site arranged at the top of each promoter for the smooth accessibility of monosaccharides, oligosaccharides and also polysaccharides. Ca^{2+} and Mn^{2+} ions (separated with a distance of 4.25 Å) are located in close proximity to carbohydrate binding site i.e., 9-13 Å, which assist in the special arrangement of amino acids that

form contact with sugar ligand. However, these ions are not directly involved in bonding with carbohydrate. Each Ca^{2+} and Mn^{2+} ions are appended to four amido acid residue side chains, out of which two of the side chains belong to aspartic acid, shared by each ion. In all legume lectins four water molecules were conserved and involved in metal binding directly or indirectly.³¹ Demetallization (mainly Ca^{2+} ions) of legume lectin resulted in significant changes in the crystallographic structure of Con A and eventually resulted in the destruction of lectin and carbohydrate interaction site.³²

B) Cereal: This sub family of simple lectins comprises of wheat germ agglutinin (WGA) and barley and rice lectins. Similar to legume lectins, they contain two identical subunits which are rich in cysteine. Generally WGA exists as a mixture of three isolectins, which moderately vary in amino acid composition. Each isolectin possess two identical subunits (weigh 17 kDa) without any metal ions. Each subunit consists of 4 sub-domains (43 amino acid residues). Further each sub-domain incorporate four identical disulfide bridges, thereby there are 16 such disulfide bridges exist in each subunit of WGA. Unlike other proteins WGA lectin lacks β sheets and α helix in its structure and it is characterized with multiple binding sites.

1.3.2 Carbohydrate binding sites on lectins and their interaction with sugar ligands

Shallow depressions on lectin surface act as combining sites. Drickamer first proposed that carbohydrate binding site on lectins be confined to particular polypeptide segments which usually are referred to as “carbohydrate Recognition Domain” (CRD).³³ Usually, one or two faces of sugar ligand connected to the corresponding lectin. Lectins are preformed, as evident by the observation of conformational changes during the lectin-sugar complex formation. Hydrogen bond formation, hydrophobic interactions and metal ions coordination play an important role in lectin-carbohydrate

interactions. Some of the important features of lectin-sugar binding include (a) the hydrogen bond formation: hydrogen bonds are formed between monosaccharide/oligosaccharide hydroxyl groups and hydroxyl, NH group and oxygen atoms of the protein. Sometimes bidentate hydrogen bonds are formed. For instance, oxygen atoms of the carboxylate of aspartic or glutamic acid residues interact with two hydroxyl moieties of the sugar ligand.³⁴ (b) even though van der Waal forces are weak, they exert considerable magnitude of impact on lectin-sugar interactions (c) ‘hydrophobic patches’ are formed on the surface of sugar ligand, due to steric configuration of hydroxyl groups. These patches can interact with hydrophobic regions of protein. For example, hydrophobic patches on monosaccharide append with the aromatic amino acid (e.g., phenylalanine and tryptophan) side chains (d) there is no possibility of charge-charge interactions between protein and sugar since all the carbohydrates are uncharged (exception: heparin-antithrombin complex) (e) sometimes water influences the lectin-sugar interactions by creating water bridges.^{35,36} Small size of water molecule transforms it to be molecular “mortar” so that it can act as hydrogen donor and acceptor.

Carbohydrate-lectin interactions in legume family:

Three amino acid residue side chains on lectin surface i.e., an aspartic acid, an asparagine^{37,38} and a aromatic amino acid (in addition some times alanine)³⁹ are crucial for lectin-sugar interactions. If there is slight variation or replacement in theses amino acids, it will lead to the destruction of lectin-carbohydrate interaction. Usually aspartic acid and asparagine coordinate with calcium ions, especially in legume family where presence of metal ion is essential for carbohydrate binding. Another characteristic feature in legume lectins is, formation of a *cis*-peptide bond between asparagine and alnine, which controls the orientation of asparagine. The most striking feature of the

lectin-sugar interactions, ‘specificity’ is achieved via the proper orientation of the monosaccharide. In other words, mannose (or glucose) scaffold orientated differently than galactose monosaccharide at mannose specific lectin combining site. In concanavalin A amino acid residue, asparagine oriented in such a way that hydrogen bonds form between O δ 1 and O δ 2 of asparagine and 6-OH and 4-OH of mannose. Similarly hydrogen bonds are formed between N δ 2 of asparagine and 4-OH of mannose (Figure 3).

Figure 3: Hydrogen bonds formation between conserved amino acid and mannose in Con A.

1.3.3 Evaluation of protein-carbohydrate binding interactions:

Due to the enormous impact of carbohydrates and lectins its high time to explore carbohydrate-lectin interactions and develop new bio-sensor techniques which can serve to simplify the carbohydrate detection processes and probing sugar-lectin interactions. According to Jelinek the primary requisites while designing a biosensor should include (a) high sensitivity and (b) reproducibility of the experiment.⁴⁰ The most diversified structural variations in carbohydrates posed several challenges in designing new biosensors. A plethora of biosensors and bioassays have been

developed to probe carbohydrate-lectin interactions, which usually rely on biochemical, electrochemical and spectroscopic methods.

Determining carbohydrate-protein binding constant is not an easy process and there has been no straightforward method readily available. Wide ranges of assays are being available for measuring the affinity of sugar-protein interaction. Some of the major techniques employed to determine the affinity constants and interactions (a) inhibition of hemagglutination (HIA) (b) enzyme linked lectin assay (ELLA) (c) isothermal titration microcalorimetry (ITM) (d) surface plasmon resonance (SPR) and (e) quartz crystal microbalance (QCM) (f) colorimetric techniques (especially fluorescence). Among these techniques much research has been carried out using colorimetric, SPR and QCM techniques.

Here are some important contributions in designing new bio-sensors for carbohydrate-lectin interactions. To understand the multivalent interactions between carbohydrates and lectins at molecular level Riguera and coworkers conducted experiments both in solution and solid surface bound with immobilized lectins with the aid of real time detection technology, surface plasmon resonance.⁴¹ SPR provides both kinetic and equilibrium data. They have studied the interaction of Concanavalin A and mannosylated GATG (gallic acid-triethylene glycol) dendrimers.⁴² Through competitive assay experiments, binding affinity of [Gn]-Man to Concanavalin A was determined in solution phase. It was determined that [G1]-Man showed 8 fold higher binding affinity towards Con A when compared to Me-Man. Similarly, [G2]-Man showed 112 and [G3]-Man showed 372 fold increase in binding affinity to Con A (Figure 4). However, when they conducted the similar experiments in solution on per sugar basis, there have not been significant affinities observed up to G3-Man. This shows that, higher dendrimer generations do not show considerable impact on sugar-

lectin affinities for solution based carbohydrate-lectin interactions. Later they conducted SPR direct binding experiments. SPR results show that the interaction of sugar and lectin goes through 3 phases. In the first phase, monovalent interactions occur between dendrimer and Con A and low affinity and low KD characterize this first phase. In the second phase, statistical effects and cross-linking leads to high affinity nonmolar mode. In this phase strong interaction exists between dendrimers and Con A. Third phase constitutes for dissociation phase where, dendrimers detach from the lectin surface resulting in a decrease in low affinity.

Figure 4: Mannosylated GATC dendrimers

Wang *et al* has studied carbohydrate-lectin interactions with the help of quartz crystal microbalance.⁴³ They utilized this technique (QCM) to detect high molecular weight bacterial targets such as *E.Coli* bacteria. Bacteria possess innumerable carbohydrate and lectin binding sites on the cell surface. Mannoside coated mass sensor (QCM) along with lectin and bacteria O-antigen provides the information of bacteria with high sensitivity. Here QCM acts as a transducer to detect *E. Coli* W1485. In this protocol, simultaneous detection of lectin-O-antigen (on bacteria) and lectin-carbohydrate (on QCM surface) facilitates enhanced rigidity and specificity that are crucial for the detection of *E.Coli* when using QCM biosensors. In order to obtain accurate QCM measurement, one should make sure that the analyte (bacteria) should be properly coupled to the QCM sensor surface, so that surface area becomes rigid (Figure 5). But, bacterial cells are not rigid and proper coupling with QCM surface may be cumbersome. Moreover, bacterial binding is associated with energy dissipation as a result of internal friction, which simultaneously results in damping of the oscillation of the crystal. Bacterial surface contains numerous pili (fimbriae) that are the tail like structures with carbohydrate binding lectin pockets. Direct coupling of bacterial

Figure 5: Schematic representation of direct *E.Coli* detection and Con A mediated *E. Coli* detection. (Reproduced with permission from Ref. 43; Copyright: American Chemical Society)

surface on QCM transducer may not be effective, as these interactions are flexible and some water molecules might be trapped in between QCM and bacterial surface. It is important to note that bacterial surface also occupied with glucoconjugates usually known as lipopolysaccharides (LPS). Each LPS is encapsulated with O-antigen core which consists of various sugar moieties capable of binding with lectins. In order to modify the surface, initially Con A was bound to O-antigen of *E. Coli* W1485. *E. Coli* along with Con A is then adhered strongly to mannose immobilized QCM surface. This sandwich type architecture or alignment (bacterial carbohydrate-Con A-mannoside on QCM) provides high rigidity on QCM surface hence enhances the QCM sensitivity by several fold. QCM detection range has been enhanced from 7.5×10^2 (mannose alone QCM sensor) to 7.5×10^7 cells/mL (mannose-Con A) QCM sensor (Figure 6).

Figure 6: Frequency change vs time curve when mannose-QCM electrodes were exposed to different concentrations of *E. Coli* from 7.5×10^2 to 7.5×10^7 cells/mL in 1 mL in 1 mL of stirred PBS with 1 mM Mn^{2+} , 1mM Ca^{2+} and 100 nM Con A. (Reproduced with permission from Ref. 43; Copyright: American Chemical Society)

Figure 7: Structures of Ru(II)glycodendrimers

Seeberger and coworkers for the first time employed logic gate operations to study lectin-dendrimer interactions. They designed and synthesized highly fluorescent tris(bipyridine)ruthenium(II) $[\text{Ru}(\text{bipy})_3]^{2+}$ derivatives, having 2,4,6 or 18 mannose or galactose units (1-12) and conducted molecular logic operations.⁴⁴ These glycodendrimers (Figure 7) are used as active components to perform logic operations. Lectins and pH are used as inputs. Analysis of photo induced electron (PET) transfer process during the interaction of glycodendrimers having fluorescent Ru(II) with lectins was investigated. In this protocol first photo electron induce process was

Figure 8: (a) relative fluorescence responses to pH and Con A as inputs (in the figure 1,2,3,4,6 denotes 4,5,6,7,8 respectively) (b) corresponding truth table (c) schematic representation of mechanism of interactions. (Produced with permission from Ref. 44; Copyright: American Chemical society)

observed between Ru(II) core and BBV (NN'-4-4'-bis(benzyl-2-bromic acid)bipyridinium dibromide). Later they conducted PET on Con A and dendrimers.

When **4-6** complexes and pH (buffer) were used as inputs, it was noticed that logic operation gave FALSE output (0), indicating that there is no interaction between lectin and dendrimers. However when AND gate was used with 4-6 complexes a high output (1) there by high quantum yield has been observed. These results could be explained on the basis of sugar decoration on ruthenium core. Only small amount of change in fluorescence has been observed when complexes **4-6** (with lower sugar decoration) interacted with Con A, leading to the weaker interactions. But, complexes **7** and **8** with high sugar density over ruthenium core showed higher interactions with Con A there by increase in the fluorescence. Finally it was observed that complexes 5 and 6 have low PET value, which shows the better affinity towards lectin (Figure 8). Thus logic gate operations can distinguish the dendrimers and may identify the suitable dendrimer that is opt to carryout lectin-sugar interaction study.

Alexander *et al* employed carbon nanotube field effect transistor (NTFET) device technology to study the carbohydrate-lectin interactions. They configured single wall carbonnanotubes into electrolyte-gated field-effect transistors (FETs) to explore binding affinities of carbohydrates and lectins.⁴⁵ Initially NTFETs (singale wall carbonnanotubes) were non-covalently functionalized with mannose, fucose and galactose glycoconjugates based porphyrin by employing click-azide chemistry (Figure 9). Noncovalant functionalization not only prevents non-specific protein adsorption but also highly specific lectin interaction. Here single wall carbonnanotubes (SWNTs) form conducting channels. These conducting channels transduce the interactions resulted because of carbohydrate-lectin interaction into electric signal. Various glycoconjugates appended to porphyrin moiety such as D- α -mannose, D- α -

fructose and β -D-galactose to specifically interact with Con A, PA-IIL and PA-IL respectively.

Figure 9: Porphyrin based glycoconjugates for non-functionalization of SWNTs.

PA-IL and PA-IIL constitute for bacteria lectin from *pseudomonas aeruginosa*. The sensitivity of this novel recognition platform found to be very good. Galactosyl functionalized NTFETs responded to PA-IL lectin with a concentration of 2nM, which is highly comparable with other methodologies. They have also measure the dissociation constant, $K_d = 6.8 \mu\text{M}$ which coincides with literature value.

Optical sensors in studying the carbohydrate-lectin interactions:

Despite many sensors and techniques have been developed to detect carbohydrate-lectin interactions, optical sensors offer additional advantages over other sensors. Some of them include low cost, simple, rapid, devoid of complicated instrumental set

up and more importantly accurate specificity. So far many optical sensors have been developed in context with carbohydrate-lectin interactions. Here are some prominent principles among optical techniques.

Scheme 1: Synthesis of mannose substituted polymer

Bunz and coworkers demonstrated that poly(*para*-phenyleneethynylene) (mPPE) acts as a good biosensor to investigate the sensitivity of lectin-carbohydrate multivalent

interactions.⁴⁶ Concanavalin A rapidly quenches the fluorescence of mannose substituted mPPEs. Mannosylated poly(*para*-phenyleneethynylene) was synthesized starting from **10** and 8-chloro-3,6-dioxaoctanol which reacts with each other in the presence of potassium carbonate in DMF to give diiodide **11**. When **11** was coupled with trimethylsilylacetylene in the presence of palladium, it furnishes **12**. Then **11** was mannosylated with the aid of $\text{BF}_3\text{Et}_2\text{O}$ in dichloromethane. In the final step **12** and **13** are coupled with $(\text{Ph}_3\text{P})_2\text{PdCl}_2/\text{CuI}$ in piperidine/THF mixture to furnish **14** (scheme 1).

Figure 10: Emission spectrum and stern-Volmer plot (inset) of **14** in the presence of Con A. (Reproduced with permission from Ref. 46; Copyright: Royal Society of Chemistry, Cambridge)

The basic principle of this technique relies on Stern-Volmer relationship, according to which the loss in fluorescence correlates with that of concentration of added quencher. They proposed two types of fluorophore quenching, i.e., dynamic quenching and static quenching. And it was deduced that quenching of mPPE follows static quenching,

where mPPE in its ground state forms a complex with Con A. At low Con A concentration the quenching process of mPPE is linear, however as the concentration of Con A increases the quenching deviates from linear path. In this experiment mPPE shows exclusive specificity and binds strongly with Con A. This was confirmed by testing mPPE with galactose specific lectin, jacalin, which has no impact on mPPE as there is no fluorescence quenching (Figure 10).

Seeberger and coworkers utilized specific carbohydrate-lectin interactions to detect pathogen bacteria.⁴⁷ They have synthesized mannose functionalized PPE [poly(p-phenylene ethynylene)] based fluorescent polymer, which is highly specific towards lectins on the cell surface of *E.Coli*. To synthesize the target polymer, 2-aminoethyl mannoside was coupled with poly(p-phenylene ethynylene) in the presence of EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) and *N,N'*-diisopropylethylamine. Subsequent quenching of the unreacted succinimide esters by adding excess of ethanolicamine provides the polymer fluorophore **15** (Figure 11).

Figure 11: Polymers used in this technique (R = OH; x:y = 0:1 and sugar = mannose and R = OH or NH (CH₂)₂OH; x:y = 1:1)

Fluorescence Resonance Energy Transfer (FRET) tests were conducted to establish the multivalent interactions between carbohydrates (mannosylated and galactosylated

PPE) and lectin (Fluor-594 labeled Con A). When mannosylated PPE was titrated against labeled Con A there was gradual decrease in fluorescence which is concentration dependent. This clearly shows the binding interaction between mannosylated PPE and Con A (Figure 12). Galactosylated PPE was titrated with Con A which does not show any decrease in fluorescence, which further confirms the high specificity of mannose to Con A.

Figure 12: Plot of the normalized fluorescence signal from addition of Alexa Fluor 594-labeled Con A to a solution of mannose functionalized polymer (dark spot) or galactose functionalized polymer (white spot). (Produced with permission from Ref. 47; Copyright: American Chemical society)

Nowadays “aggregation induced emission” (AIE) has drawn much attention and became prominent tool in carbohydrate-lectin bio sensing. Tang and coworkers observed this unique phenomenon.⁴⁸ When a faint fluorophore compound in solution state aggregates and become solid, not only its quantum yield increases by several fold but also it acquires high photo luminescence. Han *et al* employed tetraphenylethylene (TPE) based AIE and developed a method to sense lectin-carbohydrate interactions.⁴⁹ Recently “turn-on” type of luminescent sensors based on carbohydrate appended TPE

Scheme 2: synthesis of TPE based glycoconjugates

were developed to sense lectins and influenza virus. To utilize this rare phenomenon TPE based glycoconjugate was synthesized. Reaction between TPE derivative **16** with excess NaN_3 /DMF gave TPE functionalized with azide **17**. Then mannosylated azide up on click reaction with tetrakis(2-propynyloxymethyl)methane gave propargyl appended mannose cluster which was subsequently reacted with **17** to give peracetylated multivalent glycoconjugate which was further deacetylated with NaOMe/MeOH to get the desired TPE based glycocluster **22** (scheme 2). As predicted in the diluted solution state TPE-glycocluster did not show any luminescence. However, up on treatment with Con A the aqueous mixture became highly

luminescent. It was observed that 11 fold of higher fluorescence has been observed from molecular TPE-glycocluster solution to Con A binded TPE-glycocluster aggregates.

1.4 Glyconanoparticles in Carbohydrate-protein interactions

1.4.1 Goldnanoparticles

Bio-functionalized inorganic nanostructures have led to significant developments in nanoscience and bionanotechnology and pave a new way in solving new challenges associated with bioassays and interaction studies. Inorganic part of theses hybrid nanostructures allows them to possess a wide range of optical, electronic, magnetic and mechanical properties.⁵⁰ Biofunctionalized nanomaterials which act as multivalent model system,⁵¹ mimic most bio-molecules such as proteins, DNA are because of their similar size range, to employ them in biorecognition processes and . Initially, “Cluster effect”-the pioneering work of Lee⁵² has sparked many scientists to design multivalent model systems based on proteins, dendrimers, liposomes and polymer scaffolds to investigate carbohydrate-protein interactions.⁵³⁻⁵⁶

Gold nanoparticles are the most widely investigated nanoparticles among the hybrid nanoparticles.⁵⁷ In 1857 faraday first reported stable gold colloidal solution.⁵⁸ Nowadays goldnanoparticles are being used widely in nano-biosciences research due to their unique physical and chemical properties. A wide variety of biomolecules can be attached to goldnanoparticles via thiol functionality to gold surface.⁵⁹ Since 1990, numerous reports have been published on functionalization of goldnanoparticles with biomolecules i.e., proteins, peptides, antibodies and DNA.⁶⁰⁻⁶³ Soledad Penades for the first time grafted thiol terminated carbohydrate derivatives on gold surface.⁵⁹ These

goldglyconanoparticles were employed as a new multivalent tool to study the carbohydrate-carbohydrate interactions in water between oligosaccharide epitopes, which cause *in vivo* cell association. Later “glyconanotechnology strategy” has been introduced.⁶⁴ This approach utilizes a technique, where carbohydrate self-assembled monolayers (SAM) were formed on two or three dimensional nanostructure surfaces.

In 2001, these water-soluble 3D-multivalent model system based on carbohydrate functionalized gold nanoclusters were named as “glyconanoparticles (GNPs)” which were further described as “nanoparticles with chemically well defined glycocalyx-like surface and with globular carbohydrate display”, used to study the carbohydrate interactions and cell-cell- adhesion processes.⁵⁹ These chemically well defined carbohydrate coatings make these nanoparticles soluble in biological media and bestows biocompatibility and non-toxicity. In general glyconanoparticles hold additional advantages over various other carbohydrate-functionalized systems such as polymers,⁶⁵ liposomes⁶⁶ and dendrimers⁶⁷ etc. Thus, glyconanoparticle technology allows synthesizing glyconanoparticles of various carbohydrate densities and grafting different carbohydrate linkers based on flexibility, rigidity and accessibility of the ligands. The three key factors, which would effect the carbohydrate decoration and compatibility of glyconanoparticles, are (a)hydrophilicity/hydrophobicity of the spacer (b)nature of the spacer and (c)length of the spacer. The most promising part of glyconanoparticles is that a single gold cluster can accommodate a wide range of biomolecules such as carbohydrates, proteins, liposomes, DNA, RNA and fluorescent probes making it as a unique “artificial nanocell”.

Generally two methods are followed to study the carbohydrate-protein interactions through nanoparticle systems. (a) method based on colorimetric bio-sensing assay; (b) method based on aggregation and dispersion of goldnanoparticles.

Kataoka *et al* first employed gold glyconanoparticles and with the help of UV-visible spectra of corresponding colloidal metallic elements, theory disclosed the possibility of investigating carbohydrate-protein interactions.⁶⁸ This phenomenon operates on the principle that, in the presence of specific analyte GNPs aggregate together to produce a color change, evidenced by a significant shift in the surface Plasmon absorption band of the metal cluster, which can be monitored by UV-Visible spectroscopy. Kataoka has synthesized lactoside coated GNPs and combined with bivalent *Recinus communis* agglutinin (RCA₁₂₀). This lectin specifically recognizes β -galactopyranose residues. Addition of RCA₁₂₀ to lactoside GNPs causes aggregation and induces considerable change in the visible spectra, which can be evidenced, by color change from pinkish red to purple (Figure 13). Upon addition of excess of galactose, this phenomenon (aggregation of lactoside GNPs by RCA₁₂₀ lectin) recovers the initial dispersed phase along with restoration of original pinkish red color.

Figure 13: Schematic representation of reversible aggregation: Figure shows dispersion behaviour of RCA₁₂₀ lectin and galactose with change in color from pinkish-red to purple to pinkish-red. (Reproduced with permission from Ref. 68; Copyright: American Chemical society)

The degree of aggregation of lactoside GNPs is directly proportional to lectin concentration. Moreover, lactoside coating on GNPs strongly influences the aggregation by RCA₁₂₀ and it was found that >20% of the lactose density is necessary to promote the aggregation.

Kataoka's initial investigations triggered other scientists to employ glyconanoparticles to study carbohydrate-protein interactions. Influenced by Kataoka's methodology, Russel *et al* have constructed a multivalent model with mannose-modified goldnanoparticles to specifically recognize Concanavalin A (Con A). Even sub-micromolar concentrations of proteins can be determined by using this colorimetric method.⁶⁹ Later the same group replaced mercaptoethyl linker with thioctic(6,8-dithioactanoic)acid linkage to obtain enhanced nonspecific protein binding. Further they have utilized silver and goldnanoparticles and successfully detected RCA120 through colorimetric method.⁷⁰ The experiments conducted by Russel *et al* revealed that 3 factors influence the analytical accuracy of GNPs while designing them (a) length of the tether (b) nanoparticle size and (c) carbohydrate decoration density on the metal particle surface.

Chen *et al* synthesized mannose-encapsulated goldnanoparticles (m-AuNP) (scheme 3) and employed them to specifically recognize and bind to mannose specific FimH proteins on the type 1 pili of bacteria *Escherichia Coli*.⁷¹ It represents one of the best examples to label the proteins on the bacterial cell surface with the aid of carbohydrate-conjugated nanoparticiles.

Scheme 3: Synthesis of mannose encapsulated GNPs; Reaction conditions: (a) Ac_2O , Pyr, DMAP (b) HBr, HOAc (c) 4-pentylalcohol, $\text{Hg}(\text{CN})_2$, (d) HSAC, AIBN, dioxane (e) NaOMe (cat), MeOH (f) HAuCl_4 , NaBH_4 .

