MONOSACCHARIDE-BINDING OF CYSTIC FIBROSIS *PSEUDOMONAS AERUGINOSA:* GLYCOPOLYMER PREPARATION, METHODS DEVELOPMENT AND PHENOTYPE ASSESSMENT

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The undersigned, appointed by the Dean of the Graduate Faculty, have examined the dissertation entitled

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a candidate for the degree of Doctor of Philosophy,

and hereby certify that in their opinion it is worthy of acceptance.

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“All that I am, or hope to be, I owe to my angel mother”.

This work is dedicated to her memory.

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LIST OF ABBREVIATIONS

CF	Cystic fibrosis
CFTR	CF transmembrane conductance regulator
RAFT	Reversible addition−fragmentation chain-transfer
DP	Degree of polymerization
CTA	Chain transfer agent
Gal	D-Galactose
GalNAc	D-*N*-Acetylgalactosamine
GlcNAc	D-*N*-Acetylglucosamine
Neu5Ac	D-*N*-Acetylneuraminic acid
Fuc	α-L-Fucose
MEHQ	Hydroquinone monomethyl ether
AEMA	*N*-(2-Aminoethyl) methacrylamide
HEAA	*N*-(2-Hydroxyethyl) acrylamide
GAEMA	2-Gluconamidoethyl methacrylamide
LAEMA	2-lactobionamidoethyl methacrylamide
ALAEMA	2-Allolactobionamidoethyl methacrylamide
MGAEMA	6-α-D-mannopyranosyl-D-gluconamidoethyl methacrylamide
MAEMA	2-Melibionamidoethyl methacrylamide
GPC	Gel permeation chromatography
MWCO	Molecular weight cut off

DMF	Dimethylformamide
GNL	*Galanthus nivalis* lectin
*M*n	Number-average molar molecular weight
*M*w	Weight-average molar molecular weight
PAA	Polyacrylamide
PMA	Poly-methacrylamide/acrylamide
BSA	Bovine serum albumin
EPS	Exopolysaccharide
FITC	Fluorescein isothiocyanate
Rf	Retention factor
TLC	Thin layer chromatography
TEM	Transmission electron microscopy
GC-MS	Gas chromatography-mass spectrometry

Chapter I. Introduction

As a crucial step in many bacterial infections, pathogens employ their adhesins that include lectins, to bind specific carbohydrates on the surface epithelium, mucous glycoproteins, and glycolipids of the host to get attached and start colonization (1,2). In the 'notorious' respiratory infections in cystic fibrosis (CF) patients, *Pseudomonas aeruginosa*, the major bacterial pathogen, is considered to utilize two lectins, LecA and LecB (also known as PA-IL and PA-IIL), together with some other carbohydrate-binding structures (adhesins), to bind certain sugars on the CF airway surface (3-6). For several reasons, the bacterial infection in CF lungs is extremely difficult, if not impossible, to be eliminated by any current antibiotics. In the pursuit of new anti-bacterial therapeutics,

anti-adhesion therapy utilizing competitive carbohydrates to block the lectin-mediated bacterial binding offers a promising treatment for both CF and non-CF patients (7,8). Within the respiratory tract of CF patients, *P. aeruginosa* normally first appear as motile and non-mucoid, which is the typical morphology of environmental isolates. Over time, as a result of the environmental stresses and the formation of biofilms (9,10), clinically isolated *P. aeruginosa* often presents a wide range of phenotypic diversities, including the appearance of different colony morphologies, numerous physiological heterogeneities, and varied expression levels of virulence factors, to name a few (11,12). Lectins and other sugar-binding proteins are considered to be important virulence factors of *P. aeruginosa*, and their individual binding specificities have been extensively studied in the last several decades. However, the monosaccharide-binding profiles of different morphotypes haven't been thoroughly investigated on the cellular level. Due to the small sizes of the bacteria and low binding affinities of many lectins with monomeric carbohydrate ligands, studying bacterial carbohydrate-binding has always been challenging.

Therefore, the two major aims of this doctoral research project are:

1). Establish improved methodologies to investigate the lectin-mediated bacterial binding with carbohydrates.

2). Determine the monosaccharide-binding profiles of clinical CF *P. aeruginosa* isolates with different colony morphologies.

1. Cystic fibrosis

CF is one of the most common genetic disorders in Caucasians (~1 in 3000 births). As an autosomal recessive disease, CF is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR). Expressed in exocrine tissues, CFTR functions primarily as a chloride channel secreting Cl^- to the airway surface, and the inhibitor of an epithelial Na^+ channel preventing sodium reabsorption back into cells (13,14). Out of more than 1,800 known CFTR mutations (15), the most common one is an in-frame three-base deletion that leads to the loss of the amino acid phenylalanine (F) at position 508 (ΔF508) (16). This causes a folding defect of the protein, which is recognized by the quality control mechanisms in the endoplasmic reticulum, where the misfolded protein is ubiquinated and marked for degradation (17). About 87% of the CF patients in the U.S. have at least one copy of the ΔF508 mutation, and ~47% have two copies (15).

The symptoms of CF, resulted from functional disorder of exocrine tissues, include progressive infection and inflammation in airways, faulty digestion due to pancreatic insufficiency, diabetes caused by destruction of the pancreas, male infertility because of the congenital bilateral absence of the vas deferens, excessive loss of salt in sweat, etc (18). In the airway, the malfunction of CFTR causes reduced secretion of chloride into the lumen and decreased inhibition of Na^+ reabsorption. While elevated amount of salt is retained in the epithelial cells, water follows from the lumen into the cells, leaving highly dehydrated and tenacious mucus on the respiratory tract surface. Therefore, the protection offered by the mucociliary movement in normal individuals are now highly compromised, and the mucous layer, in turn, becomes a habitat for bacteria

(18,19). Albeit many other organs are affected by this genetic disorder as well, the patients' survival is primarily decided by the progression of their lung diseases (85% of the mortality), characterized by chronic inflammation and infection with a number of bacterial pathogens, including *P. aeruginosa, Staphylococcus aureus, Haemophilus influenzae, Burkholderia cepacia* (a complex of at least nine species), etc (15,20). In general, CF infants are rapidly colonized by *S. aureus* and, progressively, *P. aeruginosa* becomes predominant in the airway (21). According to the annual report by the US CF Foundation, *S. aureus* can be found in the lungs of ~70% of the CF patients under the age of 10, and only ~25% of the patients at this stage show *P. aeruginosa* infection, whereas more than 70% of the CF patients older than 25 are infected by *P. aeruginosa,* and the number for *S. aureus* infection drops to ~55%. The CF airway becomes a *P. aeruginosa* habitat for several possible reasons: permissive microenvironments within the mucous plaques anaerobic niches, increased bacterial binding to the airway surface, and compromised bacterial clearance via innate immune mechanisms (18,22).

For chronic pulmonary disease treatment, the 2013 CF Foundation guideline recommends inhaled tobramycin (an aminoglycoside antibiotic), aztreonam (a β-lactam antibiotic) and dornase alfa (recombinant human DNase) for patients with moderate to severe *P. aeruginosa* infections; the use of inhaled bronchodilator, hypertonic saline, and airway clearance techniques were also supported (23). By virtue of earlier diagnosis through newborn screening, advanced treatment and better access to health care, the prognosis for CF patients has dramatically improved over the past decades. The median predicted survival age in the U.S. has increased by almost 10 years in the last

decade—from age 31.3 in 2002 to age 41.1 in 2012 (15). However, for CF patients with end-stage respiratory inflammation and infection, lung transplantation is often required. Therefore, new CF therapies are still actively sought (24,25).

2. *P. aeruginosa* and biofilm

Not only is *P. aeruginosa* a major pathogen in CF lung infections, but also a significant cause in many other human opportunistic infections, including burn victims, urinary-tract infections in catheterized patients, hospital-acquired pneumonia in patients on respirators, etc. (26). As a ubiquitous Gram-negative rod, *P. aeruginosa* can be found in soil, water, animals and almost all man-made environments. Transmitted via environmental reservoirs and by direct contact between carriers, *P. aeruginosa* in CF lungs normally first appear as motile and non-mucoid, and can be cleared by the host, or eradicated with intensive antibiotic treatments (27). Over time, in part due to the formation of biofilms, the bacteria start to demonstrate phenotypic diversities, and it becomes extremely difficult to eliminate the infection by any antibiotics (28).

By definition, biofilm is a community formed by microorganisms that are attached to a surface and aggregate in a hydrated polymeric matrix. The matrix is composed of exopolysaccharides (EPS), lipids, extracellular nucleic acids, and proteins (28,29), components that play distinct but all essential roles in the biofilm formation (29,30). In the *P. aeruginosa* biofilm, three types of polysaccharides can be found: alginate, Psl and Pel, each of which offers different physiological properties to the biofilm matrix (31). Alginate is dominant in mucoid bacteria (32), and Pel/Psl in non-mucoid (33). The exceptional high antibiotic resistance of *P. aeruginosa* within a biofilm

is partly caused by the slow drug penetration, oxygen limitation, and low metabolic activity of the bacteria in the EPS matrix (34). Within biofilms, *P. aeruginosa* vary their phenotypes to adapt to local microenvironments. Frequent alterations include changes in surface antigens, overproduction of EPS, modulation of microaerobic metabolic pathways, etc. (35). Consequently, *P. aeruginosa* isolated from CF patients often show a remarkably diverse colony morphologies: they can be mucoid or nonmucoid, motile or nonmotile, smooth or rough, pigment-secreting or colorless, or in the form of small colony variants (36,37). Many of these characteristics are directly related to bacterial virulence and increased antibiotic resistance (38). Moreover, it has been demonstrated that phenotypic diversities of this Gram-negative bacteria can be observed after being cultured in a biofilm for 24 h (10), or contact with muco-purulent respiratory liquid from CF patients for only several hours (39). These observations offer intriguing insights into the understanding of the diverse phenotypes of *P. aeruginosa* within CF lungs and the bacteria's interactions with the hosts.

In *P. aeruginosa*, part of the biofilm formation process, along with a considerable amount of different functions, are controlled by its two intertwined quorum sensing (QS) systems–Las and Rhl (40). Employed to collectively control group behaviors, QS is a bacterial communication system that functions by producing and detecting signal molecules, namely autoinducers. *P. aeruginosa*'s QS systems are composed of transcriptional regulatory proteins–LasR, RhlR and associated autoinducer: *N*-(3-oxododecanoyl) homoserine lactone (in Las system) and *N*-butyryl homoserine lactone (in Rhl system) (41-43). The complexes formed by LasR or RhlR with their corresponding autoinducers modulate the expression of target genes (44), among which

are those controlling the development of biofilm, expression of virulence factors, and sensibility to antibiotics (45). Therefore, quorum sensing inhibitors that can potentially block biofilm formation and bacterial virulence offer latent novel antibiotics (46,47).

3. Mucus and Mucins

In addition to the formation of *P. aeruginosa* biofilm, another important reason for their persistence in CF lungs is the compromised mucociliary movement and cough clearance attributable to the dehydrated and viscous mucus in CF lungs (19,48). Mucus is a frequently-used word that has an ambiguous definition. It's generally considered as the adhesive, viscoelastic, heterogeneous gel produced by goblet cells and submucosal glands that covers and protects apical surfaces of the respiratory, gastrointestinal, and reproductive epithelial tracts (49). The presence of mucus, in cooperation with the ciliary movement, helps the clearance of invaders from our lungs. However this protection mechanism is heavily damaged in CF patients (19,48).

The two main causes for the compromised mucociliary clearance in CF are the dehydrated mucus due to the deficient ion channel CFTR and the overproduction of mucins—a major component of mucus (50). Mucins are large glycosylated proteins (up to ~10^7 Da) produced by epithelial tissues with a signature feature of tandem repeat amino acids that are rich in serine and threonine (sites for *O*-linked glycosylation) (51). The schematic structure of mucin resembles a dumbbell, composed of two hydrophobic globules at the ends and a heavily *O*-glycosylated region in the middle (52), with glycans counting for >70% of the total weight of the molecules. The three major respiratory mucins MUC2, MUC5AC and MUC5B (53) are all subject to regulation by CF

inflammatory stimuli. MUC5AC and MUC5B can be overproduced in CF lung secretion (54,55), especially following exacerbations (50,56). MUC2 gene expression can also be transcriptionally up-regulated by *P. aeruginosa*'s components (57,58). The overexpression of mucins, in turn, contributes to the airway obstruction and impairment of mucociliary clearance of the bacteria (59).