Moreover mannose-encapsulated goldnanoparticles facilitates the direct viewing of target receptor on the cell surface with electron microscopy. Earlier the same group along with Lin *et al* first time performed quantitative study of the multivalent interactions between carbohydrate grafted nanoparticles and Concanavalin A.⁷²

Chien *et al* next encapsulated goldnanoparticles with globotriose in surface plasmon resonance (SPR) competition assay, to study the interactions with the B₅ subunit of Shiga toxin I (B-Slt) that specifically bind to globotriaosylceramide, the globotriose blood group antigen.⁷³ Globotriose-GNPs were employed as multivalent probe for the purification of B-subunit from cell lysates and the affinity of globotriose-GNPs towards the protein is highly size and the tether length dependent. The high specificity of globotriose GNPs to B-Slt protein could be attributed to the binding site of B-Slt, which is highly dense and the low curvature of the large gold nanoparticle surface. This allows B-Slts binding sites to interact with globostriose-GNPs at a time.

Kamerling *et al* further provided SPR, TEM and UV evidence for the multivalent carbohydrate-protein interactions by evaluating the interaction between oligomannoside and glucosaccharides and Con A.⁷⁴

Lee group has developed a highly sensitive lectin biosensor based on quartz crystal microbalance (QCM).⁷⁵ They have employed mannoside stabilized gold nanoparticles as a signal amplifier. In this approach, gold-coated QCM electrode was modified with thiol containing mannoside, to which Con A has been added (Figure 14). As a result, there was a decrease in specific frequency due to the specific association of Con A with mannoside (Figure 14). Later mannose functionalized GNPs were introduced to this electrode so that they can combine with remaining free sites of Con A.

Figure 14: Schematic representation of role of mannose-stabilized AuNP's as a signal amplifier in bio-sensing of lectin Con A. (Reproduced with permission from Ref. 75; Copyright: Royal Society of Chemistry, Cambridge)

This resulted in a significant increase in frequency. The prominent feature of this experiment is that the signal intensity has been increased 13 times to normal detection technique. This technique is comparable with ELISA (enzyme linked immunosorbent assay) analytical technique (Figure 15).

Figure 15: Frequency response plot for the injection of Con A into the mannose SAM modified Au-QCM electrode (a) before and (b) after the addition of mannose stabilized goldnanoparticles. (Reproduced with permission from Ref. 75; Copyright: Royal Society of Chemistry, Cambridge)

Jensen and coworkers synthesized oxime and oxyamine tethered (oligo)-glucoside-GNPs to study the recognition properties of Con A.⁷⁶ N-glucosyl oximes on gold GNPs interact with Con A. In this approach, N-glucosyl oximes, which usually are in tautomeric form interacts with Con A, where as the acyclic, open chain N-glucosyl oxyamines do not interact with Con A. They also studied the interaction between glycoamylase and oxime and oxyamine-maltose –GNPs. Oxyamine-maltose GNPs were completely stable towards enzyme degradation because of their higher ligand density, which allows the easy accessibility of the sugars to the ligand. These results corroborate the findings of Penades *et al*, who reported that β -galactosidase could not cleave the galactosyl moiety of lactose-GNPs.

Carbohydrate density on the GNPs is an important factor in determining the protein-carbohydrate interaction. This is further proved by evaluating the binding between lactoside functionalized GNPs with two galactose specific proteins i.e., *E.Coli* β -galactosidase and *Viscum album* agglutinin.⁷⁷ Different rates of enzymatic hydrolysis are achieved via varying the lactose conjugate density on the surface of GNPs. This experiment emphasizes the precise selection of ligand densities and spacer lengths in accordance with the topographical requirements of the corresponding protein, while synthesizing functionalized GNPs. This is essential to prevent the hydrolysis from glycosidases.

Penades *et al* has synthesized water-soluble mannose rich multivalent mannose goldnanoparticles (manno-GNPs), which can inhibit DC-SIGN (dendritic cell specific ICAM 3-grabbing nonintegrin).⁷⁸ Interactions between glycans of HIV envelope, gp120 and dendritic cells (DCs) are mediated by DC-SIGN receptor, which is present on dendritic cells. The interaction of gp120 and dendritic cells is one of the early steps in causing the HIV viral infection. Surface Plasmon resonance experiments were conducted to calculate the inhibition potency of GNPs encapsulated with α -Man-(1 $\rightarrow$ 2)- α -Man, α -Man-(1 $\rightarrow$ 3)- α -Man, α -Man-(1 $\rightarrow$ 2)- α -Man-(1 $\rightarrow$ 2)- α -Man towards interaction of DC-SIGN to HIV encapsulated gp120 (Figure 16). Mannose rich GNPs found to have inhibited the interaction of dendritic cells and gp120. The inhibition rate was nano-molar range for multivalent oligo mannosides where as millimolar range for monovalent mannosides. Among the mannosides employed for to test the inhibition activity, disaccharide α -Man-(1 $\rightarrow$ 2)- α -Man showed 20,00 folds of higher activity (100% inhibition at 115 nM) than corresponding monomeric disaccharide (100% inhibition at 2.2 mM). Thus SPR results showed that manno-GNPs could successfully interrupt DC-SIGN and gp120 interactions.

Figure 16: structures of high mannose glycans and gold mannoglyconanoparticles.

In another attempt, interactions between sialic acid derivatives and Alzheimer's amyloid-beta ($A\beta$) peptides were studied.⁷⁹ In this approach, gold nanoparticles on carbon electrode have been modified with sialic acid derivatives by employing click chemistry strategy. AFM and electrochemistry data confirmed the interactions between sialic acid and $A\beta$ peptides. Tyrosine residue $A\beta$ peptides gave the peak-oxidation current response that is used as analytical signal.

1.4.2 Glyconanoparticles other than gold in carbohydrate-protein interactions

Glyconanoparticles other than gold have also been employed to conduct recognition experiments with proteins. Jana and Ying groups had studies such interaction by utilizing Silica coated Ag, CdSe-ZnS and Fe_3O_4 . They functionalized these nanoparticles with dextran and tested for Con A recognition (scheme 4).⁸⁰

Scheme 4: Synthesis of dextran coated silica nanoparticles.

Addition of Con A resulted in the aggregation of nanoparticles. With silver nanoparticles as the aggregation proceeds with time red shift in Plasmon absorption was noticed. Quantum Dots (QDs) caused decrease in fluorescence and absorbance

Figure 17: Con A induced nanoparticle aggregation of (A) dextran 1K-Ag and (B) dextran 40k-QD. Black line depicts absorption spectra after 0 mins. Similarly, blue line (30 min), green line (1 h) and red line (2 h). (Reproduced with permission from Ref. 80; Copyright: American Chemical Society)

over the time. While using magnetic nanoparticles Fe_3O_4 , Con A induces the aggregation by particle-particle cross-linking and the aggregates can remove by using a magnetic field. There has been no particle aggregation observed when sufficient amount of glucose in present (Figure 17).

Carbohydrate-lectin interactions are extensively studied by quantum dots. Rosenzweig *et al* has reported that carboxymethyldextran and polylysine located on nano spheres of CdSe-ZnS QDs surface, capable of interacting with glucose specific lectin Con A.⁸¹

Scheme 5: Schematic representation of water soluble β -Glc-NAc encapsulated quantum dots.

Specificity of N-acetyl-glucosamine sheathed quantum dots (QDGLNs) towards wheat germ agglutinin (WGA) has been proved by Fang and co-workers with the aid of various analytical tools such as fluorescence, TEM, dynamic light scattering (DLS) and flow cytometry.⁸² Glycosylation of protected Glc-NAc with 11-mercapto-1-undecanol and subsequent saponification and autoxidation finally furnished disulfide **37**. Then QDs encapsulated with pyridine were later treated with disulfide and NaBH_4 to

give β -Glc-NAc sheathed QDs (QDGLNs) **38**. In the similar way mannose encapsulated QDs were synthesized (QDMANs) (scheme5).

Further they have investigated the site-specific interactions of Glc-NaAc and mannose with mouse sperm. They found that QDGLNs specifically interact and bind to head part of sperm while QDMANs spread over the tile part of the sperm (Figure 18).

Figure 18: Confocal microscope imaging for (A) QDGLN-labeled sea-urchin sperm (B) QDMAN-labeled mouse sperm. (Reproduced with permission from Ref. 82; Copyright: Wiley-VCH Verlag GmbH & Co. KGaA)

Surolia and coworkers synthesized water soluble, sugar functionalized QDs and investigated in agglutination assays with different lectins.⁸³ Initially various reducing sugars such as D-melibiose, D-lactose and D-maltotriose were modified with a thiol terminated spacer arm and these neoconjugates were encapsulated on CdSe-ZnS QDs (**39**). In these carbohydrate-thiol derivatives the reducing part of the carbohydrate becomes acyclic where as the non-reducible end preserves the stereochemistry and remains in cyclic form. Thus, the synthesized neoglycoconjugate QDs resemble with dendrimer architecture (Figure 19).

Figure 19: Structure of CdSe-ZnS quantum dots encapsulated with neoglycoconjugates.

Carbohydrate-lectin multivalent interactions were studied by monitoring light scattering at 600 nm. Kinetic studies on sugar-QDs agglutination has revealed that this process occurs in step wise manner. Initially, due to specific multivalent interactions between carbohydrates and lectin, smaller soluble aggregates are formed. These smaller soluble aggregates cause turbidity increase therefore increase in scattering. As the time proceeds, the smaller aggregates promote precipitation, hence a decrease in absorption (Figure 20). They demonstrated that soybean agglutinin (SBA) specifically binds with melibiose QDs rather than lactose QDs and moreover specific deagglutination takes place upon the addition of α -galactose making this process reversible and selective. The salient feature of this approach is lectins can recognize the specific carbohydrates at nano molar concentrations.

Figure 20: Change in the absorbance of 589-MT-QD immediately after mixing with Con A as a function of time. (Reproduced with permission from Ref. 83; Copyright: American Chemical Society)

Reiger *et al* employed biodegradable nanoparticle system to study the interaction between mannosylated copolymer nanoparticles and Burkholderia cenocepacia lectin (BclA).⁸⁴ This lectin (BclA) is dimeric in nature and specifically interacts and binds to α -D-mannosepyranosyl conjugates. In the first step BclA was labeled with biotin. In order to obtain modified enzyme linked lectin assay (ELLA), nanoparticles were first incubated with biotinylated lectin, in the presence of calcium ions. Later they further incubated the assays with streptavidin phosphatase and with enzyme p-nitrophenyl phosphate. Finally absorbance of color complex measure that is formed due to the interaction of the nanoparticles with biotin labeled BclA. Isothermal titration calorimetry (ITC) was used to get the affinity constant of BclA and also to measure the enthalpy of carbohydrate-lectin complexation, which was found to be $\Delta H = -23$ kJ/mol. ITC also provided the stoichiometry of the interactions. Multiple responses were generated upon the addition of BclA to the nanoparticles in microcalorimeter cell. These responses depend on the mannose concentration and mannose density on nanoparticles.

Capobriano *et al* encapsulated lanthanide (Ln^{3+}) nanoparticles with glycoconjugates (Figure 21) and utilized the in recognizing lectins with the aid of Luminescence resonance energy transfer (LRET) technology.⁸⁵ $\text{NaGdF}_4:\text{Er}^{3+}$ and Yb^{3+} were encapsulated with poly(amidoamine) dendrimers (PAMAM). They are usually called as ‘upconverting nanoparticles’ (LnNPs). They adsorbed sugar dendrimers on upconverting nanoparticles via ligand exchange and subsequently linking thiourea moiety between amine surface and isothiocyanatophenyl α -D-mannopyranoside thereby achieving the covalent functionalization of nanoparticles.

Figure 21: Mannose coated PAMAM-LnNPs

Such resulting PAMAM-LnNPs were employed to recognize tetramethylrhodamine (RITC) conjugated with Concanavalin A. As mentioned earlier, this protocol based on luminescence resonance energy transfer (LRET), mannose encapsulated PAMAM-LnNPs act as energy donors and RITC labeled Concanavalin A act as energy acceptor. In the core experiment initially, LnNPs were excited with 980nm radiation, subsequently the green light emitted from donor LnNPs was utilized to non-radiatively excite RITC-Con A lectin (fluorophore labeled lectin). Upon this excitement at 550 nm, the acceptor RITC-Con A emits radiation at 585 nm (Figure 22). A competitive experiment was carried out to show that LRET phenomenon takes place only when mannose encapsulated LnNPs were in close proximity to RITC-Con A.

Figure 22: Schematic representation of LRET interactions between mannose encapsulated PAMAM-Ln NPs and RITC Con A (a) in absence and (b) in presence of competitor mannose. (Reproduced with permission from Ref. 85; Copyright: Royal Society of Chemistry, Cambridge)

Joseph Wang and coworkers for the first time developed nanoparticle designated recognition of carbohydrates, based on carbohydrate binding with surface immobilized lectins.⁸⁶ They immobilized peanut agglutinin (PNA) lectin on gold surface by employing EDAC-NHC coupling (Figure 23). CdS nanocrystal tracer capped with carboxy terminal alkylthiol was encapsulated with 4-aminophenyl- β -D-galactopyranoside.

Figure 23: (a) Mixed SAM on the gold template (b) covalent immobilization of the lectin (c) addition of the tagged and untagged carbohydrates (d) dissolution of the captured nanocrystals and their stripping-voltametric detection at a mercury coated carbon electrode. (Reproduced with permission from Ref. 86; Copyright: American Chemical society)

There will be competition between the target sugar and CdS labeled sugar to bind with immobilized lectin on gold surface. Sensitive electrochemical stripping due to CdS nanocrystals could monitor this process. When the CdS labeled sugar interacts with lectin there will be a precise cadmium stripping voltametric current peak. As the interaction between specific target sugar and lectin increases the voltametric peak decreases (due to the lowering of non-target sugar labeled with CdS).

1.5 Aims and goals

As mentioned earlier in the introduction section, lectins belong to protein family, which take part in innumerable biological processes. The characteristic nature of lectins i.e., binding only with specific sugar moieties could be successfully exploited to design new biosensors. Such unique molecular recognition process helps to detect disease progression there by leading a way to construct novel diagnostic tools.⁸⁷ It is highly important to investigate carbohydrate-lectin interactions to detect pathogens and to control dreaded viral or bacterial infections. Numerous biosensors have been designed to probe sugar-lectin interactions. Many suffer from various disadvantages such as (a) low LOD (limit of detection) thus low sensitivity (b) complicated instrumental set-up (c) require high technical capability. Hence there is always a need to design a biosensor system that is rapid, simple, sensitive, efficient, cost-effective and more importantly provide rapid visual evidence. Here we present a simple biosensor system based on fluorescence labeling technique, which not only provides effective probing details regarding carbohydrate-lectin binding but also proved to be highly sensitive towards sugar-lectin interaction.

Carbohydrate functionalized gold nanoparticles mimic natural presentation of cells. When these glyconanoparticles combined with lectins, exert multivalent interactions with lectins. We envisioned that the ability of boronic acid to form reversible cyclic esters with diols of monosaccharides could be exploited in designing a new biosensor. Compounds having diol moieties capable of interact with boronic acids with high affinity through reversible ester formation. Such bindings between diols and boronic acids have extensively been studied.⁸⁸ This unique phenomenon has been exploited to construct several carbohydrate sensors⁸⁹⁻⁹² and nucleotide and carbohydrate transporters,⁹³⁻⁹⁶ where boronic acid acts as recognition moiety. Boronate ester's

stability is solvent and pH dependant⁹⁶ and moreover, the factors that influence boronic acid and diol interactions have not yet clearly understood. We exploit binding interactions between carbohydrate-boronic acid to design a novel biosensor. It's highly important to note that our biosensor is completely differs from “boronic acid based fluorescent glucose sensors”,⁹⁷ which operate on either photoelectron transfer (PET) process⁹⁸ or fluorescence (or Forster) resonance energy transfer (FRET).⁹⁹

Scheme 6: Schematic representation of carbohydrate-fluorescent ligand based “turn-off” and “turn-on” system to probe carbohydrate-lectin interactions.

When a fluorescent dye tagged boronic acid couples with carbohydrate encapsulated goldnanoparticles, its fluorescence quenches. It has been proved that goldnanoparticles

are efficient quenchers of molecular excitation energy in AuNp-Chromophore composites.^{100,101} The quenching efficiency mainly depend on two parameters (a) size of the gold nanoparticles and (b) distance between chromophore and goldnanoparticles.^{102,103} Various theoretical models have been proposed for this unique phenomenon.¹⁰⁴⁻¹⁰⁶ recently it has been proved that goldnanoparticles quench fluorescence by “phase induced radiative rate suppression.”¹⁰⁷ This process can be referred to as “**turn off**” process. Subsequently when carbohydrate specific lectin is added to the carbohydrate-fluorescent boronic acid complex, the in-coming lectin would replace the fluorescent boronic acid ligand. This causes the removal of fluorescent ligand, there by regenerating the fluorescence in the system. This process could be referred as “**turn on**” process (scheme 6). We believe that this system would offer to explore the carbohydrate-lectin interactions with higher efficiency. Schematic representation of this system is given below.

2. RESULTS AND DISCUSSION

2.1 Investigation plan

A systematic study has been carried out for probing the carbohydrate-lectin interactions. At the beginning suitable thiol ended carbohydrate linkers were synthesized and encapsulated on citrate-stabilized goldnanoparticles. Later a plethora of colorimetric studies were conducted on various sugar coated goldnanoparticles and lectins by using fluorescence spectroscopy. To begin with initially four types (mannose, glucose, galactose and glucosamine) of carbohydrate linkers were synthesized.

2.2 Synthesis of glycoside linkers

Usually thiol ended hydrophilic polyethylene glycol derivatives were employed to bind the sugar moiety with gold core. Later the linkers were incubated with citrate capped goldnanoparticles which undergo ligand exchange to furnish sugar grafted goldnanoparticles. Glyconanoparticles obtained by using this procedure are water soluble, nontoxic and stable in solution. Four types of carbohydrate linkers were synthesized.

Synthesis of 2-[2-(2-chloroethoxy)ethoxy]ethyl α -D-Mannopyranoside (**44**): Treatment of peracetyl mannose pyranoside **41** with 2-[2-(2-chloroethoxy)ethoxy]ethanol in the presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in dichloromethane for 24 hrs at room temperature furnished corresponding α -chloroglycol mannosylated linker **42**. Later this linker was treated with potassium thioacetate in DMF for 24 hrs to furnish mannose thioacetate **43**. Finally mannose thioacetate linker was deprotected

with the aid of NaOMe/MeOH to furnish desired 2-[2-(2-chloroethoxy)ethoxy]ethyl α -D-Mannopyranoside **44** (Scheme 7).

Scheme 7: Synthesis of 2-[2-(2-chloroethoxy)ethoxy]ethyl α -D-Mannopyranoside.

Reaction conditions: (a) $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (5 eq), CH_2Cl_2 , RT, 24 hrs, 42% (b) KSAc (2 eq), DMF, 45 $^\circ\text{C}$, 24 hrs, 80 % (c) NaOMe (1.5 eq), MeOH, RT, 30 mins.

Synthesis of 2-[2-(2-chloroethoxy)ethoxy]ethyl β -D-glucopyranoside (**50**): 1,2,3,4,6-penta-O-acetyl-D-glucopyranose **45** was treated with methyl amine (in methanol) in THF solvent to get 2,3,4,6-tetra-O-acetyl-D-glucopyranose **46**, which was further treated with trichloroacetonitrile and DBU in dichloromethane to obtain 2,3,4,6-tetra-O-acetyl-D-glucopyranosyl trichloroacetimidate **47**. Then this trichloroacetimidate was reacted with 2-[2-(2-chloroethoxy)ethoxy]ethanol in the presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in dichloromethane for 30 mins at room temperature to furnish corresponding β -chloroglycol glucosylated linker **48**. Later this β -chloroglycol glucosylated was treated with potassium thioacetate in DMF for 24 hrs to furnish glucose thioacetate **49**. Finally glucose thioacetate linker was deprotected with NaOMe/MeOH to furnish desired 2-[2-(2-chloroethoxy)ethoxy]ethyl β -D-glucopyranoside **50** (Scheme 8).

Scheme 8: Synthesis of 2-[2-(2-chloroethoxy)ethoxy]ethyl β -D-glucopyranoside.

Conditions: (a) MeNH_2 , MeOH, THF, RT (b) CCl_3CN , DBU, CH_2Cl_2 , RT (c) 2-[2-(2-chloroethoxy)ethoxy]ethanol, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, CH_2Cl_2 , RT, 30 mins (d) KSAc, DMF, 45 °C, 24 hrs (e) NaOMe, MeOH, RT, 30 mins.

Synthesis of 2-[2-(2-chloroethoxy)ethoxy]ethyl β -D-galactopyranoside(**56**): 1,2,3,4,6-penta-O-acetyl-D-galactopyranose **51** was treated with methyl amine (in methanol) in THF solvent to get 2,3,4,6-tetra-O-acetyl-D-galactopyranose **52**, which further treated with trichloroacetonitrile and DBU in dichloromethane to obtain 2,3,4,6-tetra-O-acetyl-D-galactopyranosyl trichloroacetimidate **53**. Then this trichloroacetimidate was reacted with 2-[2-(2-chloroethoxy)ethoxy]ethanol in the presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in dichloromethane for 30 mins at room temperature to furnish corresponding β -chloroglycol galactosylated linker **54**. Later this β -chloroglycol galactosylated **54** was treated with potassium thioacetate in DMF for 24 hrs to furnish galactose thioacetate **55**. Finally galactose thioacetate linker was deprotected with NaOMe/MeOH to furnish desired 2-[2-(2-chloroethoxy)ethoxy]ethyl β -D-galactopyranoside **56** (Scheme 9).

Scheme 9: Synthesis of 2-[2-(2-chloroethoxy)ethoxy]ethyl β -D-galactopyranoside.

Conditions: (a) MeNH_2 , MeOH, THF, RT (b) CCl_3CN , DBU, CH_2Cl_2 , RT (c) 2-[2-(2-chloroethoxy)ethoxy]ethanol, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, CH_2Cl_2 , RT, 30 mins (d) KSac , DMF, 45°C , 24 hrs (e) NaOMe , MeOH, RT, 30 mins.

Synthesis of 2-[2-(2-chloroethoxy)ethoxy]ethyl 2-acetamido-2-deoxy- β -D-glucopyranoside (**62**): 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy- α -D-glucopyranose **57** was treated with methyl amine (in methanol) in THF solvent to get 2-acetamido-2,3,4,6- tri-O-acetyl-2-deoxy- α -D-glucopyranose **58**, which further treated with trichloroacetonitrile and DBU in dichloromethane to obtain 2-acetamido-3,4,6- tri-O-acetyl-2-deoxy- α -D-glucopyranosyl trichloroacetimidate **59**. Then this trichloroacetamidate was reacted with 2-[2-(2-chloroethoxy)ethoxy]ethanol in the presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in dichloromethane for 30 mins at room temperature to furnish corresponding 2-acetamido- β -chloroglycol glucosylated linker **60**. Later this 2-acetamido - β -chloroglycol glucosylated was treated with potassium thioacetate in DMF for 24 hrs to furnish 2-acetamido thioacetate **61**. Finally this thioacetate linker was deprotected with NaOMe/MeOH to furnish desired 2-[2-(2-chloroethoxy)ethoxy]ethyl 2-acetamido-2-deoxy- β -D-glucopyranoside **62** (Scheme 10).

Scheme 10: 2-[2-(2-chloroethoxy)ethoxy]ethyl 2-acetamido-2-deoxy-β-D-glucopyranoside. Conditions: (a) MeNH₂, MeOH, THF, RT (b) CCl₃CN, DBU, CH₂Cl₂, RT (c) 2-[2-(2-chloroethoxy)ethoxy]ethanol, BF₃·Et₂O, CH₂Cl₂, RT, 30 mins (d) KSAC, DMF, 45 °C, 24 hrs (e) NaOMe, MeOH, RT, 30 mins.

Synthesis of fluorescein boronic acid ligand: Toly boronic acid was refluxed in toluene with 2,2-dimethyl-propanediol to get diol protected 5,5-dimethyl-2-p-tolyl-1,3,2-dioxaborinane **64** which was later brominated with NBS to furnish 2-(4-(bromomethyl)phenyl)-5,5-dimethyl-1,3,2-dioxaborinane **65**. This compound was treated with fluorescein in K₂CO₃ DMF and following work up would furnish desired fluorescein boronic acid **66** (Scheme 11).

Scheme 11: Synthesis of fluorescein-boronic acid ligand.

2.3 Preparation of carbohydrate functionalized goldnanoparticles

Synthesis of citrate stabilized goldnanoparticles (Cit-NPs)

250 mL of ultrapure water was taken in 500 mL round bottom flask and added 1 mM HAuCl_4 . The reaction mixture was refluxed under nitrogen. Then 25 mL of 38.8 mM sodium citrate was added immediately. At this point the color of the reaction mixture changes from grey to pinkish-red. Subsequently, the contents were again refluxed for 30 min. After 30 mins, the reaction mixture was allowed to cool to room temperature and stored for further use. The average diameter of the nanoparticles obtained by this method usually is approximately 12 nm (Figure 24).

Figure 24: TEM of 1-MMNP.

Preparation of PBS buffer: Six types of PBS buffer have been prepared. Buffer 1 contains Na_2HPO_4 (10 mM), NaH_2PO_4 (10 mM) and NaCl (0.1 M) at pH 7.0. Buffer 2 contains Na_2HPO_4 (1 mM), NaH_2PO_4 (1 mM) and NaCl (0.01 M) at pH 7.0. Buffer 3 contains Na_2HPO_4 (10 mM), NaH_2PO_4 (10 mM), NaCl (0.1 M) and CaCl_2 (0.2 mM) at pH 7.0. Buffer 4 contains Na_2HPO_4 (10 mM), NaH_2PO_4 (10 mM), NaCl (0.1 M) and

Mn(NO₃)₂ (0.2 mM) at pH 7.0. Buffer 5 contains Na₂HPO₄ (10 mM), NaH₂PO₄ (10 mM), NaCl (0.1 M), CaCl₂ (0.2 mM), Mn(NO₃)₂ (0.2 mM) at pH 7.0.

Preparation of lectin solutions: Different kinds of lectins (Con A, Peanut agglutinin and Triticum vulgaris) 1mg dissolved in 1 mL of PBS buffer. In the case of Con A 1 mg is dissolved in 1 mL of buffer 5 and buffer 2, 3 or 4. Similarly peanut agglutinin and triticum vulgaris (1 mg each) dissolved in buffer 2 or buffer 5.

Synthesis of mono-modified gold nanoparticles (MMNP): Following describes the general procedure: Addition of 26 μ L of carbohydrate linker (for monnose, glucose, galactose linkers; 32 μ L for glucosamine linker) to 1 mL of ultrapure water gives approx. 97 mM of carbohydrate linker stock solution. From this solution, 32 μ L solution was added to 1 mL of Cit-NPs solution. Then the contents were stirred for 72 hrs at room temperature. Later the contents were centrifuged for 15 mins (15000 rpm) at 4 °C and supernatant was removed and re-dissolved in 1 mL ultrapure water and then centrifuged again. This process was repeated for 3 times to get different types of pure MMNP solutions. Throughout this chapter 1-MMNPs refers to monnose linker modified NPs, 2-MMNPs denotes to glucose linker modified NPs, 3-MMNPs refers to galactose linker modified NPs, 4-MMNPs indicates glucosamine linker modified NPs. The resulting MMNPs residue was dissolved in different kinds of PBS buffer and stocked for further use.

Preparation of fluorescent ligand solution: Weighed 3.5 mg fluorescein boronic acid ligand into 1 mL of ethanol, from which 0.0106 M fluorescence solution is obtained. This solution diluted with ultrapure water to obtain 1.06×10^{-4} solution, which contains 1% ethanol. This stock solution further diluted 3 times with ultrapure water to get 3.5×10^{-5} M fluorescent solution. In this solution ethanol content was calculated as

0.33%. To minimize the exposure of light, solution- containing vials were wrapped with aluminium foil and the fluorescent stock solution stored for further use.

Preparation of dis-modified gold nanoparticles (DMNP): As described above, goldnanoparticles were encapsulated with two types of carbohydrate linkers (hence dis-modified gold nanoparticles). Two kinds of dis-modified gold nanoparticles were synthesized. In both kinds, mannose is the common sugar linker whereas the other two sugar linkers are glucose and glucosamine respectively (Mannose-glucose and mannose-glucosamine). Each DMNP further classified into 3 types depending on the glucose or glucosamine concentrations. It's important to note that the over all carbohydrate amounts kept uniform throughout the synthesis of DMNPs.

- A) 1:1 Mannose-glucose DMNPs
- B) 1:5 Mannose-glucose DMNPs
- C) 1:10 Mannose-glucose DMNPs
- D) 1:1 Mannose-glucosamine DMNPs
- E) 1:5 Mannose-glucosamine DMNPs
- F) 1:10 Mannose-glucosamine DMNPs

In all the above DMNPs the total carbohydrate linker concentration was kept constant at 97 mM. After adding corresponding carbohydrate linker (either glucose or glucosamine linker) to mannose linker in 1 mL of Cit-NP solution, they were stirred for 72 hrs at room temperature. Then the contents were stirred for 72 hrs at room temperature. Later the contents were centrifuged for 15 mins (15000 rpm) at 4 °C and supernatant was removed and re-dissolved in 1 mL ultrapure water and then

centrifuged again. This process was repeated for 3 times to get different types of pure DMNP solutions. Throughout this chapter 1-DMNP refers to mannose-glucose and 2-DMNP refers to mannose-glucosamine type of DMNPs.

Characterization of MMNP through MALDI-TOF (MALDI-TOF: Shimadzu Corp. Axima ToF2 Performa): To determine whether the encapsulation of goldnanoparticles procedure with different carbohydrate ligands is successful or not, we calculated Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) mass. 1-MMNP was chosen as model and measured MALDI-TOF. The results showed that mannose linker was grafted on MMNP successfully ($M+Na^+$ ion = 351) (Figure 25).

Figure 25. MALDI-TOF of 1-MMNP showing 351 ($M+Na^+$)

Synthesis of Carbohydrate-Fluorescein bornic acid complex (Fb-MMNP): The MMNP residue obtained after centrifugation was dissolved in different kinds of PBS buffer. 1 mL of MMNP or DMNP was incubated with 40 μ L of fluorescein boronic acid ligand (3.5×10^{-5} M) for 10 hrs. Note that for bio-assay testing and to measure LOD (limit of detection), after the incubation period, carbohydrate-fluorescein complex solution was

centrifuged (15000 rpm and 15 mins at 4 °C) and supernatant solution was removed in order to get rid of the excess fluorescein boronic acid ligand (Figure 26 and 27).

Figure 26: Fluorescein boronic acid-mannose complex (Fb-1-MMNP)

Figure 27: Fluorescein boronic acid-galactose complex (Fb-3-MMNP)

2.4 UV and Fluorescence studies on Fb-MMNP complex

We envisioned that if Fb-MMNP complex has been formed the resulting complex should quench and diminish the fluorescence intensity of fluorescein. For UV experiments; For experiment 1: 100 μL of Cit-NPs solution was diluted with 300 μL ultrapure water and UV spectra was recorded which shows absorbance max at 522 nm. For experiment 2: 100 μL of 1-MMNP in PBS buffer 2 was diluted with 300 μL PBS buffer 2 and UV spectra was recorded which shows absorbance max at 520 nm. For

experiment 3: 1-MMNP was diluted with 300 μL of PBS buffer 2 and incubated with 16 μL of fluorescein ligand solution ($1.06 \times 10^{-4} \text{ M}$) and UV spectra was recorded which shows absorbance max at 522 nm. For experiment 4: 400 μL of PBS buffer 2 was added with 16 μL of fluorescein ligand solution ($1.06 \times 10^{-4} \text{ M}$) and UV spectra was recorded which shows two absorbance peaks at 454 and 478 nm (Figure 28). We chose the peak at 454 nm with strong intensity of absorption to further carryout fluorescence studies where it is used as excitation wavelength.

Figure 28: UV spectra shows 1-goldnanoparticle solution 2-1-MMNP in PBS buffer 2, 3-Fb-1MMNP solution in PBS buffer 2, 4-fluorescent ligand solution in PBS buffer 2.

2.5 Fluorescence studies of 1-MMNP and Fb-1-MMNP:

For experiment 1: 1-MMNP solution was dissolved in 100 μL of PBS buffer and measured fluorescence which did not show any peak indicating that the solution has no fluorescence. Experiment 2: 100 μL of PBS buffer was added with 1 μL of fluorescein boronic acid ligand solution ($1.06 \times 10^{-4} \text{ M}$) and the fluorescence was measured (slit width 20 nm) which showed high intensity (762 a.u) at 511 nm. Experiment 3: To

show the effect of fluorescein ligand on carbohydrate linker, 100 μL of 1-MMNP was incubated with 1 μL of fluorescein ligand solution ($1.06 \times 10^{-4} \text{ M}$) for 10 hrs. Then fluorescence was measured which shows two peaks of low intensity, one is (90 a.u) at 508 nm and another one is (102 a.u) at 581 nm (Figure 29). This provides a strong evidence that, in this system when MMNP bind with fluorescein ligand, it quenches the fluorescence.

Figure 29: Fluorescence spectra of 1-1-MMNP solution in 100 μL of PBS buffer 2; 2- 100 μL of PBS buffer was added with 1 μL of fluorescein ligand solution ($1.06 \times 10^{-4} \text{ M}$) 3-100 μL of 1-MMNP was incubated with 1 μL of fluorescein ligand solution ($1.06 \times 10^{-4} \text{ M}$) for 10 hrs.

2.6 Fluorescence studies on interaction between fluorescein boronic acid ligand and MMNP

We believe, it is highly important to prove that proper binding takes place between fluorescein boronic acid ligand and MMNP. To prove this further we have carried out fluorescence experiments. First, 100 μL PBS buffer 5 was added with 4 μL of fluorescein boronic acid solution ($1.06 \times 10^{-4} \text{ M}$) and measured the fluorescence (slit

width 10 mm), which showed intensity (213 a.u) at 514 nm of wavelength. Then 4 μL of fluorescein boronic acid (1.06×10^{-4} M) was incubated with 100 μL of 1-MMNP for 10 hrs and subsequently the solution was subjected to centrifugation (15000 rpm for 15 mins at 4 $^{\circ}\text{C}$). The resulting supernatant solution (100 μL) was taken and measured fluorescence, which gave a peak at lower intensity (174 a.u) at the same wavelength. The significant decrease in the intensity could be described as below. When the fluorescein boronic acid combines with MMNP, only limited amount of free fluorescein boronic acid will be available upon the centrifugation. If there was no binding between fluorescein boronic acid and MMNP, the intensity could have matched with the original fluorescein boronic acid i.e., 213 a.u. This clearly provides another evidence and supports our theory that upon incubation, fluorescein boronic acid interacts with MMNP (Figure 30).

Figure 30: Fluorescence of 1-fluorescein boronic acid solution and 2-Fb-1-MMNP (incubated for 10 hrs) supernatant solution.

Further to demonstrate the role of boronic acid in this system we have carried out fluorescence experiment. First, 4 μL of molecular fluorescein solution (1.06×10^{-4} M)

was added to PBS buffer 5 and measured the fluorescence (slit width 5mm) which showed high intensity peak (704 a.u) at 516 nm. Later 4 μL of molecular fluorescein solution (1.06×10^{-4} M) was added to 100 μL PBS buffer 5 and incubated for 10 hrs and subsequently centrifuged. The upper supernatant solution's (100 μL) fluorescence (slit width 5mm) showed similar intensity (694 a.u) peak (Figure 31). This proves that fluorescein molecule with out boronic acid scaffold could not combine with MMNP thus shows similar intensity as molecular fluorescein.

Figure 31: fluorescence of 1-molecular fluorescein solution and 2-supernatant solution of molecular fluorescein incubated with MMNPs for 10 hrs.

After analyzing the data of above experiments, we can understand that when equimolar concentrations of molecular fluorescein and fluorescein boronic acid interacted with MMNPs, they interact differently with MMNPs. While the fluorescein boronic acid combines strongly with MMNPs and causes significant decrease in the fluorescence intensity, molecular fluorescein has no effect on the MMNPs and the decrease in intensity is nominal.

The decrease in intensity in both cases found to be considerable. In the first case, (i.e., fluorescein boronic acid incubated MMNPs) the average decrease in fluorescence intensity calculated as 16.8% (Figure 29), where as in the second case (i.e., molecular fluorescein incubated with MMNPs) the average decrease in fluorescence intensity calculated to be only 3.5% (Figure 32).

Figure 32: Figure shows the decrease (%) in intensity of **1**-molecular fluorescein incubated with 1-MMNPs and **2**-fluorescein boronic acid incubated with-1-MMNP solution.

Upon analyzing the results of above conducted fluorescence experiments, we can deduce that: (a) when fluorescein boronic acid combined with MMNP the fluorescence diminishes (b) presence of boronic acid scaffold in fluorescein molecule is mandatory (i.e., with out boronic acid this phenomenon does not occur).

2.7 Gel electrophoresis studies on Con A and Fb-MMNP

Before beginning the core experimental part, there should be evidence that Con A would replace the fluorescein-boronic acid ligand to bind with MMNP. Hence, gel

electrophoresis studies were conducted. The following is the detailed experimental procedure for gel electrophoresis:

Preparation of SDS-PAGE for protein staining:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is generally employed technique for separating proteins based on their length of polypeptide chains or molecular weight with the ability to move within an electrical current. This is achieved by adding SDS detergent to remove protein secondary and tertiary structures and maintain protein as a polypeptide chains. SDS coats the proteins and provides negative charge across all proteins in the sample.

Casting the gel: It consists of two layers or gels: (a) separating gel (b) stacking gel

Separating gel Preparation: The separating gel is also known as resolving gel, which is more basic and has higher polyacrylamide content. The ingredients are added in a sequence of 30% acrylamide, 2M Tris pH 8.8, Distilled water, 10% SDS and the polymerizations is initiated by the addition of 10% Ammonium per sulphate (APS) and TEMED.

Table 1. 12% gel composition for 1 plate of 1mm thickness

30% Acrylamide	2.4ml
2M Tris pH 8.8	1.13ml
Distilled water	2.42ml
10%SDS	60μl
10% Ammonium per sulphate (APS)	30μl
TEMED	3μl

After the addition of all the ingredients in above mentioned volumes the gels are polymerized in a gel caster. First the separating gel is loaded and allowed to polymerize. After the addition of separating gel solution a thin layer of water or isopropanol is added to make a smooth surface of separating gel. It will take 30 to 45 min to polymerize the gel. After the gel gets polymerized a stacking gel was added and a comb is inserted to make wells for sample loading.

Stacking gel Preparation: The stacking gel has large pore size which contains 2 pH units lower than the electrophoresis running buffer (1X Tris-Glycine). These conditions provide an environment for Kohlrausch reactions determining molar conductivity as a result, the SDS coated proteins are concentrated and a thin starting zone is achieved in a few minutes.

Table 2. Stacking gel composition for 1 plate of 1mm thickness

30% Acrylamide	0.335ml
1M Tris pH 6.8	0.25ml
Distilled water	1.38ml
10%SDS	20 μ l
10%APS	15 μ l
TEMED	2.5 μ l

The ingredients as mentioned in above volumes are added in a sequence and the polymerization is initiated with 10% APS and TEMED. The gel is polymerized in a gel caster. The gel is casted over the separating gel. And a comb with 10 well is inserted in the gel caster to make the wells for sample loading.

To the Fb-MMNP residue in 1 mL of buffer 5, added 50 μL of Con A solution (1.05×10^{-5} M) and allowed to stand for 1 hr and then centrifuged (15000 rpm at 4 °C for 15 mins). Then the supernatant solution is removed and washed with PBS buffer 5. This process is repeated for 2 times in order to get rid of most of the unbound Con A. Thus obtained residue may predominantly contain Con A-MMNP complex, which is further used for gel electrophoresis. To this residue was added 2 μL of loading buffer and 1 μL of buffer 1 and subjected to heating at 90 °C for 2 mins. Later MMNP-Con A complex embedded with loading buffer is loaded into corresponding wells of the gel. Later the gel was run using 1X Tris-glycine running buffer in the sds page gel tank (Bio-Rad) at 200 volts for 45min to 60 min. The gel is stained with Coomassie Brilliant Blue (0.025%) in methanolic solution (40%) and acetic acid (7%), which is a protein stain and an anionic dye which binds with protein non specifically. Proteins in the gel are fixed by acetic acid and simultaneously stained by heating for 30 sec to 1 min. Excess dye is removed by de-staining with same solution without dye or water with several washes.

Gelelectrophoresis data shows that there is binding between Con A and 1-MMNP (lane 4) and 3-MMNP (lane 5). This also gives strong evidence that Con A binds with MMNPs. In the case of 1-MMNP (lane 4) the binding was high as indicated by the intensity of marker when compared to 3-MMNP (lane 5). Lane 6 and lane 7 act as controls (they contain fluorescein bornic acid and MMNP without Con A) for 1-MMNP and 3-MMNP respectively (Figure 33). For the sample preparation of Fb-1-MMNP (for lane 4), 1 mL of 1-MMNP was added with 40 μL of fluorescein boronic acid ligand and incubated for 10 hr. To this solution 50 μL of Con A (5.2×10^{-5} M) solution and incubated for 1 hr. This resulting solution is centrifuged (15000 rpm for 15 mins at 4 °C) and washed PBS buffer. This process is repeated for 2 times. The

final residue is combined with 2 μL of SMS buffer 5 and 2 μL of loading buffer. For 3-MMNP (lane 5) similar process is repeated.

Figure 33: Gel electrophoresis shows: Lane 1: standard Fermentas marker; lane 2: 2 μL of Con A (1.04×10^{-5} M) in PBS buffer 5 and 2 μL loading buffer; Lane 3: 2 μL of Con A (5.2×10^{-6} M) in PBS buffer 5 and 2 μL loading buffer; Lane 4: 1-MMNP with Con A and 2 μL of PBS buffer 5 and 2 μL loading buffer; Lane 5: 3-MMNP with Con A and 2 μL of PBS buffer 5 and 2 μL loading buffer; Lane 6: Fb-1-MMNP in 2 μL of PBS buffer 5 and 2 μL loading buffer; Lane 7: Fb-3-MMNP in 2 μL of PBS buffer 5 and 2 μL loading buffer.

Based on the results and evidences obtained by above conducted experiments, briefly we can summarize the results.

Step 1: In the first step we have synthesized citrate-capped gold nanoparticles (size: 12 nm) and through ligand exchange process we have grafted thiol ended carbohydrate moieties on the surface of gold core.

Step 2: To confirm the grafting of carbohydrate linkers on nanoparticles surface, MMNPs were characterized by MALDI-TOF, which shows the molecular weight of linker. This confirms the successful synthesis of MMNPs.

Step 3: Then fluorescein boronic acid ligand was incubated with MMNPs in PBS buffer in order to form Fb-MMNP complex (fluorescein bound MMNP).

Step 4: To get the evidence for the formation of Fb-MMNP complex we have conducted fluorescence experiments. Figure 28 shows that only fluorescein boronic acid ligand solution shows high intensity at 762 a.u. However when it is incubated with MMNPs for stipulated time period, the fluorescence intensity drastically decreased to 90 a.u. This data corroborates our strategy that when fluorescein boronic acid binds with MMNPs due to special characters of goldnanoparticles, they quench the fluorescence of fluorescein.

Step 5: The above stated phenomenon is further proved by additional fluorescence studies. Fluorescence was measured with Fb-MMNP and molecular fluorescein – MMNP. In the first case fluorescein boronic acid combines strongly with MMNPs and causes significant decrease in the fluorescence intensity (16.8%) while the molecular fluorescein has no effect on the MMNPs and the decrease in intensity is nominal (3.5%). This also proves that MMNPs can only combine with Fb-MMNP.

Step 6: This step is the most crucial step, where it is proved that Con A replaces fluorescein boronic acid ligand in Fb-MMNP complex to liberate fluorescein boronic acid in the system. This is confirmed by gel electrophoresis experiment.

2.8. Fluorescence studies on interaction of Con A with fluorescein boronic acid-1-MMNP complex

In order to explore the binding affinity of lectin with MMNPs we have conducted several fluorescence experiments. Con A is most widely studied lectin and it has strong affinity towards 1-MMNP, hence we chose Con A and 1-MMNP as model lectin and MMNP respectively. To begin we attempted to study the interactions between Con A and Fb-1-MMNP. In this aspect, 100 μL 1-MMNP in buffer 2 was added with 4 μL of fluorescein boronic acid (1.06×10^{-4} M) and incubated for 10 hrs. Then to this 100 μL of resulting Fb-1-MMNP solution, added 5 μL of Con A (1.04×10^{-5} M) and observed fluorescence intensity (192 a.u.) at 514 nm. It was observed that even after 1 hour there is no appreciable change in the fluorescence intensity (199 a.u.) at 514 nm (Figure 34). This suggests that Con A unable to replace fluorescein boronic acid ligand effectively from Fb-1-MMNP complex, and thereby increasing the intensity of fluorescence under these conditions.

Figure 34: Fluorescence study on interaction of Con A with Fb-1-MMNP complex. After the addition of Con A to Fb-1-MMNP system, 1 (immediately after the addition) and 2 (after 1 hr of addition) show, there is no substantial increase in fluorescence intensity.

It has been demonstrated that, metal ions play vital role in legume lectins and carbohydrate interactions. The presence of calcium and manganese ions is critical factor and these ions largely influence the binding affinities of Con A and mannose. Hence we tried to investigate the role of ions in Con A 1-MMNP interactions. Initially we employed both Ca^{2+} and Mn^{2+} ions to test the interactions. To 100 μL of Fb-1-MMNP solution in buffer 5 added 5 μL of Con A solution (1.04×10^{-5} M) and incubated for 1 hr. Then fluorescence was measured which showed a high intensity peak (764 a.u.) at 514 nm (Figure 35). Its worthy to note that upon the addition of Ca^{2+} and Mn^{2+} ions to the Fb-1-MMNP and Con A system, there has been a tremendous increase by 2.98 times in its fluorescence intensity.

To further understand and explore the individual ion roles in Con A-mannose binding we conducted additional fluorescence experiments. To 100 μL of Fb-1-MMNP solution in buffer 4 (buffer contains only Mn^{2+}) added 5 μL of Con A solution (1.04×10^{-5} M) and incubated for 1 hr. Then fluorescence was measured which showed an intensity peak (634 a.u.) at 514 nm. Even though an improvement was noticed in intensity, this intensity is lesser than the combined effect caused by both Ca^{2+} and Mn^{2+} ions. To examine the role of Ca^{2+} ions on Fb-1-MMNP, Con A system, fluorescence was measured by following the above stated experimental procedure, except, instead of buffer 4, buffer 3 (buffer contains only Ca^{2+}) was used. Surprisingly, the fluorescence intensity was further decreased to 421 a.u at 514 nm when compared to combined Ca^{2+} and Mn^{2+} ions and discrete Mn^{2+} ions (Figure 35). However, Ca^{2+} showed higher intensity when compared to Fb-1-MMNP-Con A system without any ions. This clearly demonstrates that in the presence of Ca^{2+} and Mn^{2+} ions Con A successfully replaces fluorescein boronic acid ligand from Fb-1-MMNP complex and

further corroborates the fact that, both Ca^{2+} and Mn^{2+} ions assistance is imperative in mannose-Con A binding (Figure 36).

Figure 35: Ion effect on Fb-1-MMNP-Con A system: Fluorescence measurements shows 1-when only Ca^{2+} ions employed; 2-when only Mn^{2+} ions used and 3-combined Ca^{2+} and Mn^{2+} ions effect on fluorescence intensity.