The non-reducing ends of respiratory mucin glycans are mostly composed of four types of monosaccharides: L-fucose, D-galactose, D-*N*-acetylgalactosamine, and D-*N*-acetylneuraminic acid, some of which are occasionally sulfated (60,61). Compared with healthy individuals, chronic inflammation patients often show altered levels of sulfated and sialylated carbohydrates on mucin glycans (62). Sugars at the non-reducing ends of mucin glycans are the major players involved in the lectin-mediated bacterial binding with mucins.

4. Lectins, ***P. aeruginosa*** **binding to airway surface and anti-adhesion therapy**

Respiratory mucus in CF lungs becoming a habitat of *P. aeruginosa* is partially due to the bacteria's propensities to bind mucin glycans through their lectins. Lectins are proteins that bind specifically to certain saccharides, but do not originate from immune systems or have any known enzymatic activities. The basic role of these ubiquitous proteins is to recognize carbohydrate residues on cells, glycoproteins, or glycolipids, leading to a variety of biological functions in animal, plants and microbes (63). The minimum unit of sugars that can be specifically recognized by a lectin is defined as the primary saccharide, which is normally at the non-reducing end of a glycan. And the

secondary saccharide refers to a neighboring part to the primary that can affect the lectin binding affinities.

First discovered in 1888 by Stillmark in castor bean seeds, lectins are now known to be present throughout life forms in nature. Based on the current statistics from the 3D Lectin Database, about 18% of the lectins discovered so far are from bacteria (221 out of 1241). In the microbial world, lectins tend to be called by other names, such as haemagglutinin or adhesins. Bacterial lectins mainly function as either toxins or adhesins, interacting with their host cells and tissues. A wide range of bacteria of their lectins and cognate receptors have been studied. In the book entitled "Bacterial Adhesion to Animal Cells and Tissues", edited by Itzhak Ofek et al in 2003, more than 4,000 references covering ~290 bacteria species were included (64), many of which referred to bacterial lectins. The carbohydrate-binding specificities of particular bacterial lectins are also being elucidated (65,66). The best characterized are shiga toxin produced by *Shigella dysenteria* and three pili adhesins of *Escherichia coli* (65).

P. aeruginosa produces two soluble lectins—LecA and LecB (3), which recognize α-D-galactose- and α-L-fucose-containing glycosides, respectively. They were initially considered to be located in cell plasma (67), but LecB was then proposed to be located on the outer membrane of *P. aeruginosa* (68,69). Although both lectins adopt a small jelly-roll β-sandwich fold and use Ca^{2+} in carbohydrate-binding, they don't seem to be evolutionarily related (70). Their exact functions are not very clear, but there have been many indications of their association with bacterial virulence (3,68). Besides the two lectins, *P. aeruginosa*'s type- IV pili are also considered to be an adhesin, which can specifically bind the carbohydrate sequence β-GalNAc(1–4)-β-Gal (71). Type-IV pili are

noncovalently-associated polymers of pilin monomers, and only the pilin at the tip seems to be able to bind saccharides (5). Furthermore, two components of the flagellum—flagellar cap protein and flagellin, are also adhesins involved in the binding with respiratory mucins (72,73). On a cellular level, numerous laboratory strains and clinical isolates of *P. aeruginosa* have been demonstrated to bind certain mucin glycans *in vitro* (74,75). Besides mucins, respiratory epithelial cells can be bound by the bacteria as well, especially recent daughter cells and those with CFTR mutations (76,77).

A variety of assays have been developed to explore the lectin-mediated bacterial binding with carbohydrates, including solid-phase binding assay, glycoconjugates binding tests, carbohydrate microarrays, binding inhibition tests, etc (78-80). In these methods, fluorophore- or radio-labeled materials were often used to test the binding of bacteria or purified bacterial proteins to carbohydrates, mucins or respiratory epithelial cells. In spite of the lack of consensus about the specific functions of each lectin during the binding process, a reservoir of carbohydrates that might be involved in the bacterial binding has been created (74), which can be used as a resource for our research.

With this bacterial carbohydrate-binding information on hand, researchers have been trying to use competitive carbohydrates or carbohydrate analogs to inhibit the lectin-mediated bacterial binding, and further eradicate infections. Administrations of simple sugars or glycosides as anti-*P. aeruginosa* agents have shown encouraging results *in vitro* (81), *in ex vivo* (82), and in animal models (83). Two clinical reports using this strategy have also been published. In a case reported in Germany, an infant with serious *P. aeruginosa* respiratory infection after chemotherapy for neuroblastoma, which could not be treated with antibiotics effectively, was subjected to additional D-galactose and L-

fucose inhalation (~3.6 mg of each, twice daily) and cured (8). In a clinical trial with adult CF patients, also using D-galactose and L-fucose inhalation (180 mg of each, twice daily) as additional treatment for *P. aeruginosa* infection, encouraging results were reported (7). In addition to these specific anti-adhesion targeting sugars, mannitol, xylitol (both sugar alcohols) and some polysaccharides (dextran, dextran sulphate, heparan, etc.), have been tested in animal models and in CF patients as well, showing discrepant findings (84-86). The exact functions of these carbohydrates remain unclear, but their potential anti-adhesion effects should not be excluded.

Another anti-adhesion therapy target attracting much attention is FimH—the adhesin subunit at the tip of type I pili in *E. coli*. As a key virulence factor in the urinary tract *E. coli* infection, this mannose-binding adhesin is critical in several infection stages (87). Since the first attempt to use methyl α-D-mannopyranoside as an anti-adhesion therapeutic (88), great effort has been devoted to designing α-mannose analogs with high competitive binding efficacies. Two major strategies have been adopted to increase affinities towards lectins: monovalent inhibitors with novel aglycone moieties, and glycoconjugates with multivalent carbohydrate epitopes. Both strategies have demonstrated promising results in cell/tissue cultures and animal models (6,89-93), and yet no clinical trials have been reported so far. Using multivalent glycopolymers in anti-adhesion tests may cause unfavorable cross-linking of bacteria (94). But, in the investigation of bacterial carbohydrate-binding, these polymers have been instrumental.

5. Glycopolymers: application and preparation

By definition, glycopolymers are synthetic polymers with pendant sugar residues (95). Due to the low binding affinities of some lectins to target carbohydrates (Kd in the mM range), glycopolymers that are 10^2–10^4 more potent than monomeric sugars are of great interest in studying lectins (96,97). These polymers are also being employed in several other fields, like targeted drug and gene deliveries (98-100), bio-compatible materials (101,102), and even disease treatments (103-105). Several informative reviews have been published on this subject matter (106-108).

Besides glycopolymers, a variety of glycoconjugates that afford multivalent carbohydrates have been developed, including saccharide-coated gold nanoparticles, quantum dots or nanotubes (109-111); carbohydrates-conjugated peptides and proteins (112); dendritic glycoconjugates (113), etc. Compared with other glycoconjugates, linear glycopolymers hold several advantages owing to their inherent flexibility, high efficacy, versatility in applications, and the structural comparability with mucins, making them ideal for lectin-mediated bacterial binding studies (114).

The first linear glycopolymer was synthesized in 1978 using free radical polymerizations (115). Since then, a number of polymerization techniques offering better control of the reactions have been applied in glycopolymer syntheses. One of these methods is the reversible addition–fragmentation chain-transfer (RAFT) polymerization. As a type of living polymerization, RAFT reactions employ a chain transfer agent containing a thiocarbonylthio group to control the degree of polymerization and dispersity of the products (116). First reported by Thang et al. in 1998 (117), RAFT polymerization was introduced in the glycopolymer preparation in 2003 (118), and

gained great attention. Glycopolymers with different structures have been synthesized with RAFT agents, like star-shaped dendrimers, block copolymers, statistical random copolymers, etc. (119-121). RAFT agents are popular in glycopolymer preparations for several reasons: 1) they provide predictable molecular weights and low dispersities of the products; 2) they are compatible with a variety of solvents, including aqueous environments, which is vital for synthesizing polymers with high contents of carbohydrates; 3) they are tolerant with a wide range of monomer's functionalities and reaction temperatures varying from 25–70°C.

Other than the copolymerization strategy employed in synthesizing glycopolymers, another method using preactivated polymers was also proposed by Whitesides' and Bovin's groups separately in the 1990s (122,123). Polymers preactivated by possessing active esters, like *N*-(acryloyloxy) succinimide and *p*-nitrophenol acrylate, are then conjugated with glycoside and fluorophore side chains that contain primary amine functional groups through the formation of amide bonds. Compared with the copolymerization strategy to prepare glycopolymers, this preactivated-polymer method has its own advantages. First, the length and dispersity of the polymer backbone are kept constant, if the same batch of preactivated polymer is used for all syntheses. Second, the distribution of pendant sugars along the backbone is theoretically more uniform than that of copolymerization (122,124).

In our preliminary carbohydrate-binding tests with *P. aeruginosa*, very encouraging results were obtained using commercial glycopolymers from Lectinity Holding (Moscow, Russia), which were prepared with the preactivated polymer method. However, the limited supply source and our intention to study the effects of polymer-

structural variance on the bacterial binding tests prompted us to synthesize some of the glycopolymers on our own.

In this dissertation, Chapter II describes our initial unproductive attempt to synthesize glycopolymers following the preactivated polymer method published by Bovin's group (123). Due to the absence of important reaction details in the literature, some syntheses procedures for the sugar side chains had to be developed based on other references. Our efforts of using various reaction reagents and conditions to conjugate side chains onto the polymer backbone are included.

Chapter III details the preparation of a panel of fluorescent glycopolymers using RAFT agents and the use of some of the polymers in lectin-binding tests. In the preparation, (4-cyanopentanoic acid)-4-dithiobenzoate was used as the RAFT agent because of its compatibility with polar solvents and acrylamide/methacrylamide glycomonomers. In the glycomonomers' syntheses, a procedure proposed by Narain's group to conjugate pendant sugar to acrylamide monomers was adopted with modifications (125). The binding specificities and avidities of the synthesized glycopolymers were first confirmed with corresponding plant-lectins coated on agarose beads, and then utilized in bacterial binding tests.

Chapter IV depicts our investigation of the carbohydrate-binding behaviors of a group of *P. aeruginosa* laboratory strains and clinical isolates from CF patients. Using a panel of commercial fluorescent glycopolymers containing different glycosides, together with a number of analytical techniques, the monosaccharide-binding of this Gram-negative bacterium was profiled.

Chapter V contains the discussion of the dissertation and possible future research directions of this work.

The two major aims of this work listed at the beginning of this chapter were fulfilled. Our efforts to establish an improved methodology to study bacterial carbohydrate-binding include the syntheses of the fluorescent glycopolymers and the development of methods of using these polymers with the aid of different analytical instruments. The monosaccharide-binding profiles of *P. aeruginosa* are characterized in Chapter IV. Employing the fluorescent glycopolymers and the procedures developed herein will help elucidate the carbohydrate-binding patterns of different bacterial pathogens that are of clinical significance, which is instrumental to improve our understanding of the pathogenesis of bacterial infections and provide new perspectives for the anti-adhesion therapies.

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Chapter II. Preparation of Fluorescent Glycopolymers with Preactivated Polymer Poly(*p*-nitrophenyl acrylate)

Introduction

Polyacrylamide (PAA)-based fluorescent glycopolymers (structure shown in Figure II-1), have been used in a variety of lectin-mediated carbohydrate-binding studies (1-4). Our initial tests employing PAA glycopolymers demonstrated that they could be very instrumental in *P. aeruginosa* carbohydrate-binding experiments. Though these glycopolymers were commercially available, some of the products were not attainable in the amount required for our binding studies, and the delivery of the merchandise took an

unreasonably long time. Because synthesizing some of these glycopolymers will provide us with better control over the structural variants of the glycopolymers, we decided to prepare some of the polymers.