Figure 36: “Turn-off/turn on” Fb-1-MMNP system with Con A: I: Fb-1-MMNP-Con A solution (the solution is in pinkish-red color); II: Fb-1-MMNP-Con A solution with Ca^{2+} ions (solution is faintly discolored); III: Fb-1-MMNP-Con A solution with Mn^{2+} ions (solution considerably discolored); IV: Fb-1-MMNP-Con A solution with combined Ca^{2+} and Mn^{2+} ions (solution almost discolored)

After successfully establishing this phenomenon, i.e., “turn-off/turn-on” type fluorescein boronic acid based 1-MMNP and Con A interactions, we have decided to

investigate intricate details of Con A-1-MMNP binding by estimating the effect of Con A's concentration on 1-MMNP-Con A binding. This in-turn would furnish the LOD (limit of detection) of the system. Limit of detection not only reckons the efficiency of this bio-sensor system but also determines the performance when compared to rest of the bio-sensors.

To begin with, we conducted several fluorescence experiments by employing various concentrations of Con A such as 2 mg/mL, 1 mg/mL, 0.7 mg/mL, 0.5 mg/mL, 0.1 mg/mL and finally 0.01 mg/mL. Fluorescence was measured against time factor. In the first experiment, 2mg/mL Con A was employed to investigate the Con A-1-MMNP interactions. To 100 μ L of Fb-1-MMNP solution in buffer 5 added 5 μ L of Con A solution (2.08×10^{-5} M). Then fluorescence was measured which showed, highest intensity (802 a.u.) at 514 nm. This intensity was measured, till there was no further increase in the intensity of the system with the increase in time. Fluorescence was measured at an interval of 5 mins. As the time increases fluorescence intensity gradually increases (Figure 37), which in-turn suggests that Con A binding with 1-

Figure 37: Fluorescence measurement of Con A-1-MMNP binding for 2mg/mL Con A concentration. There was no further increment in intensity observed after 120 mins.

MMNP increases with time and becomes constant at 120 mins, indicating that for specific concentration of Con A, after specified time, the binding interactions become saturated.

In the next experiment Con A concentration was changed to 1mg/mL. To 100 μ L of Fb-1-MMNP solution in buffer 5 added 5 μ L of Con A solution (1.08×10^{-5} M). Immediate fluorescence measurement of this resulting solution showed highest intensity (734 a.u.) at 514 nm after 100 mins (Figure 38). There was no further improvement in its intensity was noticed.

Figure 38: Fluorescence measurement of Con A-1-MMNP binding for 1mg/mL of Con A concentration. There was no further increase in intensity observed after 100 mins.

Later, the concentration of Con A has been changed to 0.7 mg/mL. To 100 μ L of Fb-1-MMNP solution in buffer 5 added 5 μ L of Con A solution (0.7×10^{-5} M). Fluorescence was measured for this solution, which showed highest intensity (712 a.u.) at 514 nm after 95 mins. There was no further improvement in its intensity was noticed after 95 mins (Figure 39).

Figure 39: Fluorescence measurement of Con A-1-MMNP binding for 0.7 mg/mL of Con A concentration. There was no further increase in intensity observed after 95 mins.

In the next fluorescence experiment we have used 0.5 mg/mL as concentration of Con A. To 100 μ L of Fb-1-MMNP solution in buffer 5 added 5 μ L of Con A solution (0.52×10^{-5} M). Fluorescence of this solution showed its highest intensity (388 a.u.) at 514 nm after 85 mins (Figure 40). There was no further improvement in its intensity was noticed after 85 mins.

Figure 40: Fluorescence measurement of Con A-1-MMNP binding for 0.5 mg/mL of Con A concentration. There was no further increase in intensity observed after 85 mins.

Later 0.2 mg/mL as concentration of Con A was used to test the interactions between 1-MMNP and Con A. To 100 μ L of Fb-1-MMNP solution in buffer 5 added 5 μ L of Con A solution (0.208×10^{-5} M). Fluorescence of this solution showed highest intensity (160 a.u.) at 514 nm after 20 mins (Figure 41). There was no further enhancement in fluorescence intensity noticed after 20 mins.

Figure 41: Fluorescence measurement of Con A-1-MMNP binding for 0.2 mg/mL of Con A concentration. There was no further increase in intensity observed after 20 mins.

It is important to note that in all the above conducted experiments, fluorescence was measured immediately after the addition of Con A to Fb-1-MMNP system. The over all observations noticed in the above set of experiments can be summarized as: (a) 1-MMNP-Con A interactions depend on the concentration of Con A and time of incubation period (of Fb-1-MMNP-Con A system) (b) as the concentration of Con A increases the fluorescence intensity increases. It is obvious that, as the concentration of Con A increases, more number of Con A molecules will be available for each mannosylated gold nanoparticle in the solution which in-turn would replace more

number of fluorescein boronic acid ligands, there by enhancing the fluorescence intensity of the system (c) at certain point of time, there will not be much appreciable raise in the fluorescence intensity. This shows, at this specific time, maximum numbers of fluorescein boronic acid ligands have already been replaced by Con A molecules. Hence there will be no change in fluorescence intensity (d) as the concentration of Con A decreases, fluorescence intensity also decreases suggesting that less number of Con A molecules will be available in solution to replace fluorescein boronic acid ligands from Fb-1-MMNP system (e) the decrease in concentration of Con A, would lead to lesser number of Con A molecules in the solution. However, more number of mannosylated goldnanopartilces will be available for fewer Con A molecules. Hence, Con A molecules have high opportunity to quickly access to 1-MMNP to replace fluorescein boronic acid ligands, there by decreasing the time required for Con A molecules to occupy 1-MMNPs. These results can be shown in one plot (Figure 42).

Figure 42: Fluorescence intensity pattern with Con A concentration. The curve shows the gradual increase in intensity up on the increase of Con A concentration.

Now it has been proved that, in the presence of Mn^{2+} and Ca^{2+} ions Con A successfully replaces fluoresceine boronic acid to increase the fluorescence intensity. To check this phenomenon further we have carried out a “regeneration experiment”. Now it is understood that when Con A completely replaces fluoresceine boronic acid from Fb-1-MMNP system the goldnanoparticle solution turns to colorless solution from pinkish red color (Figure 43). When we have added excess of mannose to this colorless solution, the pinkish red color again regenerated. This can be described as, when the excess mannose (2M) is added to the system, most of the bound Con A molecules (to 1-MMNP) will unbound with 1-MMNP to bind with high number of free mannose molecules. This will lead to re-union of fluorescein boronic acid and 1-MMNP, subsequently resulting in decrease in fluorescence i.e, regeneration of pinkish red color.

Figure 43: Regeneration experiment of Fb-1-MMNP system with excess of free mannose (2M). I: Fb-1-MMNP solution (pinkish red color solution); II: Fb-1-MMNP solution with Con A in the presence of Mn^{2+} and Ca^{2+} ions (solution becomes colorless) III: Fb-1-MMNP solution with Con A and excess of free mannose (colorless solution becomes pinkish red).

After examining the effect of Con A concentration, we intend to test the Limit of detection (LOD) or sensitivity of this system. As mentioned earlier LOD determines

the competence of the bio-sensor system. Lowest concentrations of Con A were employed to test the minimum amount of Con A required to replace fluorescein boronic acid from Fb-1-MMNP complex. We carried out fluorescence experiments with 0.1 and 0.01 mg/mL concentration of Con A. In this experiment, 100 μ L of 1-MMNP was centrifuged and removed the supernatant solution. This resultant residue was dissolved in 100 μ L of PBS buffer 5 solution, to which added 5 μ L of Con A solution (1.04×10^{-6} M and 1.04×10^{-7} M for 0.1 and 0.01 mg/mL Con A respectively). Fluorescence was measured and maximum fluorescence intensities observed for 1.04×10^{-6} M and 1.04×10^{-7} M of Con A are 31 a.u. and 30 a.u. at 514 nm respectively (Figure 44 & 45). Both the intensities correspond to 120 mins.

Figure 44: Fluorescence of Fb-1-MMNP-Con A system with 0.1 mg/mL concentration of Con A. 1-fluorescence intensity of blank (100 μ L of 1-MMNP in PBS buffer 5 and 5 μ L of PBS buffer 5) 2-fluorescence intensity of 1-MMNP with Con A after zero min and 3-fluorescence intensity of 1-MMNP with Con A after 120 mins.

Figure 45: Fluorescence of Fb-1-MMNP-Con A system with 0.01 mg/mL concentration of Con A. 1-fluorescence intensity of blank (100 μ L of 1-MMNP in PBS buffer 5 and 5 μ L of PBS buffer 5) 2-fluorescence intensity of 1-MMNP with Con A after zero min and 3-fluorescence intensity of 1-MMNP with Con A after 120 mins.

By analyzing the above set of experiments, limit of detection was found to be 4.9 nM. This value can be fairly comparable with other techniques generally employed for carbohydrate-lectin interactions. For example, optical microarray technique showed LOD as 1.4 nM, electrochemical surface plasmon resonance (E-SPR) gave 41 nM of detection limit, electrochemical impedance spectroscopy showed LOD at 5 nM, nanoelectronic detection using carbon nanotubes found LOD to be 2 nM and voltametric and colorimetric techniques showed detection limits 6 and 64 nM respectively.

Calculation of Limit of Detection (LOD):

Con A molecular weight is 96 KDa. Final concentration of Con A, which gives response to Fb-1-MMNP system was found to be 0.01 mg/mL. This Con A concentration (Concentration_{Con A}) is divided by molecular weight of Con A (96

KDa) to get the molar concentration of Con A solution (1.04×10^{-7} M). 5 μ L of this Con A solution was further added to 100 μ L of Fb-1-MMNP system. Hence, the final Con A concentration (limit of detection) diluted to about 20 times to give 4.9 nM (Concentration_{Con A II}).

$$\begin{aligned} \text{Concentration of Con A I} &= \frac{\text{mass of Con A in solution}}{\text{Con A molecular weight}} \\ &= \frac{0.01}{96 \times 10^3} = 1.04 \times 10^{-7} \text{ M} \\ \text{Concentration of Con A II} &= \frac{\text{concentration of Con A I}}{\text{number of times diluted}} \\ &= \frac{1.04 \times 10^{-7}}{21} = 4.9 \times 10^{-9} \text{ M} \end{aligned}$$

After successfully exploring binding interactions between 1-MMNP and Con A, we attempted to apply this system to other carbohydrates (i.e., to 2-MMNP, 3-MMNP and 4-MMNP). Initially β -galactosylated goldnanoparticles were tested for interactions with Con A. It has been studied that there is no interaction between Con A and galactose (either α or β anomer) as Con A is only specific for mannose. Hence we have chosen β -anomer of galactose and grafted over goldnanoparticles (3-MMNPs) to study interactions with Con A. Similar to 1-MMNP we conducted fluorescence experiments to find out the interactions. To 100 μ L 3-MMNP in buffer 5 was added with 4 μ L of fluorescein boronic acid (1.06×10^{-4} M) and incubated for 10 hrs. Then to this 100 μ L of resulting Fb-3-MMNP solution, added 5 μ L of Con A (1.04×10^{-5} M) and surprisingly we have observed increase in the intensity of fluorescence. Highest fluorescence intensity was found to be 444 a.u. at 514 nm after 95 mins (Figure 46). This is unusual phenomenon, which we have observed through our turn-off/turn on system (Figure 47). This clearly demonstrates that β -galactose capable of interacting

with Con A to some extent. To the best of our knowledge no bio-sensor technique has showed the interaction of β -galactose with Con A.

Figure 46: Fluorescence measurement of Con A-3-MMNP binding for 1 mg/mL of Con A concentration. Unusual increase in intensity till 444 a.u.. There was no further increase in intensity observed after 95 mins.

Figure 47: “Turn-off/turn on” Fb-3-MMNP system with Con A: I: Fb-3-MMNP-Con A solution (the solution is in pinkish-red color); II: Fb-3-MMNP-Con A solution with Ca^{2+} ions (solution is faintly discolored); III: Fb-3-MMNP-Con A solution with Mn^{2+} ions (solution considerably discolored); IV: Fb-3-MMNP-Con A solution with combined Ca^{2+} and Mn^{2+} ions (solution almost discolored).

Since the β -anomer of galactose showed considerable interactions with Con A we performed the “regeneration experiment” with Fb-3-MMNP-Con A system also. As we predicted, upon the addition of excess of free mannose (2M) all the Con A molecules detach with 3-MMNP and bind with highly populated free mannose molecules (Figure 48). Hence, fluorescein boronic acid molecules will again bind with 3-MMNP causing decrease in fluorescence. This will regenerate the pinkish red color.

Figure 48: Regeneration experiment of Fb-3-MMNP system with excess of free mannose (2M). I: Fb-3-MMNP solution (pinkish red color solution); II: Fb-3-MMNP solution with Con A in the presence of Mn^{2+} and Ca^{2+} ions (solution becomes colorless) III: Fb-3-MMNP solution with Con A and excess of free mannose (colorless solution becomes pinkish red).

After observing this surprising result with β -galactose (3-MMNP), we further studied the effect of 2 and 4-MMNPs (β -glucose and β -glucosamine) on Con A. Similar fluorescence experiment was conducted (as mentioned in β -galactose) by employing 2-MMNP and 4-MMNP with 1 mg/mL Con A. However, the effect of 2-and 4-MMNPs on Con A was found to be nominal (or negligible interaction with Con A). The overall results can be summarized as below. (a) 1-MMNP (α -mannosylated goldnanoparticles) show high binding affinities with Con A (b) interestingly, 3-MMNP (β -galactosylated MMNPs) showed even though low still considerable

interactions with Con A (c) 2-MMNP (β -glucosylated goldnanoparticles) showed negligible interaction and 4-MMNP (β -glucosaminylated goldnanoparticles) showed extremely low interactions with Con A. These results can be shown in a plot (Figure 49).

Figure 49: Interaction of different carbohydrates with Con A: Results of fluorescence measured after each MMNP (1, 2, 3 and 4-MMNP) was diluted with PBS buffer 5 and added with 5 μ L of Con A (1mg/mL) and incubated for 1 hr. In the plot I shows interaction of 4-MMNP with Con A (with 61 a.u. as highest intensity); II shows interaction of 2-MMNP with Con A (with 10 a.u. as highest intensity); III shows interaction of 3-MMNP with Con A (with 331 a.u. as highest intensity); IV shows interaction of 1-MMNP with Con A (with 615 a.u. as highest intensity); Note: highest intensity measured from the blank (Fb-MMNP in PBS buffer 5 without Con A) intensity.

Then we intend to apply this system to hybrid nanoparticle system. That is, goldnanoparticles grafted with more than one carbohydrate. The advantages of employing dis-modified goldnanopartilces to study the carbohydrate-lectin interactions

are (a) dis-functionalized or modified goldnanoparticles mimic natural cell systems (cells contain more than one sugar on their surface) hence it would be advantageous to understand lectin-sugar interactions thoroughly and the investigation results will be more accurate (b) in this case concentration of the lectin assists to estimate the amount of different carbohydrates adsorbed on each DMNP which in turn useful in estimating the degree of carbohydrate presence on a cell.

2.9 Investigations on DMNP and Con A interactions:

We have employed 3 kinds of mannose-glucose DMNPs. They are (a) 1:1 mannose-glucose DMNP (1:1-1-DMNP) (b) 1:5 mannose-glucose DMNP and (1:5-1-DMNP) (c) 1:10 mannose-glucose DMNP (1:10-1-DMNP). DMNPs are synthesized in such a way that the total amount of carbohydrates while incubating with goldnanoparticles kept uniform or constant. For example 1:1 mannose-glucose DMNP contains 16 μL of mannose (with concentration ca. 97 mM) and 16 μL of glucose (with concentration of ca. 97 mM). 1:5 mannose-glucose DMNP contains 5.3 μL of mannose (with concentration ca. 97 mM) and 26.6 μL of glucose (with concentration of ca. 97 mM). 1:10 mannose-glucose DMNP contains 2.9 μL of mannose (with concentration ca. 97 mM) and 29 μL of glucose (with concentration of ca. 97 mM).

In the first experiment, 3 mg/mL Con A was employed to investigate the Con A-1:1-1-DMNP interactions. To 100 μL of Fb-1:1-1-DMNP solution in buffer 5 added 5 μL of Con A solution (3.12×10^{-5} M). Then, fluorescence was measured which showed, highest intensity (431 a.u.) at 514 nm after 60 mins. Similarly, fluorescence intensity for 2.08×10^{-5} M and 1.04×10^{-5} M Con A concentrations showed heist intensity peaks at 171 and 116 a.u (after 60 mins) respectively (Figure 50).

Figure 50: Fluorescence intensity for interactions between 1:1-1-DMNP and different concentrations of Con A. **1**-concentration of Con A 1.04×10^{-5} M ; **2**- concentration of Con A 2.08×10^{-5} M and **3**- concentration of Con A 3.12×10^{-5} M. Note that increase in intensity calculated from blank.

Figure 51: Fluorescence intensity for interactions between 1:5-1-DMNP and different concentrations of Con A. **1**-concentration of Con A 1.04×10^{-5} M ; **2**- concentration of Con A 2.08×10^{-5} M and **3**- concentration of Con A 3.12×10^{-5} M. Note that increase in intensity calculated from blank.

In the next experiment, we have utilized 1:5-1-DMNP. First, 3 mg/mL Con A was employed to investigate the Con A-1:5-1-DMNP interactions. To 100 μL of Fb-1:5-1-DMNP solution in buffer 5 added 5 μL of Con A solution (3.12×10^{-5} M). Then, fluorescence was measured which showed, highest intensity (295 a.u.) at 514 nm after 60 mins. Similarly, fluorescence intensity for 2.08×10^{-5} M and 1.04×10^{-5} M Con A concentrations showed heist intensity peaks at 116 and 85 a.u (after 60 mins) respectively (Figure 51).

Finally, we have employed 1:10-1-DMNP to test the interactions with Con A. As described in the above experiments, initially, 3 mg/mL Con A was employed to investigate the Con A-1:10-1-DMNP interactions. To 100 μL of Fb-1:10-1-DMNP solution in buffer 5 added 5 μL of Con A solution (3.12×10^{-5} M). Then, fluorescence was measured which showed, highest intensity (311 a.u.) at 514 nm after 60 mins. Similarly, fluorescence intensity for 2.08×10^{-5} M and 1.04×10^{-5} M Con A

Figure 52: Fluorescence intensity for interactions between 1:10-1-DMNP and different concentrations of Con A. **1**-concentration of Con A 1.04×10^{-5} M ; **2**-concentration of Con A 2.08×10^{-5} M and **3**- concentration of Con A 3.12×10^{-5} M. Note that increase in intensity calculated from blank.

concentrations showed highest intensity peaks at 132 and 109 a.u (after 60 mins) respectively (Figure 52).

Fluorescence intensity tendency from 1:1 to 1:10-1-DMNP revealed some interesting phenomenon. The fluorescence tendency from 1:1 to 1:5 is normal. In this case the fluorescence intensity decreases as expected because of the decrease of mannose concentration on DMNP. In the next case, to our surprise, the tendency from 1:5 to 1:10 has reversed. It was expected that, due to extremely low concentration of mannose on 1:10-DMNP, the fluorescence intensity should be lower than that of 1:5-DMNP. However, fluorescence intensity has been increased (for 3mg/mL concentration intensity has been increased from 295 to 311 a.u). This phenomenon is quite unusual and need to do several molecular level experiments to understand the complex interactions between mannose and Con A.

Next we intend to investigate the interactions between mannose-glucosamine grafted hybrid system with Con A. We have employed 3 kinds of mannose-glucosamine DMNPs. They are (a) 1:1 mannose-glucosamine DMNP (1:1-2-DMNP) (b) 1:5 mannose-glucosamine DMNP (1:5-2-DMNP) and (c) 1:10 mannose-glucosamine DMNP (1:10-2-DMNP). Mannose-glucosamine DMNPs are synthesized in such a way that the total amount of carbohydrates while incubating with goldnanoparticles kept uniform or constant. The ratios are similar to that of mannose-glucose DMNPs. 1:1 mannose-glucose DMNP contains 16 μ L of mannose (with concentration ca. 97 mM) and 16 μ L of glucose (with concentration of ca. 97 mM). 1:5 mannose-glucose DMNP contains 5.3 μ L of mannose (with concentration ca. 97 mM) and 26.6 μ L of glucose (with concentration of ca. 97 mM). 1:10 mannose-glucose DMNP contains 2.9 μ L of

mannose (with concentration ca. 97 mM) and 29 μL of glucose (with concentration of ca. 97 mM).

Figure 53: Fluorescence intensity for interactions between 1:1-2-DMNP and different concentrations of Con A. **1**-concentration of Con A 1.04×10^{-5} M; **2**- concentration of Con A 2.08×10^{-5} M and **3**- concentration of Con A 3.12×10^{-5} M. Note that increase in intensity calculated from blank.

In the first experiment, similar to mannose-glucose DMNP, 3 mg/mL Con A was employed to investigate the Con A-1:1-2-DMNP interactions. To 100 μL of Fb-1:1-1-DMNP solution in buffer 5 added 5 μL of Con A solution (3.12×10^{-5} M). Then, fluorescence was measured which showed, highest intensity (412 a.u.) at 514 nm after 60 mins. Similarly, fluorescence intensity for 2.08×10^{-5} M and 1.04×10^{-5} M Con A concentrations showed highest intensity peaks at 173 and 97 a.u (after 60 mins) respectively (Figure 53).

In the second experiment, 1:5-2-DMNPs were employed and calculated the Con A-1:5-2-DMNP interactions by initially using 3 mg/mL Con A. To 100 μL of Fb-1:5-2-

DMNP solution in buffer 5 added 5 μL of Con A solution (3.12×10^{-5} M). Then, fluorescence was measured which showed, highest intensity (283 a.u.) at 514 nm after 60 mins. Similarly, fluorescence intensity for 2.08×10^{-5} M and 1.04×10^{-5} M Con A concentrations showed highest intensity peaks at 123 and 86 a.u (after 60 mins) respectively (Figure 54).

Figure 54: Fluorescence intensity for interactions between 1:5-2-DMNP and different concentrations of Con A. **1-**concentration of Con A 1.04×10^{-5} M; **2-** concentration of Con A 2.08×10^{-5} M and **3-** concentration of Con A 3.12×10^{-5} M. Note that increase in intensity calculated from blank.

Finally in the third fluorescence experiment, 1:10-2-DMNPs were employed and explored the Con A-1:10-2-DMNP interactions by using 3 mg/mL Con A. To 100 μL of Fb-1:10-2-DMNP solution in buffer 5 added 5 μL of Con A solution (3.12×10^{-5} M). Then, fluorescence was measured which showed, highest intensity (291 a.u.) at 514 nm after 60 mins. Similarly, fluorescence intensity for 2.08×10^{-5} M and 1.04×10^{-5} M Con A concentrations showed highest intensity peaks at 129 and 92 a.u (after 60 mins) respectively (Figure 55).

Figure 55: Fluorescence intensity for interactions between 1:10-2-DMNP and different concentrations of Con A. **1**-concentration of Con A 1.04×10^{-5} M; **2**-concentration of Con A 2.08×10^{-5} M and **3**- concentration of Con A 3.12×10^{-5} M. Note that increase in intensity calculated from blank.

We further surprised to notice that, the same pattern of results were repeated with both 1-DMNP and 2-DMNP in the case of tendency from 1:5 to 1:10. In the case of 2-DMNP, fluorescence tendency from 1:1 to 1:5 is usual and decreases as expected because of the decrease of mannose concentration on 2-DMNP. In the next case, with 2-DMNPs also we found the same phenomenon has repeated. The tendency from 1:5 to 1:10 has changed and it was expected that, due to extremely low concentration of mannose and high concentration of glucosamine on 1:10-2-DMNP, the fluorescence intensity should be lower than that of 1:5-2-DMNP. However, fluorescence intensity has been increased (for 3mg/mL concentration intensity has been increased from 283 to 291 a.u).

Figure 56: Fluorescence intensity response curves (each curve is obtained by plotting different concentrations 1:0, 1:1, 1:5 and 1:10) of 2-DMNPs with different concentrations of Con A. **1**-Con A concentration 1.04×10^{-5} M and **2**- 2.08×10^{-5} M (after 30 mins of time).