Two decades ago, Nicolai Bovin's group in Moscow published a synthesis method for fluorescent glycopolymers (5). In the protocol, which was claimed to be "very simple to carry out" (2), the authors prepared the *p*-nitrophenyl acrylate polymer backbone first, and then attached side chains containing primary amine functional groups onto the polymers through the formation of amide bonds. Different side chains include glycosides, fluorescent tags, and ethanolamine (spacer on the polymer). But due to the inadequate description of glycosides side chains preparations and the selection of fluorescent tags in the Bovin's paper, the glycoside side chains syntheses were adopted from other literature sources and a series of fluorophores were tested in the reactions.

The resultant glycopolymers were analyzed by gel permeation chromatography (GPC) for their molecular weights and dispersities, the sugar contents of the polymers were determined, and their lectin-mediated bacterial binding efficacies were tested with *P. aeruginosa,* using corresponding commercial glycopolymers as a positive control.

Materials and Methods

Materials

Fluorophores were purchased from Life Technologies (Carlsbad, California). The remaining chemicals were purchased from Sigma-Aldrich Chemicals (St Louis, MO). Unless mentioned otherwise, all chemicals were used as received. Bacteria strains were obtained from ATCC (Manassas, VA).

Synthesis of 2-aminoethyl 2-acetamido-2-deoxy-β-D-galactopyranoside (see Schemes II-1)

2-(Benzyloxycarbonyl)aminoethyl-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galacto-pyranoside was prepared according to published methods with some modifications (6,7). In brief, 1.0 g of β-D-galactosamine pentaacetate and 1.25 g of *N*-Cbz-ethanolamine were dissolved in 10 mL of dry acetonitrile under Ar atmosphere. The mixture was cooled to 0°C and 360 μL of $SnCl_4$ was added dropwise. The reaction was allowed to warm to room temperature and stirred at 75°C for 16 h. The mixture was then allowed to cool to room temperature, quenched with 2 mL of triethylamine and concentrated under vacuum. The residue was dissolved in 50 mL of CH_2Cl_2 and washed with water (3 x 30 mL), and the organic phase was dried over $MgSO_4$ and evaporated. The products were separated by column chromatography (EtOAc/hexane 80:20 on silica), deacetylated with sodium methoxide, and hydrogenated with Pd/C to obtain 2-aminoethyl 2-acetamido-2-deoxy-β-D-galactopyranoside (0.39 g, 58% yield, three steps).

Synthesis of 1-amino-1-deoxy-melibiitol (see Schemes II-2)

To 20 mL aqueous solution of 4.0 g of melibiose was added 7.5 mL of NH_4OH and 6.65 mL of glacial acetic acid. The mixture was stirred at 65°C for 1.5 h before 8.0 g of sodium cyanoborohydride were added. The reaction was stirred at 65^0C for another 3 h when TLC (1% acetic acid in MeOH) showed no free sugar left. The volume of the reaction was then reduced under vacuum and a mixture of 100 mL of MeOH and 10 mL of isopropanol were added to precipitate the product, which was then washed twice with cold MeOH and dried under vacuum to yield the product as syrup (6.0 g). The product 1-

amino-1-deoxy-melibiitol was obtained by preparative TLC using *n*-butanol: acetic acid: water (2:1:1) as the solvent system (Rf=0.44, 1.58 g, 39% yield). ESI-MS (in methanol: water 50:50): calculated *m/z* for $C_{12}H_{25}O_{10}N+Na^{+}$, 366.13707; found m/z 366.13638.

Synthesis of *p*-nitrophenyl acrylate and poly(*p*-nitrophenyl acrylate) (see Schemes II-3)

p-Nitrophenyl acrylate was prepared according to a published method (8). Briefly, *p*-nitrophenol (21 g), 21 mL of triethylamine, and 150 mL of 2-butanone were mixed in a 1 L flask and 13 mL of acryloyl chloride was added dropwise to the mixture that was vigorously stirred in an ice bath. The reaction was stirred for 1 h in the ice bath and another 16 h at room temperature. The mixture was then evaporated and washed sequentially with water, 1% NaOH, very dilute acetic acid and once again water. *p*-Nitrophenyl acrylate was obtained from recrystallization from hexane (24 g, 83% yield).

Poly(*p*-nitrophenyl acrylate) was synthesized based on a published method (5). To a 1 M solution of *p*-nitrophenyl acrylate in dry benzene were added 2,2′-azobis(2-methylpropionitrile) (3% w/w to the monomer) under a stream of nitrogen and stirred at 70°C for 50 h. The benzene solution was then decanted and the brown residues were dissolved in dimethylformamide (DMF) to obtain a 1% solution. The polymers were re-precipitated with five volumes of methanol twice, and the clean white residue was washed again with methanol and dried to obtain the products (83% yield).

Synthesis of PAA-Glucamine-Fluor (see Schemes II-4)

To a solution of 7.7 mg of poly (*p*-nitrophenyl acrylate) (40 μmol of phenol groups) in 2 mL of DMF, were added a solution of glucamine (1.45 mg) in 1.5 mL of DMF, fluorescence tags (molar ratio 1% to phenol groups, structures shown in Figure II-2) in 0.5 mL of DMF, and 40 μL of triethylamine. The mixture was stirred at room temperature for 16 h in the dark, and a 50-fold molar excess of ethanolamine (120 μL) was then added. Following another 16 h of stirring at room temperature, the solution was dialyzed against distilled water (5 x 2 L) for 24 h (MWCO=7,500) and freeze-dried to obtain the products (6.5 mg), which were used without further purification.

Synthesis of PAA-Sugar-Fluor (see Schemes II-5)

DMF **s**olutions (2 mL) containing various amount of 2-aminoethyl 2-acetamido-2-deoxy-β-D-galactopyranoside or 1-amino-1-deoxy-melibiitol (molar ratio 20-50% to phenol groups) and different fluorescence tags (molar ratio 1-4% to phenol groups, structures shown in Figure II-2) were added to a solution of 7.7 mg of poly (*p*-nitrophenyl acrylate) (40 μmol phenol groups) in 2 mL of DMF, respectively. Equivalent amount of triethylamine or diisopropylethylamine to the glycosides was then added. The mixtures were stirred at room temperature or 60°C for 16–72 h in the dark, and 120 μL of ethanolamine was then added. Following another 16 h of stirring at room temperature, the solutions were then either dialyzed against distilled water 5 x 2 L for 24 h (MWCO=7,500) and freeze-dried, or precipitated in acetone and dried under vacuum. Polyacrylamide-based fluorescent glycopolymers possessing different ratios of pendant

α-D-galactoside (α-Gal) or β-D-N-acetylgalactosamine (β-GalNAc) and fluorophores were obtained and used without further purification.

Analysis of glycopolymers

Synthesized glycosides, dissolved in MeOH/water 50:50 (v/v) were subjected to direct electrospray mass spectrometry analyses, using a Thermo Scientific LTQ Orbitrap XL hybrid Fourier transform mass spectrometer. Molecular weights and the dispersities of the glycopolymers were derived against a calibration curve from polyethylene glycol standards using a Waters Alliance HPLC system equipped with a refractive index detector (Waters e2695, 2414) and TOSOH TSK-GEL G4000 PWxl and a TSK-GEL G4000 SW GPC columns using 0.1M Tris/0.1M sodium chloride buffer (pH=7) as eluent at a flow rate of 0.6 mL/min. Total carbohydrate content of the synthesized glycopolymers was analyzed according to a published protocol (9).

Bacterial binding tests using synthesized fluorescent glycopolymers

P. aeruginosa ATCC 39018 cultured on trypticase soy agar for 24 h were lifted and suspended in 20 mL of binding solution (155 mM NaCl, 1 mM $CaCl_2$, and 1% bovine serum albumin) to attain a bacterial suspension with an optical density of 1.0 at 600 nm. For each binding experiment, 100 µg of the fluorescent glycopolymers dissolved in 100 µL sodium phosphate buffer (0.3 M, pH=7.4) were added to 1.0 mL of bacteria suspension, respectively. Following 2 h of incubation at 35°C in the dark, the mixtures were centrifuged (8,000 x g, 10 min), and the pellets were washed three times with 1.2 mL of rinsing solution (155 mM NaCl, 1 mM $CaCl_2$). The final pellets were

then resuspended in 100 μL of PBS (pH=8.0), and aliquots were taken for analysis with fluorescence microscopy and fluorimetry.

Results and discussion

Based on the results from bacterial binding tests with commercial PAA-α-Gal-Fluor and PAA-β-GalNAc-Fluor, *P. aeruginosa* can specifically bind these two sugars. However, the positive binding results could not be repeated with fluorescent glycopolymers synthesized in our laboratory.

To investigate possible reasons of non-binding with bacteria, we tested various reaction conditions and reagents in the step of attaching the glycosides onto the polymer backbone that were reported by the Bovin's group in different publications (5,10,11). These variants include different reaction times and temperatures, use of triethylamine or diisopropylethylamine, different stoichiometry of the reagents and purification methods. However, positive binding was still not observed. Several reasons for the unsuccessful synthesis attempt can be speculated.

First, the efficiency of attaching glycosides onto the polymer backbone might be lower than expected. This speculation is supported by the fact that during the quantification of the total carbohydrate contents in the synthesized polymers, the actual molar ratios of the sugar side chains were always lower than the theoretical stoichiometric values. For instance, only a molar ratio of ~7% of sugar side chains containing α-Gal was detected in the sugar profile test for the synthetic polymers from a reaction where a 20% molar ratio was used. It should also be noted that due to the small

quantity of the available commercial PAA polymers, we were unable to confirm the content of sugar side chains to be 20% in their glycopolymers as claimed by the supplier.

Second, the removal of the nitrophenol groups from the backbone might not be complete in the reactions, which could potentially interfere with the lectin-mediated bacterial binding with the glycopolymers. During the characterization of the resultant glycopolymers, a new GPC column (TOSOH TSK-GEL G4000 PWxl) that was utilized to measure the molecular weights of the synthesized glycopolymer subsequently became dysfunctional, likely due to the minor presence of nitrophenol groups on the polymers or other reaction byproducts that could not be removed.

Third, many details of the chemical syntheses, especially for the preparation of the glycoside side chains and the choice of fluorophores, were not included in any of the published manuscripts from Bovin's group. The difficulties of chemically analyzing the resultant glycopolymers and the absence of the required reaction details resulted in significant difficulties in the preparation of glycopolymers. This unsuccessful preparation compelled us to adopt another synthesis strategy that is described in Chapter III.

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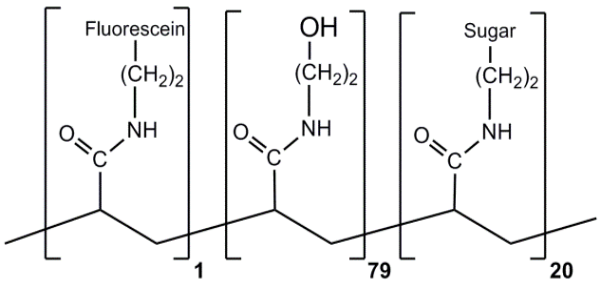


Figure II-1. Schematic structure of PAA glycoconjugates.