Our aim is to determine the percentage of each type on carbohydrate present on each MMNP or DMNP or unknown cell system or scaffold. It is highly beneficial to measure the amount of particular carbohydrate on a cell system (or artificial cell system such as carbohydrate encapsulated nanoparticles/quantum dots which mimic natural cell system) in understanding molecular level cellular communication and interaction. Our system (turnoff/turn on system) may provide ample information of carbohydrate percentage on each scaffold. For example, for an unknown DMNP (that has unknown percentage of mannose) when treated with known Con A concentration, it is feasible to calculate the percentage of mannose on 2-DMNP. As shown in Figure 56 the corresponding slopes of 2-DMNP with 2.08×10^{-5} M Con A are different from the corresponding slopes of 2-DMNP with 1.04×10^{-5} M Con A concentration. Unknown concentration of DMNP would give a specific intensity. The line drawn from this particular intensity on corresponding 2.08×10^{-5} M curve would give the

percentage of mannose on that DMNP. In case, if this specific intensity provides two values on 2.08×10^{-5} M curve, then we can measure the intensity of unknown DMNP mannose percentage by using other concentration of Con A for example, 1.04×10^{-5} M, which will also provide two values on corresponding DMNP curve (Figure 55). It is important to note that in this system, change in variation accordingly with different concentration of Con A. Because of this, for unknown sample, only one value (of 1.04×10^{-5} M) would match with value on 2.08×10^{-5} M curve. This value is taken as correct value to calculate the percentage of mannose on DMNP. In summary, even though we end up with two values on high Con A concentration i.e., 2.08×10^{-5} M curve, we can detect the correct value which would in turn will give correct mannose percentage on 2-DMNP. In both concentration curves the mean variation differs with each other (for example mean variation for high concentration of Con A is higher than low concentration Con A). Hence, even though we end up with 2 values on corresponding curves, only one point will match with each other.

Later we tested 1-MMNP with different lectins to understand the interactions between mannose and lectins. We employed peanut agglutinin and triticum vulgaris. Both lectins did not show any response to 1-MMNP. To 100 μ L of Fb-1-MMNP solution in buffer 5 added 5 μ L of peanut agglutinin (PNA) (or triticum vulgaris, WGA) solution (1.04×10^{-5} M) and incubated for 1 hr. Then fluorescence was measured which showed a no improvement in intensity even after one hour (Figure 57).

Figure 57: Fluorescence intensity response when Fb-1-MMNP interacted with WGA, PNA and Con A. WGA and PNA did not show any response where as Con A showed excellent response after 1 hr.

2.10 Bioassay testing:

Biofunctionalized goldnanoparticles can deliver various molecules into the cell. We believe our Fb-1-MMNP system could be utilized to transfer fluorescence into the cell there by acting as fluorescence probe. Hence we have carried out some bio-assay experiments to investigate the behavior of Fb-1-MMNP system towards cell cultures.

Methods and experimental procedure:

Cell line culture. Jurkat human T cell lymphoma were purchased from American Type Cell Collection, ATCC (Rockville, MP). Jurkat was cultured in Roswell Park Memorial Institute Medium-1640 (RPMI) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) Penicillin/Streptomycin (PS) (Hyclone, Logan, UT). The cells were cultured in humidified 95% O₂/ 5% CO₂ atmosphere incubator at 37 °C. The cell culture solution was removed by centrifugation and washed by using PBS buffer 5 for

3 times. Then cells were dissolved in 5×RPMI medium. We have used diluted RPMI solution because at high concentration of RPMI goldnanopartilces are not stable. RPMI solution does not contain glucose moieties; hence it won't interrupt lectin mannose interactions. Cell cultures were visualized using Zeiss LSM 510 Meta confocal miscroscope. In the first step, cells were incubated with Fb-1-MMNP for 30 mins in glass bottom plate. Then after 30 mins confocal images were taken. Confocal images showed that the absorbance of fluorescein bornonic acid by the cells. This can be explained as, when cells were incubated with Fb-1-MMNP system, Con A present on the cell surface would replace fluorescein boronic acid and there will be binding between cell surface Con A and 1- MMNP. Further, the released free fluorescein boronic acid enters into the cell cytoplasm and lightens up the cell (at 454 nm) (Figure 58).

Figure 58: Multiplexed confocal analysis of Jurkat cells incubated with Fb-1-MMNP (A) Jurkat cells after incubating with Fb-1-MMNP under confocal at 454 nm (B) cell culture (c) overlay of cell culture with fluorescence.

To understand the bio-assay analysis and to confirm that fluorescence in the cells is due to Fb-1-MMNP complex, we performed one control experiment. In control

experiment we have employed only fluorescein-boronic acid ligand. Fluorescein-boronic acid ligand has been added to the cell culture (instead of Fb-MMNP) and observed the cells under confocal microscopy. However, there was no fluorescence in the cells, indicating that fluorescein boronic acid alone could not show any fluorescence effect on the cells (Figure 59). The combination of fluorescein boronic acid-1-MMNP (Fb-1-MMNP) could show fluorescence in the cells. This is because, we assume that when Fb-1-MMNP comes in contact with cell surface, Con A present on the cell surface may assist or cause Fb to enter cell. However, elaborate experiments and examinations have to be conducted to understand the intricate details of molecular mechanism. We just tried to show “proof of concept”. This strongly supports our strategy. Bio-assay results show that Fb-MMNP system act as delivery vehicle to transport fluorescein into the cell. Thus system could be a potential “fluorescent probe”. Further studies are going on in our laboratory to study more extensively about this bio-sensor system.

Figure 59: Multiplexed confocal analysis of Jurkat cells incubated with fluorescein boronic acid ligand only (A) Jurkat cells after incubating with fluorescein boronic acid ligand and cells under confocal at 454 nm (B) cell culture (c) overlay of cell culture in which no fluorescence is noticed.

3. CONCLUSION

Goldnanoparticles functionalized with carbohydrates mimic natural cell systems and act as multivalent models which can exert multivalent interactions with lectins. We designed a novel “**turn-off**” and “**turn-on**” biosensor system to explore carbohydrate-lectin interactions. We have synthesized several mono-modified goldnanoparticles (MMNPs) and combined with fluorescein boronic acid ligand (Fb-MMNP complex) and exploited the characteristic feature of carbohydrate-boronic acid binding. Due to the intrinsic properties of goldnanoparticles, the fluorescence intensity of Fb-MMNP complex has been quenched (**turn-off**). Upon the addition of lectin to Fb-MMNP system, fluorescence intensity again increases (**turn-on**). The strong specificity and affinity of monosaccharides (MMNP in this case) towards lectins replaces fluorescein boronic with lectin. Thus the removed fluorescein boronic acid increases the fluorescence intensity again. Mannosylated goldnanoparticles (1-MMNP) showed high affinity and specificity towards Concanavalin A (Con A). The most striking feature of this bio-sensor system is limit of detection which is 4.9 nM. This sensitivity value is ahead of most reported bio-sensor systems and could be comparable with the best bio-sensor systems. To our surprise, β -galactose encapsulated goldnanoparticles (3-MMNP) showed considerable affinity with Con A. We also synthesized hybrid carbohydrate functionalized goldnanoparticles (dis-modified goldnanoparticles DMNP) and tested for lectin interactions. Fluorescence experiments with DMNP provided useful information, such as, to calculate the percentage of mannose on each DMNP. This could be highly beneficial in estimating the carbohydrate amount on the cell surface. We employed this system successfully on bio system. Bio-assay results showed that Fb-1-MMNP system could be employed as fluorescence delivery vehicle and fluorescent probe.

4. EXPERIMENTAL

All reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise stated. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010–0.063 mm). Chromatograms were visualized by staining using base solution of potassium permanganate. Technical grade solvents were used for chromatography and distilled prior to use. NMR spectra were recorded at room temperature on 400 MHz Bruker DPX 400 spectrometers. The residual solvent signals were taken as the reference (7.26 ppm and 4.78 ppm for ^1H NMR spectra and 77.0 ppm and 49.1 ppm for ^{13}C NMR spectra in CDCl_3 and methanol- d_4 respectively. Chemical shift (δ) is reported in ppm, coupling constants (J) are given in Hz. The following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved. LCMS (ESI) spectra were recorded on a Waters Q-Tof premierTM mass spectrometer.

2-[2-(2-Chloroethoxy)ethoxy]ethyl, 2,3,4,6-tetraacetate- α -D-mannopyranoside:

6.39 mL of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (7.2 g, 5 eq, 51.2 mmol) was added drop wise to a solution of 2-[2-(2-chloroethoxy)ethoxy]ethanol (2.2 mL, 1.5 eq, 15.3 mmol) and 1,2,3,4,6-peracetylated mannose (4g, 1eq, 10.2 mmol) in dry dichloromethane (20 mL) stirring at 0 °C. Later the mixture was allowed to come to room temperature and stirred at room temperature for 24 hrs. Then the reaction mixture was extracted with 30 mL of dichloromethane, washed with sat. NaHCO_3 (2×20 mL) and dried over Na_2SO_4 . Then the crude product was purified by using column chromatography using (Hexane/DCM/Acetone=10:0.5:1) to furnish desired 2-[2-(2-chloroethoxy)ethoxy]ethyl, 2,3,4,6-tetraacetate- α -D-mannopyranoside. Yield = 2.4 g (40%) ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 1.20 s, 3H), 2.00 (s, 3H), 2.10 (s, 3H), 2.14 (s, 3H), 3.16-3.66 (m, 10H), 4.05-4.11 (m, 2H), 4.28 (dd, J = 4.5, 11.8 Hz, 1H), 4.86 (s, 1H), 5.25-5.30 (m, 2H), 5.34 (dd, J = 3.6, 10.0 Hz, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.6, 20.7, 20.7, 20.9, 42.7, 62.4, 66.1, 67.4, 68.4, 69.0, 69.6, 70.0, 70.6, 70.7, 71.4, 76.7, 77.0, 77.3, 97.7, 169.7, 169.9, 170.0, 170.7 ppm. LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{20}\text{H}_{31}\text{ClO}_{12}\text{Na}$ 521.90 Found 521.72.

2-[2-(2-Acetylthio)ethoxy]ethyl, 2,3,4,6-tetraacetate- α -D-mannopyranoside:

2-[2-(2-Chloroethoxy)ethoxy]ethyl, 2,3,4,6-tetraacetate- α -D-mannopyranoside (1.3 g, 2.61 mmol) and potassium thioacetate (0.89 g, 7.83 mmol) were taken in round bottom flask and dissolved in 20 mL of dry DMF and the contents were heated at 45 °C for 24 hrs. Later, the reaction mixture was diluted with ethyl acetate (50 mL) and washed with water (2×20 mL), NaHCO₃ (1×20 mL) and finally with brine solution (1×20 mL). The organic layer was dried over Na₂SO₄ and the crude product was purified by column chromatography using Hexane/EtOAc (1:1) as eluent. Yield 1.2g (85%); ¹H NMR (400 MHz, CDCl₃, 25 °C) δ : 1.91 (s, 3H), 1.98 (s, 3H), 2.04 (s, 3H), 2.10 (s, 3H), 2.30 (s, 3H), 3.04 (t, *J* = 6.3 Hz, 2H), 3.53-3.63 (m, 9H), 3.73-3.80 (m, 1H), 4.04 (d, *J* = 11.6 Hz, 1H), 4.24 (dd, *J* = 4.8, 12.0 Hz, 1H), 4.82 (s, 1H), 5.21-5.25 (m, 2H), 5.30 (dd, *J* = 3.6, 10 Hz, 1H) ppm; ¹³C NMR (100 MHz, CDCl₃) δ : 20.6, 20.6, 20.7, 20.8, 28.8, 30.5, 62.4, 66.1, 67.3, 68.3, 69.0, 69.5, 69.8, 70.0, 70.3, 70.6, 76.8, 77.1, 77.4, 97.7, 169.7, 169.8, 169.9, 170.6, 195.4 ppm. LC-MS *m/z* [M+Na]⁺ Calcd for C₂₂H₃₄O₁₃SNa 561.56 Found 561.25. HRMS (ESI) *m/z*: Calcd for C₂₂H₃₅O₁₃S: 539.1798 [M+H]⁺; found: 539.1797.

2-[2-(2-Chloroethoxy)ethoxy]ethyl α -D-Mannopyranoside: **44**

In a round bottom flask 2-[2-(2-acetylthio)ethoxy]ethyl 2,3,4,6-tetraacetate- α -D-mannopyranoside (0.2 g, 0.37 mmol) was dissolved in 5 mL of methanol. To this NaOMe (0.03 g, 0.55 mmol) was added and stirred at room temperature for 30 mins. It is important to note that in this reaction small amounts of disulfides were also formed. As the reaction time proceeds, due to the air oxidation, the amount of disulfides increases. Yields were not calculated for this reaction. The solvent was evaporated and the crude compound (includes minute amount of disulfide) was purified by column chromatography by using EtOAc/MeOH (10:0.2) to get almost pure 2-[2-(2-chloroethoxy)ethoxy]ethyl α -D-mannopyranoside. Compounds were formed in this reaction in high yields (80%). ^1H NMR (400 MHz, MeOD, 25 °C) δ : 2.72 (t, J = 6.0 Hz, 2H), 3.64-3.78 (m, 13 H), 3.87-3.91 (m, 3H), 4.86 (s, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 23.2, 61.5, 66.4, 67.2, 69.8, 70.0, 70.1, 70.7, 71.2, 72.7, 73.2, 110.3 ppm; LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{12}\text{H}_{24}\text{O}_8\text{SNa}$ 351.37 Found 351.17. HRMS (ESI) m/z : Calcd for $\text{C}_{12}\text{H}_{25}\text{O}_8\text{S}$: 329.1270 $[\text{M}+\text{H}]^+$; found: 329.1277.

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranose: **58**

A solution of methylamine in methanol (2 M, 4 ml) was added to a solution of 2-Acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy- α -D-glucopyranose (1.62 g, 4.2 mmol) in THF (20 mL) at room temperature and the mixture was stirred for 2 hr. The mixture was concentrated *in vacuo* and the residue was purified by flash column on silica gel (EtOAc/CH₂Cl₂=1:1 to 3:1) to give the desired compound as a colorless oil (1g, 70%). ¹H NMR (400 MHz, CDCl₃, 25 °C) δ : 1.94 (s, 3H), 1.99 (s, 3H), 2.00 (s, 3H), 2.01 (s, 3H), 4.06-4.11 (m, 1H), 4.17-4.27 (m, 3H), 5.07-5.12 (m, 2H), 5.21 (t, *J* = 3.7 Hz, 1H), 5.27 (t, *J* = 10.1 Hz, 1H), 6.10 (d, *J* = 9.4 Hz, 1H) ppm; ¹³C NMR (100 MHz, CDCl₃) δ : 20.6, 20.7, 23.0, 52.3, 62.1, 67.3, 69.3, 71.0, 91.4, 169.5, 170.6, 171.0, 171.4 ppm.

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranosyl trichloroacetimidate:

To a stirred solution of 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranose (0.98g, 2.8 m.mol) in dry dichloromethane (15 mL) were added trichloroacetonitrile (1:12 mL, 11.2 mmol) and DBU (0.08 mL, 0.56 mmol). The reaction mixture was stirred for 3 hrs at room temperature. The crude compound was then concentrated and purified by flash column chromatography on silica gel (EtOAc/hexane =1:1) to give imidate **59** as a light yellow solid (1 g, 75%). ¹H NMR (400 MHz, CDCl₃, 25 °C) δ : 1.93 (s, 3H), 2.04-2.09 (s, 9H), 4.08-4.15 (dd, *J* = 4.8, 12.9 Hz, 1H), 4.55 (ddd, *J* = 9.8, 9.8, 3.6 Hz, 1H), 5.21-5.35 (m, 2H), 5.66 (d, *J* = 9.0 Hz, 1H), 6.36 (d, *J* = 3.6 Hz,

1H), 8.79 (br, s, 1H)ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.6, 20.7, 23.0, 51.6, 61.4, 67.3, 70.2, 90.8, 94.8, 160.3, 169.1, 170.0, 170.6, 171.6 ppm.

2-Acetamido-2-[2-(2-chloroethoxy)ethoxy]ethyl 3,4,6-triacetate- β -D-glucopyranoside:

To a stirred solution of 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranosyl trichloroacetimidate (1 eq, 1g, 2.03 mmol) and 2-[2-(2-chloroethoxy)ethoxy]ethanol (1.5 eq, 0.44 mL, 3.04 mmol) in dry dichloromethane (10 mL) was added $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (1 eq, 0.25 mL, 2.03 mmol). The reaction mixture was stirred for 30 mins at room temperature. When the reaction is completed, the reaction mixture was evaporated. The crude compound was purified by column chromatography using hexane/ CH_2Cl_2 /acetone = 10:0.5:1 as eluent to afford pure compound. Yield=0.63 g (62%); ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 1.92 (s, 3H), 1.96 (s, 3H), 1.97 (s, 3H), 2.03 (s, 3H), 3.56-2.72 (m, 10H), 3.76-3.86 (m, 4H), 4.03-4.08 (m, 2H), 4.20 (dd, J = 4.8, 12 Hz, 1H), 4.75 (d, J = 8.8 Hz, 1H), 5.04 (q, J = 9.6 Hz, 2H), 6.45 (d, J = 9.2 Hz, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.6, 20.6, 20.7, 23.1, 42.8, 53.9, 62.2, 68.5, 68.5, 68.6, 70.3, 70.5, 71.2, 71.7, 73.3, 76.8, 77.1, 77.4, 101.9, 169.3, 170.5, 170.7, 170.8 ppm; LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{20}\text{H}_{32}\text{ClNO}_{11}\text{Na}$ 520.90 Found 520.83. HRMS (ESI) m/z : Calcd for $\text{C}_{20}\text{H}_{33}\text{O}_{11}\text{ClN}$: 498.1742 $[\text{M}+\text{H}]^+$; found: 498.1745.

2-Acetamido-2-[2-(2-acetylthio)ethoxy]ethyl 3,4,6-tetraacetate- β -D-glucopyranoside:

61

Same procedure is followed as described for the synthesis of **43**; Yield (66%); ^1H NMR (400 MHz, CDCl_3 , 25 $^\circ\text{C}$) δ : 1.91 (s, 3H), 1.95 (s, 6H), 2.03 (s, 3H), 2.30 (s, 3H), 3.02-3.12 (m, 2H), 3.54-3.68 (m, 10H), 3.69-3.80 (m, 2H), 3.81-3.83 (m, 2H), 4.02-4.09 (m, 2H), 4.21 (dd, $J = 4.8, 12.2$ Hz, 1H), 4.74 (d, $J = 8.4$ Hz, 1H), 5.05 (q, $J = 9.2$ Hz, 2H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.6, 20.6, 20.7, 23.1, 28.3, 30.5, 33.9, 62.2, 68.6, 69.7, 70.1, 70.3, 71.6, 71.7, 73.2, 76.7, 77.1, 77.4, 101.9, 169.3, 170.4, 170.7, 170.8, 195.3 ppm; LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{22}\text{H}_{35}\text{NO}_{12}\text{Na}$ 560.57 Found 560.18. HRMS (ESI) m/z : Calcd for $\text{C}_{22}\text{H}_{36}\text{O}_{11}\text{SN}$: 522.2009 $[\text{M}+\text{H}]^+$; found: 522.2004.

2-[2-(2-Chloroethoxy)ethoxy]ethyl 2-acetamido-2-deoxy- β -D-glucopyranoside:

62

Same procedure is followed as described for the synthesis of **44**; Yield (78%); ^1H NMR (400 MHz, CDCl_3 , 25 $^\circ\text{C}$) δ : 2.00 (s, 3H), 2.70 (dd, $J = 6.4, 14.0$ Hz, 2H), 3.00 (br s, 1H), 3.34 (d, $J = 8.8$ Hz, 1H), 3.56-3.83 (m, 14 H), 3.91 (br s, 1H), 3.93-3.94 (m,

1H), 4.56 (d, $J = 8.0$ Hz, 1H), 4.92 (br s, 1H), 5.38 (br s, 1H), 7.35 (br s, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 23.3, 24.0, 56.3, 61.5, 68.9, 70.1, 70.3, 70.4, 70.7, 72.9, 75.0, 75.7, 101.6, 172.6 ppm; LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{14}\text{H}_{27}\text{NO}_8\text{Na}$ 592.43 Found 592.16. HRMS (ESI) m/z : Calcd for $\text{C}_{14}\text{H}_{28}\text{O}_8\text{SN}$: 370.1536 $[\text{M}+\text{H}]^+$; found: 370.1543.

2,3,4,6-Tetra-O-acetyl- α -D-glucopyranose:

Same procedure is followed as described for the synthesis of **58**; 80% yield; ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 2.01 (s, 3H), 2.03 (s, 3H), 2.08 (s, 3H), 2.09 (s, 3H), 3.44 (br s, 1H), 4.11-4.14 (m, 1H), 4.21-4.28 (m, 2H), 4.89 (dd, $J = 3.6, 9.9$ Hz, 1H), 5.08 (t, $J = 9.9$ Hz, 1H), 5.45-5.46 (m, 1H), 5.53 (t, $J = 9.9$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.6, 20.7, 61.9, 67.2, 68.5, 69.8, 71.0, 90.1, 169.6, 170.1, 170.2, 170.8 ppm.

Synthesis 2,3,4,6-tetra-O-acetyl - α -D-glucopyranosyl trichloroacetimidate:

Same procedure is followed as described for the synthesis of **59**; (86%). ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 2.01 (s, 3H), 2.02 (s, 3H), 2.04 (s, 3H), 2.07 (s, 3H), 4.12 (dd, $J = 1.7, 12.3$ Hz, 1H), 4.19-4.22 (m, 1H), 4.26 (dd, $J = 3.9, 12.3$ Hz, 1H), 5.12 (dd, $J = 3.9, 10.0$ Hz, 1H), 5.17 (t, $J = 10.0$ Hz, 1H), 5.55 (t, $J = 10.0$ Hz, 1H), 6.55 (d, $J = 3.6$ Hz, 1H), 8.69 (br s, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.4, 20.5, 20.6, 61.3, 67.8, 69.7, 69.8, 70.0, 90.6, 92.9, 160.8, 169.5, 169.8, 170.0, 170.5 ppm.

2-[2-(2-Chloroethoxy)ethoxy]ethyl, 2,3,4,6-tetraacetate- β -D-glucopyranoside:

Same procedure is followed as described for the synthesis of **60**; 46% yield; ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 1.95 (s, 3H), 1.97 (s, 3H), 1.99 (s, 3H), 2.00 (s, 3H), 3.60-3.62 (m, 8H), 3.65-3.73 (m, 4H), 3.87-3.92 (m, 1H), 4.10 (dd, $J = 2.4, 12.0$ Hz, 1H), 4.21 (dd, $J = 4.8, 12.4$ Hz, 1H), 4.57 (d, $J = 8.0$ Hz, 1H), 4.94 (dd, $J = 8.0$ Hz, 1H), 5.03 (t, $J = 9.6$ Hz, 1H), 5.16 (t, $J = 9.6$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.5, 20.6, 20.6, 20.7, 42.7, 61.9, 68.4, 69.0, 70.4, 70.6, 71.2, 71.3, 71.7, 72.8, 76.7, 77.1, 77.4, 100.8, 169.3, 169.4, 170.2, 170.6 ppm; LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{20}\text{H}_{31}\text{ClO}_{12}\text{Na}$ 521.90 Found 521.15. HRMS (ESI) m/z : Calcd for $\text{C}_{20}\text{H}_{32}\text{O}_{12}\text{Cl}$: 499.1582 $[\text{M}+\text{H}]^+$; found: 499.1584.

2-[2-(2-Thioacetyl)ethoxy]ethyl, 2,3,4,6-tetraacetate- β -D-glucopyranoside:

Same procedure is followed as described for the synthesis of **43**; 86% yield; ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 1.97 (s, 3H), 1.20 (s, 3H), 2.01 (s, 3H), 2.05 (s, 3H), 2.31 (s, 3H), 3.10 (t, $J = 6.5$ Hz, 2H), 3.54-3.62 (m, 8H), 3.65-3.71 (m, 1H), 3.73 (dd, $J = 4.0, 6.8$ Hz, 1H), 3.90-3.93 (m, 1H), 4.10 (dd, $J = 2.4, 12.2$ Hz, 1H), 4.23 (dd, $J = 4.8, 12.4$ Hz, 1H), 4.60 (d, $J = 8.0$ Hz, 1H), 4.96 (dd, $J = 8.0, 9.5$ Hz, 1H), 5.10 (t, $J = 9.7$ Hz, 1H), 5.17 (d, $J = 9.6$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.5, 20.5, 20.6, 20.7, 28.7, 30.4, 61.9, 68.3, 68.7, 69.7, 70.2, 20.3, 20.5, 71.2, 71.7, 72.7, 76.7, 77.0, 77.3, 100.7, 169.2, 169.3, 170.2, 170.6, 195.3 ppm; LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{22}\text{H}_{34}\text{O}_{13}\text{SNa}$ 561.56 Found 561.16. HRMS (ESI) m/z : Calcd for $\text{C}_{22}\text{H}_{35}\text{O}_{13}\text{S}$: 539.1798 $[\text{M}+\text{H}]^+$; found: 539.1796.

2-[2-(2-Chloroethoxy)ethoxy]ethyl β -D-glucopyranoside:

Same procedure is followed as described for the synthesis of **44**; 75% yield; ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 1.66 (t, $J = 8$ Hz, 1H), 2.70 (dd, $J = 6.4, 14.4$ Hz, 2H), 3.2

(br s, 1H), 3.31 (d, $J = 8.0$ Hz, 1H), 3.39 (t, $J = 8.0$ Hz, 1H), 3.49-3.76 (m, 1H), 3.83 (s, 2H), 3.99-4.02 (m, 1H), 4.35 (d, $J = 7.6$ Hz, 1H), 4.87 (br s, 1H), 4.96 (br s, 1H), 5.20 (br s, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 24.1, 61.5, 68.7, 69.4, 70.0, 70.2, 72.9, 73.3, 75.8, 76.3, 76.7, 77.0, 77.3, 103.0 ppm; LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{12}\text{H}_{24}\text{O}_8\text{SNa}$ 351.37 Found 351.13. HRMS (ESI) m/z : Calcd for $\text{C}_{12}\text{H}_{25}\text{O}_8\text{S}$: 329.1270 $[\text{M}+\text{H}]^+$; found: 329.1271.

2-[2-(2-Chloroethoxy)ethoxy]ethyl, 2,3,4,6-tetraacetate- β -D-galactopyranoside:

Same procedure is followed as described for the synthesis of **60**; 52% yield; ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 1.98 (s, 3H), 2.04 (s, 3H), 2.05 (s, 3H), 2.14 (s, 3H), 3.62-3.69 (m, 7H), 3.72-3.78 (m, 3H), 3.89-3.98 (m, 2H), 4.10-4.19 (m, 2H), 4.57 (d, $J = 8.0$ Hz, 1H), 5.01 (dd, $J = 4.0, 10.4$ Hz, 1H), 5.20 (dd, $J = 8.0, 10.4$ Hz, 1H), 5.38 (d, $J = 3.2$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.5, 20.6, 20.7, 42.7, 61.2, 67.0, 68.9, 69.0, 70.3, 70.6, 70.6, 70.9, 71.3, 76.7, 77.1, 77.4, 101.3, 169.4, 170.1, 170.2, 170.3 ppm; LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{20}\text{H}_{31}\text{ClO}_{12}\text{Na}$ 521.90 Found 521.13.

2-[2-(2-Thioacetyl)ethoxy]ethyl, 2,3,4,6-tetraacetate- β -D-galactopyranoside:

Same procedure is followed as described for the synthesis of **43**; 85% yield; ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 1.90 (s, 3H), 1.97 (s, 3H), 1.99 (s, 3H), 2.07 (s, 3H), 2.27 (s, 3H), 3.03 (t, J = 4.0 Hz, 2H), 3.50-3.59 (m, 8H), 3.66-3.71 (m, 1H), 3.86-3.89 (m, 2H), 4.00-4.09 (m, 2H), 4.51 (d, J = 8.0 Hz, 1H), 4.95 (dd, J = 4.0, 10.8 Hz, 1H), 5.13 (dd, J = 8.0, 10.4 Hz, 1H), 5.30 (d, J = 3.6 Hz, 1H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 20.5, 20.6, 20.7, 20.9, 28.7, 30.5, 60.2, 61.2, 67.0, 68.8, 69.0, 69.7, 70.2, 70.3, 70.5, 70.6, 70.8, 76.8, 77.1, 77.4, 101.2, 169.3, 170.0, 170.1, 170.2, 195.3 ppm; LC-MS m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{22}\text{H}_{34}\text{O}_{13}\text{SNa}$ 561.56 Found 561.18.

2-[2-(2-Chloroethoxy)ethoxy]ethyl β -D-galactopyranoside:

Same procedure is followed as described for the synthesis of **44**; 78% yield; ^1H NMR (400 MHz, MeOD, 25 °C) δ : 2.78 (t, J = 4.0 Hz, 2H), 3.59-3.65 (m, 3H), 3.68-3.90 (m, 1H), 3.96 (d, J = 2.4 Hz, 1H), 4.11-4.16 (m, 1H), 4.38 (d, J = 7.2 Hz, 1H) ppm; ^{13}C NMR (100 MHz, MeOD) δ : 23.3, 47.3, 47.5, 47.8, 48.0, 48.2, 61.1, 68.2, 68.9, 69.7,

70.1, 70.1, 71.1, 72.7, 73.4, 75.2, 103.6 ppm; LC-MS m/z $[M+Na]^+$ Calcd for $C_{12}H_{24}O_8SNa$ 351.37 Found 351.16. HRMS (ESI) m/z : Calcd for $C_{12}H_{25}O_8S$: 329.1270 $[M+H]^+$; found: 329.1271.

5,5-Dimethyl-2-p-tolyl-1,3,2-dioxaborinane:

To a stirred solution of tolyl boronic acid (1 eq, 5.6 g, 41.1 mmol) in 50 mL of toluene was added 2,2-dimethyl-propane diol (1.05 eq, 4.49 g, 43.2 mmol). The reaction mixture containing round bottom flask was fitted with dean stark apparatus to collect H_2O formed in the reaction. Later the contents were refluxed for 12 hrs. When the reaction completed, solved was removed to get pure white color compound. (7.2 g, 86%). 1H NMR (400 MHz, $CDCl_3$, 25 °C) δ : 1H NMR (400 MHz, $CDCl_3$, 25 °C) δ : 0.89 (s, 6H), 2.25 (s, 3H), 3.60 (s, 4H), 7.06 (d, $J = 7.6$ Hz, 2H), 7.61 (d, $J = 7.6$ Hz, 2H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ : 21.7, 21.9, 31.9, 72.3, 128.4, 134.0, 140.7 ppm; LC-MS m/z $[M+H]^+$ Calcd for $C_{12}H_{18}BO_2$ 205.08 Found 205.15.

2-(4-(Bromomethyl)phenyl)-5,5-dimethyl-1,3,2-dioxaborinane:

To a stirred solution of **64** (1 eq, 5g, 24.5 mmol) in CCl_4 was added NBS (1.1 eq, 4.7 g, 26.9 mmol) and the contents for refluxed for 12 hrs. When the reaction is completed, solvent was removed and the crude compound purified by column chromatography by using hexane/EtOAc = 10:1 to give the desired compound (6.2 g, 89%). ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ : 1.01 (s, 6H), 3.76 (s, 4H), 4.49 (s, 2H), 7.37 (d, J = 8.0 Hz, 2 H), 7.77 (d, J = 8.0 Hz, 2H) ppm; ^{13}C NMR (100 MHz, CDCl_3) δ : 21.9, 31.8, 33.5, 72.3, 128.1, 134.3 ppm; LC-MS m/z $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{12}\text{H}_{17}\text{BBrO}_2$ 283.97 Found 283.76.

4-Fluoresceinmethyl-phenylboronic acid:

To a solution of fluorescein (1 eq, 0.1 g, 0.30 m.mol) in DMF was added 2-(4-(bromomethyl)phenyl)-5-5-dimethyl-1,3,2-dioxaborinane (2 eq, 0.164 g, 0.60 mmol) and K_2CO_3 (3 eq, 0.127 g, 0.92 mmol) and the contents were stirred at room temperature for 24 hrs. After the reaction time, the contents were poured in ice water. To it added 3 M HCl slowly until it reached pH 3 to 4. This acidic solution was stirred 30 mins at room temperature. Finally acidic solution was extracted with EtOAc (3× 20 mL) and the combined organic layers were dried over Na_2SO_4 and concentrated. Crude compound was purified by using hexane/EtOAc (1:1) as eluent. Yield = 0.75g, 52%; ^1H NMR (400 MHz, MeOD, 25 °C) δ : 4.58 (s, 2H), 5.19 (s, 2H), 6.57 (m, 2H), 6.89-

6.98 (m, 2H), 7.02 (s, 1H), 7.22-7.33 (m, 4H), 7.60-7.77 (m, 4H), 8.05-8.06 (m, 1H) ppm; 29.3, 70.8, 101.0, 103.0, 113.6, 125.4, 126.0, 127.1, 127.6, 129.3, 129.7, 130.8, 133.2, 154.1, 171.4 ppm; HRMS (ESI) m/z : Calcd for $C_{27}H_{20}O_7B$: 467.1320 $[M+H]^+$; found: 467.1306.

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CHAPTER 2

**Development of new promoters for
stereoselective green glycosylation**

PART A

Green glycosylation promoted by reusable biomass carbonaceous solid acid: An easy access to β -stereoselective terpene galactosides

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1. INTRODUCTION

Carbohydrates are integral part of glycolipids, glycoproteins and key constituents of cell membrane and extra-cellular matrix. They are closely associated with various biological processes such as cell-cell communication, cell adhesion, cell protein interaction and molecular recognition of antibodies and hormones¹⁻⁴. Glycosylation provides a unique platform to synthesize an array of carbohydrate derived novel therapeutic agents⁵⁻⁷. Glycosylation has gained much attention and witnessed a vast development ever since the historical development of Koenigs-Knorr method and its modified versions⁸. Over the past few decades, there has been significant amount of development in glycosyl donors⁹⁻¹⁴ and activators. Its essential to choose appropriate donor and employ suitable promoter to attain regio and stereo selective glycoside synthesis. Most common activating agents include strong and highly moisture sensitive Lewis acids such as $\text{BF}_3 \cdot \text{Et}_2\text{O}$,¹⁵ TBSOTf ,¹⁶ Tf_2O ,¹⁷ ZnBr_2 ,¹⁸ LiClO_4 ,¹⁹ LiOTf ,²⁰ AgOTf ²¹ and TMSOTf .²²⁻²⁵ These promoters require dry reaction conditions and most of the times reactions were carried out at low temperatures. Moreover due to the oxophilic nature of Lewis acid catalysts, stoichiometric amounts of catalytic loading is mandatory.

Most of the promoters furnish glycosides as α/β anomers. When using mineral and Lewis acid as catalysts, the final glycoside isolation requires aqueous work-up and neutralization steps, which subsequently results in the disposal of massive amounts of hazardous waste. Most of the times cost of the pollutants significantly greater than the cost of the product. The principle of “Green Chemistry” or “Clean Technology” emphasizes on the usage of environmentally benign catalysts/chemicals in order to have minimal deteriorate effects on the human-beings and environment.^{26, 27} As an alternative to homogenous catalysis, nowadays environmentally sustainable nontoxic solid acid catalysts with strong acidic sites became more prominent in organic transformations. Several solid acid catalysts have been reported in the literature such as high silica zeolites,^{28, 29} ion exchange resins^{30, 31} and

mesoporous materials.^{32, 33} Solid acid catalysts have got advantages^{26, 33-35} over liquid acids such as (a) no work-up procedures (b) easy separation and (c) easy recovery and reusability.

To date several silica and alumina supported solid acid catalysts have been developed and employed in glycosylation.^{36, 37} For example Toshima and coworkers performed glycosylation of mannopyranosyl sulfoxides and alcohols by using a sulfated zirconia as an activator.³⁸ The same group later reported the usage of montmorillonite K-10 as solid acid in glycosylation reactions.³⁹ Corma et al first reported the Fischer glycosylation by using zeolites.⁴⁰ Till today a wide variety of zeolites as heterogeneous catalysts have been employed in the glycosylation.⁴¹⁻⁴³ Unfortunately, many of the reported solid acids does not control the stereoselectivity at anomeric centre hence provide glycosides as anomeric mixture. More importantly most of the solid acids are mechanically unstable in various solvents, therefore practically they are not reusable.

In order to address the above mentioned practical difficulties and in our continuous effort to explore new glycosylation protocols⁴⁴⁻⁴⁸ herein we wish to describe glycosylation of various unprotected, unactivated monosaccharides and protected, activated glycosyl imidates promoted by sulfonated biomass carbonaceous material, which is inexpensive, moisture stable and environmentally benign with high catalytic performance. Most striking feature of this catalyst is that its remarkable reusable capacity and when employed with activated sugars it delivered desired *O*-glycosides with exclusive anomeric selectivity. In a process to practice green protocols, recently we reported Freidal crafts alkylation of indoles with the aid of sulfonated bio-mass chaocoal.⁴⁹ Incomplete carbonization and subsequent sulfonation of natural carbohydrates such as glucose and starch would result in the desired amorphous carbon solid acid catalyst.^{50, 51} The sulfonated carbonaceous solid acid material was synthesized according to given literature procedure.⁵¹ It consists of flexible polycyclic carbon sheets, which bear phenolic hydroxyl (-OH), carboxylic acid (-COOH) and sulfonic acid (-SO₃H) groups. Elemental analysis of solid acid revealed it's sulfur content, which was found to be

1.13%. Since all sulfur atoms in the carbon material were in the form of SO₃H groups, the density of SO₃H groups in solid acid was thus estimated to be 0.35 mmol/g. The solid acid was further characterized by X-ray diffraction (XRD). The XRD pattern is in good agreement with literature, showing that the prepared solid acid is in the form of amorphous carbon.

2. RESULTS AND DISCUSSION

To begin with, in our first set of experiments, we tried to test the catalytic efficiency of the solid acid by performing glycosylation on unprotected and unactivated sugars. Fischer first reported the acid catalyzed glycosylation of naked glucose with methanol to give methylglycoside.⁵² Glycosylation of unprotected alcohols^{53, 54} remains particularly challenging due to self-condensation, ring chain tautomerism and in-situ anomerization.⁵⁵ In a greener prospective, glycosylation of unprotected sugars particularly reduce the stodgy protection-deprotection steps thus leading to high atom economy and E-factor. Moreover direct synthesis of *O*-glycosides from *O*-unprotected donors accounts for (a) enhancing the reactivity (b) allows generating a different stereoselectivity at anomeric centre or controls the α/β selectivity and (c) making the process modifications with more ease by reducing the number of synthetic steps. Enzymatic methods such as glycosynthase⁵⁶ and transglycosydase⁵⁷ may accomplish this task but they draw on fluoro and nitro phenyl activated sugars respectively. A systematic study of glycosylation was undertaken by performing neat glycosylation between D-glucose (1 eq) and allyl alcohol (10 eq) to test the catalytic activity and performance. Glycosylation was sluggish when the reaction was carried out at room temperature. With 1 mol% of the catalytic loading, at room temperature neat glycosylation resulted in desired allyl glycoside (α/β = 66/34) in 26% yield. However, when the temperature has been raised to 80 °C, we were intrigued by the formation of the glycoside in 82 % yield (Table1, entry 2). A detailed probe into the optimization showed that the reaction time required for complete conversion of monosaccharide to corresponding glycoside is inversely proportional to the amount of **1a** loaded and temperature (Table1, entries 13). A series of experiments were conducted to compare the efficiency of solid acid catalyst against commonly used Lewis acid catalysts. Under the same conditions (5 mol %) Lewis acid catalyst and at 80 °C) glycosylation was carried out using the model substrates i.e., glucose and allyl alcohol. The results are summarized in table 1. It was observed that all the Lewis acids could not successfully converted the monosaccharide in to glycoside even after prolonged reaction times except

Part A Green glycosylation promoted by reusable biomass carbonaceous solid acid: An easy access to β -stereoselective terpene galactosides

Table 1 Catalytic efficiency of solid acid compared against various Lewis acids^a

Entry	Catalyst	Catalyst loading (mol%)	Temp. (°C)	Time (h)	Yield (%) ^b	α/β ratio ^c
1	Solid acid	1	25	24	26	73:27
2	Solid acid	1	80	10	82	73:27
3	Solid acid	5	80	8	84	73:27
4	ZnCl ₂	5	80	24	42	65:35
5	BF ₃ ·OEt ₂	5	80	24	71	63:37
6	FeCl ₃	5	80	24	24	69:31
7	AgOTf	5	80	24	-	-
8	Sc(OTf) ₂	5	80	24	49	73:27
9	InCl ₃	5	80	24	-	-
10	AuCl ₃	5	80	24	41	70:30
11	Yb(OTf) ₃	5	80	24	45	68:32

^a The reaction was conducted with 1 eq. of D-glucose and 10 eq. of allylic alcohol. ^b Isolated yields. ^c Determined by ¹H NMR.

BF₃OEt₂ which gave allyl glycoside (α/β = 63/37) in acceptable yields (71%). To our surprise AgOTf and InCl₃ proved to be inefficient candidates to carry out the glycosylation transformation (Table 1, entries 7 and 9). However, Lanthanide triflates such as Sc(OTf)₂ and Yb(OTf)₃ are able to furnish the allyl glycoside in moderate yields albeit with good selectivity (α/β = 73:27 and 68:32 respectively). This could be attributed to the oxophilicity of the corresponding metals and dearth of basicity and nucleophilicity of the triflates. This study clearly shows the preeminent catalytic efficiency of bio-mass solid acid over Lewis acids in terms of yields, reaction time and stereoselectivity when exerted with unprotected and unactivated sugars.

Part A Green glycosylation promoted by reusable biomass carbonaceous solid acid: An easy access to β -stereoselective terpene galactosides

Pleased by this result we began to probe the breadth of this catalytic application on various unprotected and unactivated monosaccharides. In the second set of the experiments, the scope of the neat glycosylation on various sugar moieties such as D-galactose, D-mannose, D-fructose, fucose, D-xylose, D-ribose, 2-deoxyribose, glucosamine, N-acetyl glucosamine was investigated. We chose allyl alcohol as aglycone because of the fact that, allyl glycosides find substantial applications in the synthesis of oligosaccharides, for example (a) reducing sugars were attained via the deprotection of anomeric *O*-allyl moiety on allyl glycoside at any length of the saccharide chain by using various reagents⁵⁸ (b) reducing sugars could be coupled with proteins to obtain corresponding glycoconjugates⁵⁹ (c) with the assistance of a spacer-arm, anomeric *O*-allyl group could be availed to synthesize glycoconjugates⁶⁰ and (d) asymmetric functionalization of *O*-allyl moiety paves a new way to synthesize enantiomerically pure compounds, thereby contributing to the asymmetric organic synthesis.⁶¹ Under the standard experimental conditions except glucosamine (Table 2, entry 8) all the monosaccharides furnished corresponding allyl glycosides in good to excellent yields (68-96%, Table 2, entries 5 to 13). As predicted, mannose gave allyl glycoside as pure α – diastereomer (Table 2, entry 10), but it is worthy of note that fucose, N-acetyl glucosamine and 2-deoxy-D-ribose also afforded the corresponding allyl glycosides as pure α – anomers (Table 2, entries 7, 9 and 13 respectively). It's important to note that all the glycosylation reactions are distinguished with the exclusive formation of thermodynamically stable pyranosides than the corresponding kinetically favored furanosides. We contemplate that the formation of the sole pyranoside could be explained on the basis of the general Fischer glycosylation mechanism, that is first formation of open chain hemiacetal, subsequent nucleophilic cyclization from C-4 hydroxyl group leads to the formation of kinetically favored glycofuranoside, which ultimately expands back to form thermodynamic *O*-allyl glycopyranoside. The selectivity could be attributed to the acidic sites on the solid acid and topology of the flexible polycyclic carbon sheets of solid acid catalyst. Meanwhile, galactose, fructose, xylose and ribose afforded respective allyl glycosides in good to excellent yields with

Table 2 Neat glycosylation of unprotected and unactivated glycosyl donor with acceptors^a

Entry	Glycosyl donor	Acceptor	Time (h)	Yield (%) ^b	ratio ^c
1			8	62	44:56
2			8	68	56:44
3			8	55	71:29
4			8	70	68:32
5			8	72	60:40
6			8	82	90:10
7			8	75	50:50
8			8	94	100:0
9			8	90	70:30
10			8	96	75:25
11			8	80	85:15

^a The reaction was conducted with 1 eq. of glycosyl donor and 10 eq. of glycosyl acceptor. ^b Isolated yields. ^c Determined by ¹H NMR.

good selectivities (Table 2, entries 5, 6, 11 and 12 respectively). Having synthesized various synthetically useful allyl glycosides we extended the scope of the catalytic activity by synthesizing long chain glycosides. In synthetic point of view, there has been enormous interest in alkylpolyglycosides (APG),⁶² which are a part of carbohydrate derived surfactants. APGs are known to play significant role food and cosmetic industry, as they are preferred candidates to serve as non-ionic surfactants for cosmetics and as food emulsifiers.⁶³ These factors encouraged us to synthesize n-butyl glycopyranoside and n-octyl glycopyranoside through direct glycosylation. Both the APGs were isolated in good yields with acceptable anomeric selectivities (Table 1, entries 2 and 3). Finally we embarked on checking the recyclable ability of the solid acid catalyst, which is the most crucial factor that reckons its practical utility while employing in glycosylation reactions. After recovering the carbonaceous solid acid *via* a simple filtration and removing the volatile solvents under vacuum, the catalyst was reused without further activation in catalyzing the reaction between unprotected mannose and allyl alcohol.

Figure 1 Catalytic performance of recovered solid acid.

To our delight glycosylation proceeded smoothly even after 6 runs, without any extension of reaction time or marked loss in yield (Figure 1). In each experiment mannose has maintained α -anomeric selectivity. Finally we scrutinized solid acid's catalytic ability in the glycosylation of glycosyl trichloroacetimides and its potential in delivering highly stereoselective glycosides. Due to the ease of synthesis, Schmidt's trichloroacetimides have been proven to be

the most popular among glycosyl donors.¹⁵ Many promoters have been reported to activate the glycosyl trichloroacetimides, which include the promoters that were mentioned earlier in this article. Some of the moisture stable promoters were also been reported such as acid washed 4 °A molecular sieves,⁶⁴ I₂/Et₃SiH,⁶⁵ HClO₄/silica,⁶⁶ Amberlyst-15⁶⁷ and some transition metal catalysts such as Pd(PhCN)₂(OTf)₂.⁶⁸ Most of the catalysts work well with this protocol but lack reusability and end up with curtail selectivities. In the fourth and final set of experiments, we began to investigate the catalytic efficacy of the solid acid by glycosylating glycosyl trichloroacetimide with various terpenoid alcohols to synthesize biologically active carbohydrate containing terpenoids and steroids. Nowadays the Diversity Oriented Synthesis (DOS) for synthesizing oxygen rich small molecules with definite stereochemistry has gained much attention.^{69, 70} Terpenes possess a significant amount of biological activity⁷¹ and synthesis of exclusive β selective terpene and steroidal galactose analogues are unknown. Synthesis of terpene glycosides generally relies on enzymatic methods.⁷² Due to their potential candidature as antimicrobial, anti-fungal, antiparasitic, antitoxigenic and anticancer agents we envisaged to synthesize β-terpene and steroidal galactosides with the aid of solid acid catalyst. Glycosylation between glycosyl trichloroacetimidate (1 eq) and citronellol (1.5 eq) in the presence of 5 mol% solid acid catalyst at 80 °C in toluene for 4 hrs afforded desired citronellol galactoside in excellent yield (82 %) with exclusive β anomeric selectivity (Table 3, entry 5). Encouraged by this result, we tried to construct glycosyl bond between various terpenes and galactose. Endo-fenchol, borneol and menthol gave the corresponding galactosyl derivatives in excellent yields with absolute stereo selectivity (Table 3, entries 1, 2 and 3). Pregnenolone, a steroid hormone involved in the steroidogenesis of progesterone, also successfully transformed into steroidal β galactoside (Table 3, entry 4). To broaden the substrate scope and to show the catalytic utility we have synthesized protected serine galactoside and bulky moieties containing galactosides such as adamantane and ferrocene (Table 3, entries 6 to 8). To corroborate solid acid's practical reusability as glycosylation promoter, we employed recycled catalyst in the above set of experiments. First recycled catalyst has been used with menthol

and pregnenolone, while second generated recycled catalyst used for the synthesis of citronellol, adamantane, ferrocene and Fmoc-Ser-OMe galactoside analogues.