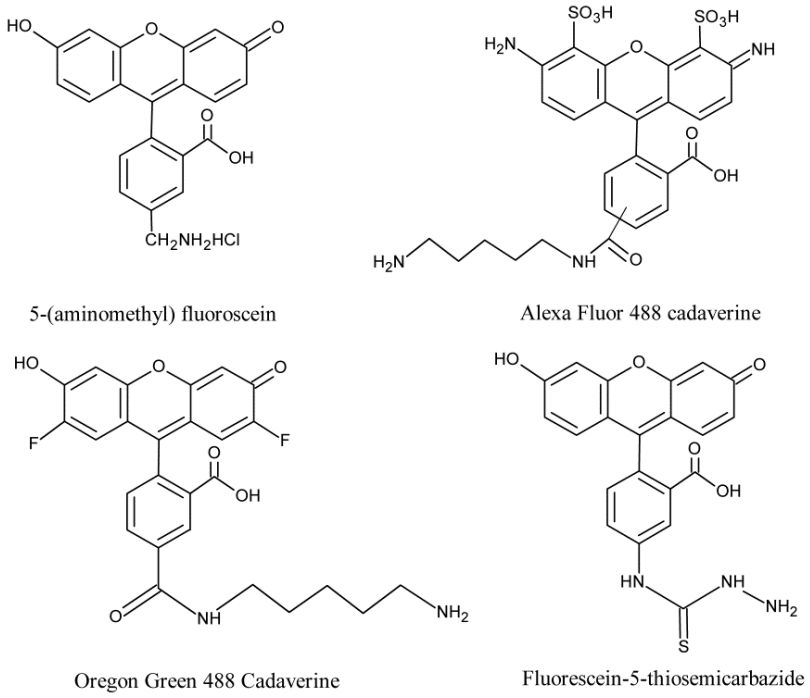
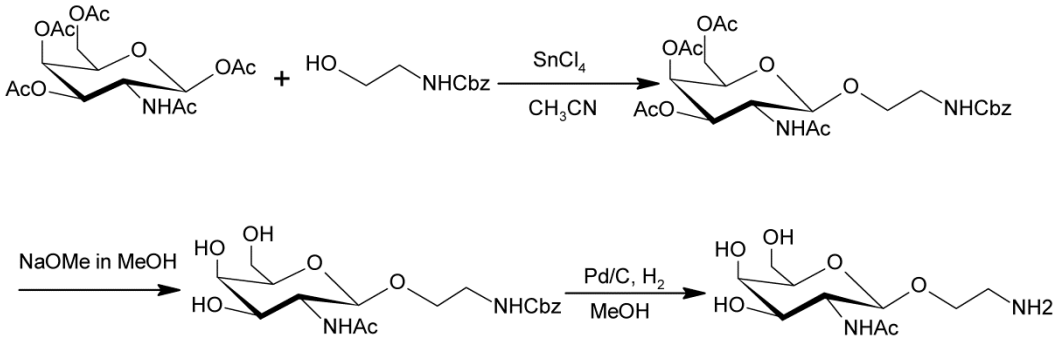
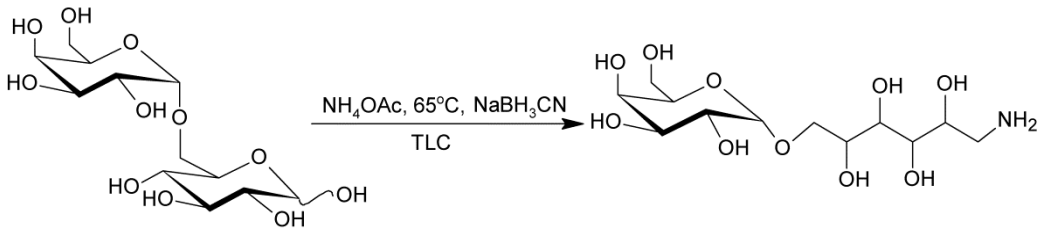


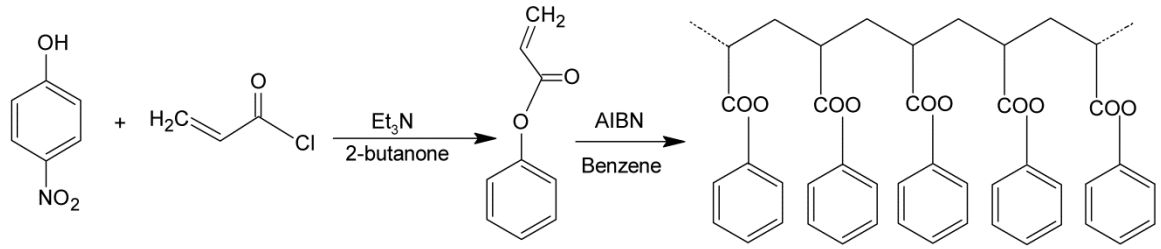
Figure II-2. Fluorescent tags possessing primary amine functional groups used in this study.



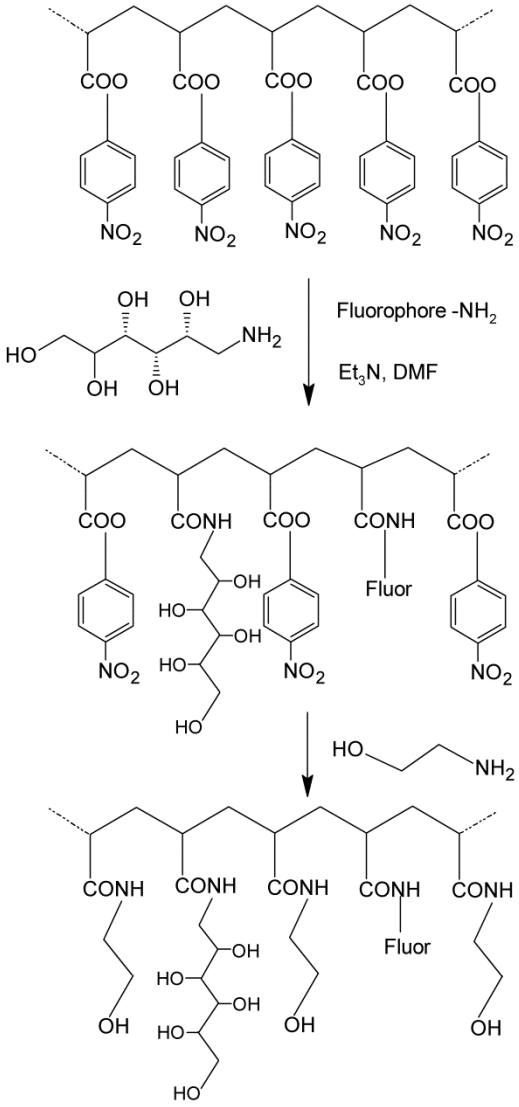
Schemes II-1. Synthesis of 2-aminoethyl 2-acetamido-2-deoxy-β-D-galactopyranoside.