Table 3 Glycosylation of glycosyl trichloroacetimidates with biologically reactive alcohols^a

Entry	Aglycone	Product	Time (h)	Yield (%) ^b
1			4	88
2			4	82
3 ^c			2	94
4 ^c			8	76
5 ^c			4	82
6 ^c			6	92
7 ^c			6	94
8 ^{c,d}			8	85

^a The reaction was conducted with 1 eq. of glycosyl donor and 1.5 eq. of acceptor. ^b Isolated yields. ^c Recovered catalyst was used. ^d Dichloromethane was used as solvent.

Part A Green glycosylation promoted by reusable biomass carbonaceous solid acid: An easy access to β -stereoselective terpene galactosides

3. CONCLUSION

In conclusion, we have demonstrated the remarkable catalytic efficiency of environmentally sustainable carbonaceous solid acid as glycosylating promoter. Various unprotected and unactivated glycosyl donors, which are usually prone to self-condensation and *in-situ* anomerization while performing the glycosylation, were successfully glycosylated to afford corresponding glycosides. This catalyst also employed for the glycosylation of glycosyl trichloroacetimides. Some of the salient features of the catalyst are (a) derived from naturally existing D-glucose hence quite economical to synthesize (b) air and moisture stable therefore easy to handle (c) reaction procedure is operationally simple which precludes the stringent and inert conditions; use of molecular sieves can be avoided (d) easy recovery of the catalyst upon completion of the reaction through simple filtration thereby avoiding hectic aqueous work ups and neutralization steps (e) high reusable capacity; catalyst is reusable up to 6 times while persisting the high catalytic activity (f) affords galactosides with exclusive β anomeric selectivity when used with glycosyl trichloroacetimides. With these advantages in hand, we strongly believe solid acid promoter could become an alternative to other glycosylating promoters. Its synthetic assets coupled with green principles could benefit the glyco-chemists in their regular laboratory usage.

4. EXPERIMENTAL

GENERAL

All reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise stated. Evaporation of organic solutions was achieved by rotary evaporation with a water bath temperature below 40 °C. Product purification by flash column chromatography was accomplished using silica gel 60 (0.010–0.063 mm). Chromatograms were visualized by staining using base solution of potassium permanganate. Technical grade solvents were used for chromatography and distilled prior to use. NMR spectra were recorded at room temperature on 400 MHz Bruker DPX 400 spectrometers. The residual solvent signals were taken as the reference (7.26 ppm and 4.78 ppm for ^1H NMR spectra and 77.0 ppm and 49.1 ppm for ^{13}C NMR spectra in CDCl_3 and methanol- d_4 respectively. Chemical shift (δ) is reported in ppm, coupling constants (J) are given in Hz. The following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved. HRMS (ESI) spectra were recorded on a Waters Q-ToF premierTM mass spectrometer. Elemental analysis was measured on Perkin Elmer Series II CHNS/O Analyzer 2400. Powder X-ray diffraction (XRD) was collected from Shimadzu 6000 diffractometer. Carbonization of D-glucose was carried out on Carbolite 1200 °C three zone tube furnace.

Preparation of carbon-based solid acid catalyst (1a)

D-Glucose powder (2 g) was heated at 400 °C for 15 h under N₂ flow to produce a black carbon solid. The solid was ground to fine powder and heated in 20 mL of conc. H₂SO₄ (>96%) at 150 °C under N₂. After heating for 15 h and then cooling to room temperature, the mixture was diluted with 100 mL of distilled water. The black precipitate was collected by filtration and washed repeatedly with hot distilled water (>80 °C) until pH 7 was observed in filtrate. The resulting black solid was then washed with methanol (30 mL) followed by diethyl ether (30 mL). It was further dried at 60 °C *in vacuo* prior to use. Elemental analysis revealed that sulfur content is 1.13 wt% which is equivalent to 0.35 mmol SO₃H per gram of catalyst.

Figure 1 XRD profile for the sulfonated carbon based solid acid

A typical procedure for neat glycosylation of monosaccharides with aglycones

To a mixture of monosaccharide (1 eq) and aglycone (10 eq) was added 5 mol% solid acid catalyst and the reaction mixture was heated at 80 °C and stirred for 8 hrs. When the monosaccharide is completely consumed (TLC monitored), the reaction mixture was diluted with ethyl acetate and subjected to centrifugation. After the centrifugation, the remaining supernatant solution was decanted and this process is repeated for 3 times. The combined organic solutions were removed under reduced pressure followed by purification of the residue by silica gel chromatography to give the desired glycosides. The remaining solid acid catalyst was dried under reduced pressure to remove all the volatile components, and then reused in the next run. The NMR data of all the glycosides is in accordance with the reported literature data.

A typical procedure for glycosylation of galactosyl trichloroacetimidates with acceptors

To a mixture of galactosyl trichloroacetimidate (1 eq) and acceptor (1.5 eq) in toluene (3 mL) was added 5 mol% solid acid catalyst and the reaction mixture was heated at 80 °C and stirred for appropriate amount of time (table 2) and the extent of reaction was monitored by TLC analysis. The reaction mixture was diluted with ethyl acetate and subjected to centrifugation. After the centrifugation, the remaining supernatant solution was decanted and this process is repeated for 3 times. The combined organic solutions were removed under reduced pressure followed by purification of the residue by silica gel chromatography to give the desired β -galactosides. The remaining solid acid catalyst was dried under reduced pressure to remove all the volatile components, and then reused in the next run.

Characterization of galactosides:

Fenchyl-2,3,4,6-tetraacetyl-*O*- β -D-galactopyranoside

R_f (50% EtOAc/Hexane) 0.46; $[\alpha]_D^{24}$ -7.3 ($c = 0.1$, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 5.37 (d, $J = 2.7$ Hz, 1H), 5.21 (dd, $J = 8.0, 10.3$ Hz, 1H), 5.00 (dd, $J = 3.3, 10.3$ Hz, 1H), 4.32 (d, $J = 8.0$ Hz, 1H), 4.09-4.14 (m, 2H), 3.85 (t, $J = 6.3$ Hz, 1H), 3.03 (s, 1H), 2.16 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.66-1.59 (m, 4H), 1.26-1.43 (m, 3H), 0.96 (s, 3H), 0.85 (s, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 170.5, 70.4, 170.3, 169.4, 103.0, 94.6, 71.0, 70.4, 69.2, 67.3, 61.7, 49.1, 48.0, 41.0, 39.2, 29.7, 26.1, 25.6, 21.6, 20.9, 20.7, 20.6, 19.4; IR (neat) $\nu_{\max}/\text{cm}^{-1}$ 1718, 1367, 1217, 1097; HRMS (ESI) m/z $[M + Na]^+$ calcd for C₂₄H₃₆O₁₀Na 507.2206, found 507.2200.

Bornyl-2,3,4,6-tetraacetyl-*O*- β -D-galactopyranoside

R_f (50% EtOAc/Hexane) 0.46; $[\alpha]_D^{24}$ -14.5 ($c = 0.1$, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 5.19 (t, $J = 9.4$ Hz, 1H), 5.08 (t, $J = 9.6$ Hz, 1H), 5.00 (dd, $J = 8.1, 10.5$ Hz, 1H), 4.45 (d, $J = 8.1$ Hz, 1H), 4.26 (dd, $J = 4.8, 12.1$ Hz, 1H), 4.11 (dd, $J = 2.5, 12.1$ Hz, 1H), 3.95 (d, $J = 9.4$ Hz, 1H), 3.63-3.66 (m, 1H), 2.00-2.17 (m, 12 H), 1.85-1.88 (m, 1H), 1.60-1.70 (m, 2H), 1.17-1.25 (m, 2H), 1.05-1.07 (m, 1H), 0.81-0.94 (m, 7H); ¹³C NMR (100 MHz, CDCl₃): δ 170.7, 170.4, 169.5, 169.2, 99.7, 84.1, 72.8, 71.6, 71.4, 68.8, 62.2, 49.1, 48.0, 44.9, 36.1, 28.3, 26.4, 20.7, 20.7, 20.6, 19.7, 18.9, 13.3. IR (neat) $\nu_{\max}/\text{cm}^{-1}$ 1755, 1367, 1222, 1039; HRMS (ESI) m/z $[M + Na]^+$ calcd for C₂₄H₃₆O₁₀Na 507.2206, found 507.2190.

Menthyl-2,3,4,6-tetraacetyl-*O*- β -D-galactopyranoside

R_f (50% EtOAc/Hexane) 0.40; $[\alpha]_D^{24}$ -240.2 (c = 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 5.20 (t, J = 9.5 Hz, 1H), 5.04 (t, J = 9.7 Hz, 1H), 4.93 (dd, J = 8.0, 9.6 Hz, 1H), 4.54 (d, J = 8.0 Hz, 1H), 4.18 (dd, J = 5.5, 12.0 Hz, 1H), 4.11 (dd, J = 2.7, 12.0 Hz, 1H), 3.67 (ddd, J = 2.7, 5.5, 9.9 Hz, 1H), 3.38 (dt, J = 4.3, 10.7 Hz, 1H), 2.21-2.23 (m, 1H), 2.05 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.93-1.96 (m, 1H), 1.60-1.64 (m, 2H), 1.58 (s, 3H), 1.31-1.35 (m, 1H), 1.17-1.25 (m, 1H), 0.91 (d, J = 6.6 Hz, 4 H), 0.86 (d = 6.6 Hz, 3H), 0.77-0.81 (m, 2H), 0.72 (d, J = 6.9 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 170.7, 170.4, 169.5, 169.3, 98.8, 79.1, 77.2, 73.1, 71.6, 71.5, 68.9, 62.5, 47.4, 40.8, 34.2, 31.5, 25.0, 23.0, 22.3, 20.9, 20.7, 20.7, 20.6, 20.6, 15.4; IR (neat) $\nu_{\max}$ /cm⁻¹ 1749, 1367, 1226, 1037; HRMS (ESI) m/z [M + Na]⁺ calcd for C₂₄H₃₈O₁₀Na 509.2328, found 509.2332.

Pregnenolonyl-2,3,4,6-tetraacetyl-*O*- β -D-galactopyranoside

R_f (50% EtOAc/Hexane) 0.42; $[\alpha]_D^{24}$ +46.96 (c = 0.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 5.36 (m, 2H), 5.18 (dd, J = 8.0, 10.4 Hz, 1H), 5.01 (dd, J = 3.4, 10.4 Hz, 1H), 4.54 (d, J = 8.0 Hz, 1H), 4.18 (dd, J = 6.6, 11.2 Hz, 1H), 4.10 (dd, J = 7.1, 11.2 Hz, 1H), 3.88 (t, J = 6.6 Hz, 1H), 3.54-3.55 (m, 1H), 2.52 (t, J = 9.6 Hz, 1H), 2.19-2.25 (m, 3H), 2.14 (s, 3H), 2.12 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 1.98 (s, 3H), 1.85-1.91 (m, 2H), 1.60-1.67 (m, 3H), 1.43-1.52 (m, 3H), 1.21-1.29 (m, 4H), 1.03-1.19 (m, H), 0.93-1.01 (m containing s, 4H), 0.89 (t, J = 7.0 Hz, 1H), 0.62 (s, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 209.5, 170.4, 170.4, 170.2, 169.4, 140.3, 121.9, 100.3, 80.2, 71.0, 70.6, 69.1, 67.0, 63.7, 61.3, 56.9, 50.0, 44.0, 38.9, 38.8, 37.2, 36.7, 31.8, 31.8, 31.6, 29.5, 24.5, 22.8, 21.1, 20.8, 20.7, 20.6, 19.4, 13.2. IR (neat) $\nu_{\max}$ /cm⁻¹ 1749, 1367, 1217, 1055; HRMS (ESI) m/z [M + Na]⁺ calcd for C₃₅H₅₀O₁₁Na 669.3251, found 669.3242.

Part A Green glycosylation promoted by reusable biomass carbonaceous solid acid: An easy access to β -stereoselective terpene galactosides

Citronellyl-2,3,4,6-tetraacetyl-*O*- β -D-galactopyranoside

R_f (50% EtOAc/Hexane) 0.37; $[\alpha]_D^{24} +3.9$ ($c = 0.1$, CHCl_3); ^1H NMR (400 MHz, CDCl_3): δ 5.19 (t, $J = 9.5$ Hz, 1H), 5.08 (t, $J = 9.6$ Hz, 2H), 4.97 (dd, $J = 8.0, 9.5$ Hz, 1H), 4.48 (d, $J = 8.0$ Hz, 1H), 4.26 (dd, $J = 4.7, 12.3$ Hz, 1H), 4.13 (dd, $J = 2.3, 12.3$ Hz, 1H), 3.89-3.94 (m, 1H), 3.68 (ddd, $J = 2.3, 4.7, 9.9$ Hz, 1H), 3.46-3.52 (m, 1H), 2.09 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.9-1.99 (m, 2H), 1.49-1.67 (m containing s, 5H), 1.25-1.37 (m, 2H), 1.11-1.18 (m, 1H), 0.86 (d, $J = 6.6$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 170.7, 170.4, 169.4, 169.3, 131.3, 124.6, 101.0, 72.9, 71.7, 71.3, 68.5, 68.5, 62.0, 37.2, 36.3, 29., 25.7, 25.4, 20.7, 20.6, 20.6, 19.1, 17.6; IR (neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1749, 1371, 1226, 1056; HRMS (ESI) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{24}\text{H}_{38}\text{O}_{10}\text{Na}$ 509.2363, found 509.152352.

Adamantanemethanyl-2,3,4,6-tetraacetyl-*O*- β -D-galactopyranoside

R_f (50% EtOAc/Hexane) 0.55; $[\alpha]_D^{24} -86.16$ ($c = 0.2$, CHCl_3); ^1H NMR (400 MHz, CDCl_3): δ 5.37 (d, $J = 3.2$ Hz, 1H), 5.21 (dd, $J = 8.2, 10.4$ Hz, 1H), 5.00 (dd, $J = 3.2, 10.4$ Hz, 1H), 4.37 (d, $J = 7.9$ Hz, 1H), 4.12-4.17 (m, 2H), 3.86 (t, $J = 6.8$ Hz, 1H), 3.50 (d, $J = 9.5$ Hz, 1H), 2.97 (d, $J = 9.6$ Hz, 1H), 2.14 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 1.98 (s, 3H), 1.94 (m, 2H), 1.69-1.72 (m, 3H), 1.60-1.62 (m, 4H), 1.46-1.52 (m, 6H); ^{13}C NMR (100 MHz, CDCl_3): δ 170.4, 170.4, 170.2, 169.3, 102.5, 81.1, 70.9, 70.5, 69.0, 67.1, 61.2, 39.3, 37.1, 33.9, 28.1, 20.8, 20.7, 20.6; IR (neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1751, 1369, 1224, 1053; HRMS (ESI) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{25}\text{H}_{36}\text{O}_{10}\text{Na}$ 519.2206, found 519.2198.

Ferrocenemethyl 2,3,4,6-tetraacetyl-*O*- β -D-galactopyranoside

R_f (50% EtOAc/Hexane) 0.42; $[\alpha]_D^{24} +22.9$ ($c = 0.6$, CHCl_3); ^1H NMR (400 MHz, CDCl_3): δ 5.36 (d, $J = 3.2$ Hz, 1H), 5.18 (dd, $J = 8.0, 10.2$ Hz, 1H), 4.96 (dd, $J = 3.2, 10.2$ Hz, 1H), 4.63

Part A Green glycosylation promoted by reusable biomass carbonaceous solid acid: An easy access to β -stereoselective terpene galactosides

(d, $J = 11.8$, 1H), 4.49 (d, $J = 7.9$ Hz, 1H), 4.43 (d, $J = 11.8$ Hz, 1H), 4.11-4.19 (m, 9H), 3.86 (t, $J = 6.4$ Hz, 1H), 2.14 (s, 3H), 2.07 (s, 3H), 1.96 (s, 3H), 1.95 (s, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 170.4, 170.3, 170.2, 169.4, 99.4, 82.0, 71.0, 70.6, 69.3, 69.0, 68.9, 68.8, 68.6, 68.5, 68.5, 67.4, 67.1, 61.3, 20.8, 20.7, 20.7, 20.6; IR (neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1749, 1369, 1222, 1053; HRMS (ESI) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{27}\text{H}_{36}\text{FeO}_{10}\text{Na}$ 599.1556, found 599.1558.

N-9-fluorenylmethoxycarbonyl-O-(2,3,4,6-tetra-O-acetyl- β D-galactopyranosyl)-L-serine methylester

R_f (50% EtOAc/Hexane) 0.25; $[\alpha]_{\text{D}}^{24} -7.3$ ($c = 0.1$, CHCl_3); ^1H NMR (400 MHz, CDCl_3): δ 7.77 (d, $J = 7.4$ Hz, 2H), 7.71 (m, 2H), 7.40 (m, 2H), 7.34 (t, $J = 7.3$ Hz, 2H), 5.74 (d, $J = 4.7$ Hz, 1H), 5.63 (d, $J = 8.6$ Hz, 1H), 5.42 (s, 1H), 5.02 (dd, $J = 3.0, 6.7$ Hz), 4.53 (d, $J = 8.6$ Hz, 1H), 4.30-4.41 (m, 2H), 4.22-4.29 (m, 3H), 4.08-4.14 (m, 2H), 4.0 (dd, $J = 2.2, 9.6$ Hz, 1H), 3.78 (s, 3H), 2.04-2.10 (m, 12H); ^{13}C NMR (100 MHz, CDCl_3): δ 170.4, 170.4, 170.0, 169.7, 155.9, 143.8, 143, 141.3, 127.7, 125.1, 121.0, 95.5, 73.9, 71.4, 69.2, 67.3, 65.8, 63.1, 61.3, 53.9, 52.7, 47.1, 23.5, 20.7, 20.5; IR (neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1747, 1367, 1217, 1060; HRMS (ESI) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{33}\text{H}_{37}\text{NO}_{14}\text{Na}$ 694.2112, found 694.2082.

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Part A Green glycosylation promoted by reusable biomass carbonaceous solid acid: An easy access to β -stereoselective terpene galactosides

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PART B

ZnCl₂/Alumina impregnation catalyzed Ferrier rearrangement: An expedient synthesis of pseudoglycosides

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1. INTRODUCTION

2,3-Unsaturated-*O*-glycosides or pseudoglucals are known as versatile chiral intermediates in the synthesis of antibiotics, nucleosides, complex carbohydrates, and various natural products.¹ Moreover, the unsaturated part of the sugar ring allows many straightforward modifications such as hydrogenation, hydroxylation, epoxidation, and aminohydroxylation, which contribute to their diversities and complexities. To date, many continuous efforts to develop expedient syntheses of 2,3-unsaturated-*O*-glycosides have been extensively made. One of the most common procedures to achieve pseudoglucals is the acid-catalyzed allyl rearrangement of glucals, which was discovered by Ferrier *et al.*² The Ferrier rearrangement is a reliable procedure for the formation of unsaturated glycoside derivatives, which has seen expensive development over decades. This rearrangement typically occurs by treatment of glucals and alcohols with Lewis acids or oxidants such as ZnCl_2 ,³ H_3PO_4 ,⁴ InCl_3 ,⁵ SnCl_4 ,⁶ $\text{Yb}(\text{OTf})_3$,⁷ FeCl_3 ,⁸ montmorillonite K-10,⁹ LiBF_4 ,¹⁰ $\text{Dy}(\text{OTf})_3$,¹¹ DDQ,¹² I_2 ,¹³ $\text{I}(\text{Coll})_2\text{ClO}_4$,¹⁴ CAN ,¹⁵ $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$,¹⁶ $\text{HClO}_4\text{-SiO}_2$,¹⁷ and *N*-iodosuccinamide.¹⁸ These methods could generate rearrangement products in one-step from glycoside derivatives, but they often suffer from incomplete stereoselectivity and the limited uses of acid-labile glugal donors and acceptors. The oxidants are usually required in stoichiometric amounts, and stringent conditions, long reaction times, and high temperatures are unavoidable. Thus, any further improvement must be judged against the vast background of current knowledge. Here, we wish to report a new synthetic component of Ferrier reaction, which delivers the desired products with exclusive α -stereoselectivity in high yield and in a short reaction time.

2. RESULTS AND DISCUSSION

A facile and highly stereoselective preparation of 2,3-unsaturated-*O*-glycoside derivatives from 3,4,6-tri-*O*-acetyl-D-glucal was initially investigated by examining the incorporation of Lewis acid on different solid supports. Pursuing the environmental impact, all reactions were carried out with minimal solvent and at ambient temperature. We surveyed the catalytic activity of various Lewis acids in the glycosylation of glucal by employing ethanol as a glucal acceptor and Al₂O₃ as a solid media (Table 1). Among them, ZnCl₂ was found to be superior to other catalysts in terms of yield, reaction profile and selectivity. However, strong Lewis acids [FeCl₃, SnCl₂, AlCl₃ and BF₃·OEt₂] also provided pseudoglucal products, albeit with moderate yields and selectivities (Table 1, Entries 1-4). To study the effect of the solid media, a wide range of solid supports such as silical gel, MgO, 4Å molecular sieves, MnO, and Al₂O₃ were examined by mixing with 1 equivalent of ZnCl₂. The results showed that silica gel and molecular sieves had no impact on the rearrangement, whereas MgO and MnO found to be less efficient in both conversion and anomeric selectivity (Table 1, Entries 6-9). ZnCl₂ impregnated on activated alumina was conclusively the best combination in this synthetic transformation giving rise to a good anomeric ratio ($\alpha:\beta$ = 20:1) and short reaction time (10 min) as shown in Table 1, Entry 5.

According to the published results, most of the Ferrier reactions of 3,4,6-tri-*O*-acetyl-D-glycal conducted in the solution phase³ and the miscellaneous system¹⁷ were sluggish (12 h) and showed the low level of the anomeric selectivity. Moreover, some reactions required considerable care when using toxic and moisture sensitive catalysts. These cause problems, the tedious procedures and work- up and require special equipments. On the other hand, the solvent free ZnCl₂/Al₂O₃ system¹⁹ acts as a potential tool for synthesis of 2,3-unsaturated glycosides. The purification could be

Table 1 Optimized conditions for Ferrier rearrangement with Lewis acids / solid supports^a

Entry	Lewis Acid / Solid Support ^b	Time	% Yield (α/β) ^c
1	FeCl ₃ ·6H ₂ O / Al ₂ O ₃	30 min	86 (1:1)
2	SnCl ₂ / Al ₂ O ₃	45 min	32 (6:1)
3	AlCl ₃ / Al ₂ O ₃	80 min	60 (8:1)
4	BF ₃ ·OEt ₂ / Al ₂ O ₃	30 min	56 (8:1)
5	ZnCl ₂ / Al ₂ O ₃	10 min	92 (20:1)
6	ZnCl ₂ / SiO ₂	2 h	48 (6:1)
7	ZnCl ₂ / MgO	6 h	72 (2:1)
8	ZnCl ₂ / 4Å MS	4 h	Trace
9	ZnCl ₂ / MnO ₂	6 h	75(1:1)

^a 10 equivalents of ethanol was used. ^b 1 equivalent of Lewis acid on 5.3 equivalents of solid support was used per 1 equivalent of tri-*O*-acetyl-D-glucal. ^c α/β ratio was confirmed by the anomeric proton ratio on 500 MHz NMR.

easily done after the completion of the reaction (10-30 mins) by silica gel column chromatography.

Having the optimized conditions for the glycosylation of glucal over ZnCl₂/Alumina impregnation in hand we proceeded to screen a wide range of alcohols and thiols (Table 2). In a typical reaction, a mixture of glucal and alcohol was added to the ZnCl₂/Alumina impregnation and stirred. In order to maintain the proper stirring of

Table 2 Ferrier reaction of 2,3-tri-*O*-acetyl-D-glucal with alcohols in the presence of the ZnCl₂/Al₂O₃ reagent system^a

Entry	Product	Time (min)	Yield (%)	α/β^b
1 ^c		10	92	20:3 ^{2C}
2 ^d		20	94	α
3		20	83	α^{11}
4		30	78	α
5		10	88	α^{11}
6		20	82	α
7		45	70	α
8		10	85	α
9		10	85	α
10		10	85	α^{20}

11		20	82	α
12		15	90	α^{11}
13		10	75	α^{21}
14		10	62	α
15		10	65	α^{22}
16 ^d		20	94	20:1
2p				

^a Unless otherwise noted, 3 equivalents of nucleophiles were used. ^b α/β ratio was confirmed by the anomeric proton ratio on ¹H NMR spectra. ^c 10 equivalents of methanol was used. ^d As nucleophile is solid, glucal, 1 equivalent of alcohol and ZnCl₂/Al₂O₃ impregnation were mixed and grinded by using a mortar.

the reaction mixture excess of alcohol was used (3 equiv.). When the reaction was completed, the solid mass was simply filtered off, rinsed with dichloromethane (or ethyl acetate). Evaporation of the solvent and subsequent elution of the crude product on the short column chromatography afforded the desired 2,3-unsaturated glycosides (**2a-p**) in excellent yields. All reactions gave best yields when 1 equivalent of ZnCl₂ used with 5.3 equivalents of activated alumina. Using methanol and ethanol as

Part B ZnCl₂/Alumina impregnation catalyzed Ferrier rearrangement: An expedient synthesis of pseudoglycosides

nucleophiles gave a mixture of anomeric diastereomers (up to α/β 20:1), while others irrespective of size and nature furnished desired α -glycosides. Moreover, this compliment could be cooperated with a glucose derivative to give the corresponding disaccharide **2p** in 94% yield and 20:1 of α/β ratio (Entry 16).

Table 3 Ferrier reaction of 2,3-tri-*O*-acetyl-D-glucal with various natural products alcohols in the presence of the $\text{ZnCl}_2/\text{Al}_2\text{O}_3$ reagent system^a

Entry	Natural Product Alcohols	Product	Time (min)	Yield (%)	α/β
1	S-(-)- β -Citronellol	3a	15	85	α^{26}
2	L-Menthol	3b	10	92	α
3	(+)-Borneol	3c	10	80	α
4	(+)-endo-Fenchol	3d	15	84	α
5	Pregnenolone	3e	20	88	20:1

^a As these nucleophiles are solids, 1 equivalent of them were mixed with glucal, $\text{ZnCl}_2/\text{Al}_2\text{O}_3$ impregnation were mixed and grinded by using a mortar.

Most of the glycosylated products were obtained in exclusively pure diastereomers as resulted in Table 2. The significance of the highly stereoselective glycosylation has been highlighted in this report. We then explored the scope of this concise route for the synthesis of pseudoglucals connected to various biologically

important natural products. For example, citronellol,²³ L-menthol,²⁴ borneol,²⁴ endo-fenchol, and pregnenolone²⁵ glycosides, these all have served as challenging targets to study and transform because of their diversity of the biological activities and their structural complexity.

Some of them were generally synthesized by enzymatic²⁶ as well as Koenigs-Knorr-Zemplen²⁴ methods, which are expensive, lengthy and tedious procedures. We envisioned that our solid support system would offer an easy access to natural products containing α -selective sugar moiety. As expected, transformation of glucal to the corresponding 2,3-unsaturated glycosides was rapid and the desired compounds were formed exclusively with α -anomeric selectivity within 10-15 minutes of reaction time (Table 3, Entries 1-4).

Pregnenolone glycoside, which was first isolated from *Nerium Odorum*,²⁵ operates as a powerful neurosteroid in the brain, modulating the transmission of messages from neuron to neuron and strongly influencing learning and memory. The synthesis of pregnenolone 2,3-unsaturated glycoside was also success with excellent α -selectivity (88% yield and 20:1 α/β selectivity) as described in Table 3, entry 5. Furthermore, its structure was elucidated by NMR spectroscopic data and confirmed by X-ray crystallography (Figure 1).

Figure 1 X-ray structure of pregnenolone 2,3-unsaturated glycoside **3e**.

3. CONCLUSION

We have demonstrated a new protocol for forming a glycosidic linkage in high diastereoselective fashion. Many complex alcohols and thiols could be incorporated with glucal donor in a single process. 11 new compounds, which include 5 natural product-like compounds, were successfully prepared. The main advantages of this method concern high anomeric selectivity, rapid reaction time, and friendly environmental conditions. Low cost reagents and no aqueous work-up are required and the alumina could be recycled up to 3 times.

4. EXPERIMENTAL

Product purification by flash column chromatography was accomplished using silica gel 60 (0.010-0.063 mm). The ^1H and ^{13}C NMR data was obtained on a 300 MHz Bruker ACF 300 and 500 MHz Bruker AMX 500 NMR spectrometers. For ^1H NMR, chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane and are, in all cases, referenced to the residual proton resonance peaks: δ 7.26 for CHCl_3 . The ^{13}C NMR chemical shifts were reported in ppm relative to the center peak of the multiplet for deuterated solvents: δ 77.0 (t) for CDCl_3 . Coupling constants (J) for all spectra are reported in Hz. Following abbreviations classify the multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, br = broad signal. Infrared spectra were measured on a Bio-RAD FTS 165 FT-IR Spectrometer as thin films on sodium bromide plates and are recorded in units of cm^{-1} . HRMS (ESI) spectra were recorded on a Finnigan/MAT LCQ quadrupole ion trap mass spectrometer, coupled with the TSP4000 HPLC system and the Crystal 310 CE system. X-ray crystallographic data was collected by using a Bruker X8Apex diffractometer with Mo $\text{K}\alpha$ -radiation (graphite monochromator).

General procedure for preparation 2,3-unsaturated glycosides

To a stirred mixture of tri-*O*-acetyl-D-glucal (100 mg, 0.37 mmol) and an alcohol (1 equiv) was added 250 mg of $\text{ZnCl}_2\text{-Al}_2\text{O}_3$ at ambient temperature. The mixture was stirred for appropriate time (Table 2 and 3), and completion of the reaction was monitored by TLC analysis. The reaction mixture was then filtered and washed with dichloromethane, and the combined organic extract was concentrated under vacuum. All the products were purified by silica gel column chromatography (30% EtOAc/hexane).

Nonadecanyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (2b)

R_f (30% EtOAc/hexane) 0.67; $[\alpha]_D^{24} +83.3$ (c 0.7, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 5.84 (d, $J = 10$ Hz, 1H), 5.80 (d, $J = 10$ Hz, 1H), 5.29 (d, $J = 10$ Hz, 1H), 5.0 (s, 1H), 4.23 (dd, $J = 12.1, 5.4$ Hz, 1H), 4.15 (dd, $J = 12.1, 2.2$ Hz, 1H), 4.10-4.08 (m, 1H), 3.75 (dt, $J = 9.4, 6.9$ Hz, 1H), 3.48 (dt, $J = 9.5, 6.6$ Hz, 1H), 2.07 (s, 3H), 2.06 (s, 3H), 1.61-1.52 (m, 2H), 1.29-1.23 (m, 30H), 0.86 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 170.7, 170.2, 128.9, 127.9, 94.3, 69.0, 66.8, 65.3, 63.0, 32.8, 31.9, 29.7, 29.6, 29.6, 29.4, 29.3, 26.2, 26.0, 25.7, 22.6, 20.9, 20.7, 14.1; IR (NaCl neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1747, 1226, 1039; HRMS (ESI) m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{28}\text{H}_{50}\text{O}_7\text{Na}$ 505.3505, found 505.3509.

2-(Allyloxy)ethanyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (2d)

R_f (30% EtOAc/hexane) 0.35; $[\alpha]_D^{24} +272.2$ (c 0.4, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 5.90 (ABX system, $J_{\text{AX}} = 17.2$ Hz, $J_{\text{BX}} = 9.6$ Hz, $J_{\text{AB}} = 5.4$ Hz, 1H), 5.85 (br s, 2H), 5.30 (d, $J = 9.7$ Hz, 1H), 5.26 (dd, $J = 17.2, 1.6$ Hz, 1H), 5.16 (d, $J = 9.5$ Hz, 1H), 5.06 (s, 1H), 4.23 (dd, $J = 12.1, 5.2$ Hz, 1H), 4.15 (dd, $J = 12.1, 2.3$ Hz, 1H), 4.12-4.10 (m, 1H), 4.0 (d, $J = 5.0$ Hz, 2H), 3.89 (dt, $J = 11.0, 4.5$ Hz, 1H), 3.72-3.68 (m, 1H), 3.61 (t, $J = 4.8$ Hz, 2H), 2.07 (s, 3H), 2.05 (s, 3H); ^{13}C NMR (125 MHz,

CDCl₃) δ 170.7, 170.2, 134.6, 129.1, 127.7, 117.1, 94.6, 72.1, 69.2, 67.8, 66.8, 65.2, 62.9, 20.9, 20.7; IR (NaCl neat) $\nu_{\max}/\text{cm}^{-1}$ 1743, 1317, 1234, 1047; HRMS (ESI) m/z [M+Na]⁺ Calcd for C₁₅H₂₂O₇Na 337.1263, found 337.1252.

3-Phenylprop-2-ynyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (2f)

R_f (50% EtOAc/hexane) 0.52; $[\alpha]_{\text{D}}^{24}$ +196.0 (*c* 0.5, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 7.46-7.42 (m, 2H), 7.32-7.29 (m, 3H), 5.93 (d, *J* = 17.5 Hz, 1H), 5.42-5.33 (m, 2H), 4.53 (s, 1H), 4.28 (dd, *J* = 12.1, 5.1 Hz, 1H), 4.20 (dd, *J* = 12.1, 2.4 Hz, 1H), 4.19-4.11 (m, 1H), 2.09 (s, 3H), 2.07 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 170.7, 170.2, 131.8, 129.6, 128.6, 128.4, 128.3, 127.4, 122.3, 92.7, 86.5, 67.2, 65.2, 62.8, 55.8, 20.9, 20.7; IR (NaCl neat) $\nu_{\max}/\text{cm}^{-1}$ 1743, 1371, 1234, 1037; HRMS (ESI) m/z [M+Na]⁺ Calcd for C₁₉H₂₁O₆Na 345.1338, found 345.1339.

3-Pentanyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (2h)

R_f (50% EtOAc/hexane) 0.66; $[\alpha]_{\text{D}}^{24}$ +313.9 (*c* 0.2, CHCl₃); ¹H NMR (300 MHz, CDCl₃) δ 5.85 (d, *J* = 10.5 Hz, 1H), 5.82 (d, *J* = 10.5 Hz, 1H), 5.29 (dt, *J* = 9.3, 1.4 Hz, 1H), 5.11 (s, 1H), 4.24 (dd, *J* = 12.1, 5.7 Hz, 1H), 4.11-4.12 (m, 3H), 3.56 (p, *J* = 5.9 Hz, 1H), 2.08 (s, 3H), 2.07 (m, 3H), 1.60-1.55 (m, 3H), 0.94 (t, *J* = 7.4 Hz, 3H), 0.91 (t, *J* = 7.4 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 170.8, 170.3, 128.7, 128.2,

93.4, 81.2, 66.9, 65.3, 63.1, 27.1, 26.2, 20.9, 20.7, 10.0, 9.3; IR (NaCl neat) $\nu_{\max}/\text{cm}^{-1}$ 1743, 1369, 1230, 1033; HRMS (ESI) m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{15}\text{H}_{24}\text{O}_7\text{Na}$ 323.1471, found 323.1462.

Cyclohex-2-enyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (2i)

R_f (30% EtOAc/hexane) 0.50; $[\alpha]_D^{24} +38.7$ (c 0.5, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 5.83-5.82 (m, 3H), 5.77-5.76 (m, 1H), 5.29 (d, $J = 8.8$ Hz, 1H), 5.19 (d, $J = 17.4$ Hz, 1H), 4.25-4.20 (m, 2H), 4.19-4.17 (m, 2H), 2.09 (d, $J = 2.7$ Hz, 3H), 2.08 (d, $J = 1.3$ Hz, 2H), 2.07-2.03 (m, 2H), 1.97-1.88 (m, 2H), 1.83-1.66 (m, 2H), 1.91-1.53 (m, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 170.8, 170.3, 131.5, 128.9, 128.7, 128.4, 128.3, 94.0, 72.7, 66.8, 65.3, 63.1, 30.1, 25.0, 21.0, 19.2; IR (NaCl neat) $\nu_{\max}/\text{cm}^{-1}$ 1743, 1371, 1230, 1033; HRMS (ESI) m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{16}\text{H}_{22}\text{O}_6\text{Na}$ 333.1314, found 323.1310.

Cyclooctanyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (2k)

R_f (50% EtOAc/hexane) 0.54; $[\alpha]_D^{24} +156.7$ (c 1.0, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 5.83 (d, $J = 10.2$ Hz, 1H), 5.76 (dt, $J = 10.2, 2.3$ Hz, 1H), 5.26 (dd, $J = 9.6, 1.4$ Hz, 1H), 5.09 (s, 1H), 4.20 (d, $J = 12.1$ Hz, 1H), 4.12 (dd, $J = 12.1, 5.9$ Hz, 1H), 4.16-4.11 (m, 1H), 3.83 (h, $J = 4.2$ Hz, 1H), 2.07 (s, 3H), 2.05 (s, 3H), 1.86-1.83 (m,

1H), 1.78-1.63 (m, 4H), 1.59-1.40 (m, 9H); ^{13}C NMR (125 MHz, CDCl_3) δ 170.7, 170.2, 128.6, 128.4, 92.8, 78.6, 72.6, 66.8, 66.4, 64.5, 63.5, 63.2, 33.0, 31.2, 27.3, 25.3, 23.0, 20.7; IR (NaCl neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1743, 1373, 1234, 1036; HRMS (ESI) m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{18}\text{H}_{28}\text{O}_7\text{Na}$ 363.1784, found 345.1771.

2-Methoxybenzenethainyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (2n)

R_f (30% EtOAc/hexane) 0.45; $[\alpha]_D^{24}$ +262.6 (c 0.5, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 7.42 (dd, J = 7.5, 1.6 Hz, 1H), 7.24 (m, 1H), 6.8 (m, 2H), 6.40 (d, J = 5.8 Hz, 1H), 5.13 (dd, J = 10.1, 4.5 Hz, 1H), 4.94 (t, J = 5.8 Hz, 1H), 4.54 (m, 1H), 4.38 (dd, J = 12.2, 4.4 Hz, 1H), 4.32 (dd, J = 12.2, 2.1 Hz, 1H), 3.88 (s, 1H), 2.06 (s, 6H), 1.47 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 170.7, 169.8, 158.7, 144.7, 133.6, 129.1, 122.9, 120.8, 111.0, 98.7, 70.8, 69.8, 62.3, 55.8, 40.4, 20.7, 19.8; IR (NaCl neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1743, 1370, 1232, 1037; HRMS (ESI) m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{17}\text{H}_{20}\text{O}_6\text{SNa}$ 375.0878, found 375.0871.

Benzyl-O-(4',6'-di-O-acetyl-2'-3'-dideoxy- α -D-erythro-hex-2'-enopyranosyl)-(1 $\rightarrow$ 6)-2,3,4-tri-O-benzyl- α -D-glucopyranoside (2p)

R_f (30% EtOAc/hexane) 0.45; $[\alpha]_D^{24} +20.8$ (c 1.0, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 7.34-7.21 (m, 20H), 5.85 (s, 2H), 5.30 (d, $J = 9.6$ Hz, 1H), 5.14 (s, 1H), 4.96-4.90 (m, 4H), 4.81-4.76 (m, 2H), 4.67-4.62 (m, 3H), 4.51 (d, $J = 7.8$ Hz, 1H), 4.13-4.07 (m, 2H), 3.84-3.71 (m, 1H), 3.68-3.64 (m, 2H), 3.60-3.55 (m, 1H), 3.51-3.45 (m, 2H), 2.07 (s, 3H), 2.06 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 170.8, 170.3, 135.5, 138.4, 137.4, 129.0, 128.5, 128.5, 128.4, 128.4, 128.2, 128.1, 128.1, 127.9, 127.9, 127.8, 127.8, 127.7, 127.7, 127.7, 102.8, 102.5, 94.8, 84.7, 84.5, 82.4, 78.0, 77.6, 75.7, 75.1, 75.1, 75.0, 74.9, 74.6, 71.6, 71.2, 67.2, 67.0, 65.2, 62.8, 62.0, 60.4, 21.0, 20.8; IR (NaCl neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1733, 1455, 1259, 1061; HRMS (ESI) m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{44}\text{H}_{48}\text{O}_{11}\text{Na}$ 775.3094, found 775.3085.

S-(-)- β -Citronellyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (3a)

R_f (30% EtOAc/hexane) 0.64; $[\alpha]_D^{24} +41.5$ (c 0.5, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 5.86 (d, $J = 10.4$ Hz, 1H), 5.87 (d, $J = 10.4$ Hz, 1H), 5.29 (dd, $J = 9.6, 1.1$ Hz, 1H), 5.08 (t, $J = 7.0$ Hz, 1H), 5.01 (s, 1H), 4.23 (dd, $J = 12.1, 5.4$ Hz, 1H), 4.16 (dd, $J = 12.1, 2.2$ Hz, 1H), 4.10-4.07 (m, 1H), 3.82 (dd, $J = 7.4, 2.2$ Hz, 1H), 3.55-3.50 (m, 1H), 2.08 (s, 3H), 2.07 (s, 3H), 1.99-1.93 (m, 2H), 1.67-1.65 (m, 4H), 1.59-1.55 (m, 4H), 1.41-1.30 (m, 2H), 1.17-1.12 (m, 1H), 0.88 (d, $J = 6.6$ Hz, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 170.8, 170.2, 131.2, 128.9, 127.9, 124.6, 94.3, 67.0, 66.9, 65.3, 63.0, 37.2, 36.5, 29.5, 25.7, 25.4, 20.9, 20.7, 19.3, 17.6; IR (NaCl neat) $\nu_{\text{max}}/\text{cm}^{-1}$

1743, 1371, 1232, 1035; HRMS (ESI) m/z $[M+Na]^+$ Calcd for $C_{20}H_{32}O_6Na$ 391.2097, found 391.2086.

L-Menthyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (3b)

R_f (30% EtOAc/hexane) 0.61; $[\alpha]_D^{24}$ +142.5 (c 0.5, $CHCl_3$); 1H NMR (500 MHz, $CDCl_3$) δ 5.85 (s, 2H), 5.26 (d, J = 10 Hz, 1H), 5.08 (s, 1H), 4.18 (dd, J = 12.3, 6.7 Hz, 1H), 4.17-4.14 (m, 2H), 3.39 (dt, J = 10.6, 4.4 Hz, 1H), 2.17 (d, J = 4.0 Hz, 1H), 2.09 (s, 3H), 2.08 (s, 3H), 1.67-1.59 (m, 2H), 1.44-1.37 (m, 1H), 1.25-1.20 (m, 1H), 1.03 (dd, J = 23.2, 12.2 Hz, 1H), 0.95 (dd, J = 12.1, 3.0 Hz, 1H), 0.92-0.87 (m, 7H), 0.85-0.79 (m, 1H), 0.75 (d, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8, 170.3, 128.5, 128.0, 96.1, 81.0, 66.6, 65.3, 63.3, 48.8, 43.3, 34.2, 31.7, 25.6, 23.1, 22.3, 21.1, 20.9, 20.8, 16.2; IR (NaCl neat) ν_{max}/cm^{-1} 1743, 1369, 1234, 1035; HRMS (ESI) m/z $[M+Na]^+$ Calcd for $C_{20}H_{32}O_6Na$ 391.2097, found 391.2089.

(+) Bornyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (3c)

R_f (30% EtOAc/hexane) 0.61; $[\alpha]_D^{24}$ +72.6 (c 0.8, $CHCl_3$); 1H NMR (500 MHz, $CDCl_3$) δ 5.84-5.79 (m, 2H), 5.27 (d, J = 9.8 Hz, 1H), 4.99 (s, 1H), 4.21 (dd, J = 12.2, 5.6 Hz, 1H), 4.12 (dd, J = 12.2, 2.2 Hz, 1H), 4.10-4.09 (m, 1H), 3.82 (dt, J = 6.6, 2.1 Hz, 1H), 2.24-2.22 (m, 1H), 2.07 (s, 3H), 2.06 (s, 3H), 1.96-1.90 (m, 1H), 1.70-1.64

(m, 2H), 1.59 (t, $J = 4.5$ Hz, 1H), 1.23-1.17 (m, 2H), 1.11 (dd, $J = 13.4, 3.4$ Hz, 1H), 0.94-0.82 (s, 3H), 0.81(s, 3H), 0.80 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 170.7, 170.2, 128.4, 128.2, 96.1, 85.8, 66.8, 66.7, 64.1, 48.8, 47.7, 46.6, 38.9, 28.2, 28.2, 26.6, 20.9, 20.7, 19.7, 13.6; IR (NaCl neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1747, 1369, 1230, 1041; HRMS (ESI) m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{20}\text{H}_{30}\text{O}_6\text{Na}$ 389.1940, found 389.1938.

(+)-Endo-fenacholyl 4,6-di-O-acetyl-2,3-dideoxy- α -D-erythro-hex-2-enopyranoside (3d)

R_f (30% EtOAc/hexane) 0.61; $[\alpha]_D^{24} +48.7$ (c 0.5, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 5.83 (s, 2H), 5.25 (d, $J = 10$ Hz, 1H), 4.95 (s, 1H), 4.20 (dd, $J = 12.2, 5.2$ Hz, 1H), 4.13-4.09 (m, 2H), 3.42 (d, $J = 1.5$ Hz, 1H), 2.06 (s, 3H), 2.05 (2, 3H), 1.65-1.61 (m, 3H), 1.45 (dd, $J = 10.1, 1.5$ Hz, 1H), 1.08 (s, 3H), 1.07-1.06 (m, 1H), 1.01 (s, 3H), 0.96-0.94 (m, 1H), 0.85 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ_C 170.8, 170.2, 128.5, 127.9, 94.1, 90.1, 66.8, 65.2, 63.1, 48.8, 48.7, 41.3, 39.4, 31.8, 25.9, 21.1, 20.9, 20.8, 19.7; IR (NaCl neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1732, 1456, 1259, 1064; HRMS (ESI) m/z $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{20}\text{H}_{30}\text{O}_6\text{Na}$ 389.1940, found 389.1935.

Pregnenolonyl 4,6-di-O-acetyl-2,3- dideoxy- α -D-erythro-hex-2-enopyranoside (3e)

R_f (30% EtOAc/hexane) 0.40; $[\alpha]_D^{20} +162.4$ (c 0.5, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 5.85 (d, $J = 10.4$ Hz, 1H), 5.78 (d, $J = 10.4$ Hz, 1H), 5.32 (s, 1H), 5.26 (d, $J = 10.0$ Hz, 1H), 5.14 (s, 1H), 4.20 (dd, $J = 12.2, 5.9$ Hz, 1H), 4.17-4.13 (m, 2H), 3.57-3.51 (m, 1H), 2.50 (t, $J = 8.0$ Hz, 1H), 2.38-2.34 (m, 1H), 2.32 (d, $J = 11.0$ Hz, 1H), 2.19-2.14 (m, 1H), 2.09 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 2.02 (d, $J = 9.5$ Hz, 1H), 1.99-1.95 (m, 1H), 1.88-1.83 (m, 2H), 1.60-1.42 (m, 8H), 1.24-1.18 (m, 1H), 1.17-1.12 (m, 1H), 1.06-1.03 (m, 1H), 0.98-0.95 (m, 4H), 0.60 (s, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 209.5, 170.8, 170.3, 140.7, 128.9, 128.3, 121.5, 92.8, 78.0, 66.8, 65.3, 63.6, 63.1, 56.8, 49.9, 43.9, 40.3, 38.8, 37.1, 36.6, 31.8, 31.7, 31.5, 28.1, 24.4, 22.8, 21.0, 20.9, 20.8, 19.3, 13.2; IR (NaCl neat) $\nu_{\text{max}}/\text{cm}^{-1}$ 1745, 1699, 1371, 1228, 1035; HRMS (ESI) m/z : Calcd for $\text{C}_{31}\text{H}_{44}\text{O}_7\text{Na}$: 551.2985 $[\text{M}+\text{Na}]^+$; found: 551.2994.

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