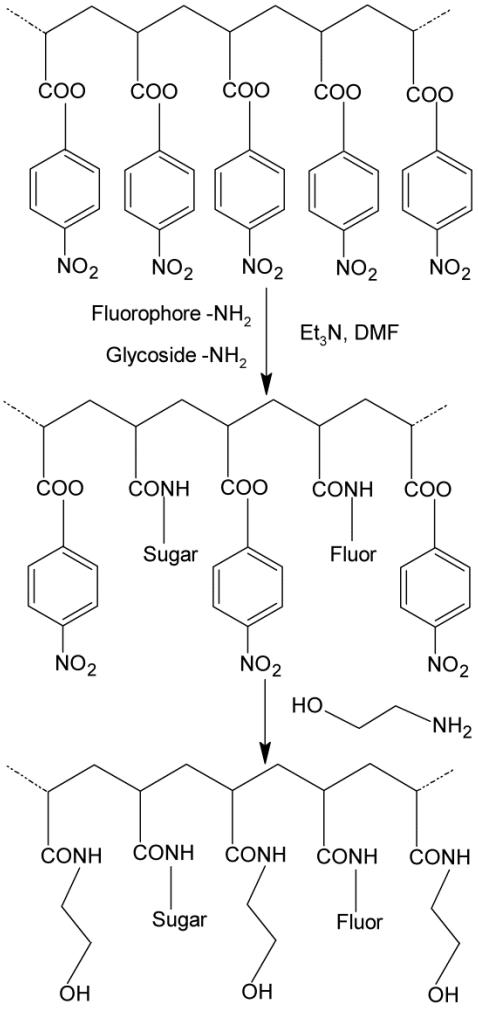
Schemes II-2. Synthesis of 1-amino-1-deoxy-melibiitol.



Schemes II-3. Synthesis of *p*-nitrophenyl acrylate and poly(*p*-nitrophenyl acrylate).



Schemes II-4. Synthesis of PAA-Glucamine-Fluor.



Schemes II-5. Synthesis of PAA-Sugar-Fluor.

Chapter III. RAFT-based Tri-component Fluorescent Glycopolymers: Synthesis, Characterization and Application in Lectin-mediated Bacterial Binding Study

Abstract

A group of fluorescent statistical glycopolymers, prepared via reversible addition-fragmentation chain-transfer (RAFT)-based polymerizations, were successfully employed in lectin-mediated bacterial binding studies. The resultant glycopolymers contained three different monomers: *N*-(2-hydroxyethyl) acrylamide (HEAA), *N*-(2-aminoethyl) methacrylamide (AEMA), and *N*-(2-glyconamidoethyl)-methacrylamides possessing

different pendant sugars. Low dispersities (≤1.32) and predictable degrees of polymerization were observed among the products. After the polymerization, the glycopolymers were further modified by different succinimidyl ester fluorophores targeting the primary amine groups on AEMA. With their binding specificities being confirmed by testing with lectin coated agarose beads, the glycopolymers were employed in bacterial binding studies, where polymers containing α-galactose or β-galactose as the pendant sugar were specifically bound by two clinically important pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus,* respectively. This is the first report of using RAFT-based glycopolymers in bacterial binding studies, and the ready access to tri-component statistical glycopolymers also warrants further exploration of their utility in other glycobiological applications.

Introduction

In the past two decades, the use of synthetic glycopolymers in biochemical and biomedical research areas has acquired widespread attention. Various types of glycoconjugates were applied in studies of lectin recognition processes (1-3), drug and gene delivery systems (2,4,5), disease treatments (6-9), to note a few. Among different designs of glycoconjugate structures, linear glycopolymers with pendant carbohydrate moieties have certain advantages because of the inherent flexibility, efficacy, and their structural comparability with some natural glycoproteins, making them very suitable for cell-carbohydrates recognition studies (3). In our clinical research, we are particularly interested in the lectin-carbohydrate interactions of key pathogenic bacteria with surface carbohydrates that are found on human tissue cell surfaces and extracellular matrices, a

process that is considered to be highly involved in the initial step of bacterial adhesion and the persistence of some infections (10,11). Many important human respiratory pathogenic bacteria, like *Pseudomonas aeruginosa, Staphylococcus aureus, Helicobacter pylori, Escherichia coli,* etc., have been widely studied in their lectin binding behaviors (12). Due to the naturally low binding affinities of many lectins to target carbohydrates, especially monosaccharides, multivalent glycoconjugates possessing higher lectin binding affinities are of great advantage in studying bacterial lectins (3). Our initial success with the use of commercially available polyacrylamide-based glycoconjugates in the phenotypic expression of lectins of human respiratory pathogenic bacteria prompted us to develop a facile approach to well-defined polymers that would allow for varied pendant carbohydrates, along with different fluorescent labels.

In this pursuit, we turned our attention to a type of living or controlled radical polymerization using reversible addition−fragmentation chain-transfer agents (RAFT) (13) that have been successfully applied in the synthesis of glycopolymers. Specifically, the RAFT reactions demonstrate tolerance of a diversity of participating monomers and reaction conditions, as well as, the low dispersity of the products (14-16), all of which are desirable characteristics. Several excellent reviews have been published on the utilization of RAFT in preparing glycopolymers (16-18). Tri-component glycopolymers have also been synthesized through RAFT-based reactions, however, most of them either used stepped polymerizations to prepare tri-block copolymers (19-21) or did not possess anomeric pendant sugars appropriate for lectin-binding studies (22). To the best of our knowledge, this is the first report of well-defined statistical tri-component glycopolymers prepared via one-step RAFT reactions, which possess varied pendant carbohydrates, the

versatility of post-modification with varied fluorescent labels, and demonstrated utility in targeted lectin-mediated bacterial binding.

Materials and methods

Materials

Unless mentioned otherwise, all chemicals were used as received. *N*-(2-aminoethyl) methacrylamide hydrochloride was purchased from Polysciences (Warrington, PA). Texas Red-X succinimidyl ester and 5-(and-6)-carboxyfluorescein succinimidyl ester were bought from Molecular Probes (Grand Island, NY). *Marasmius oreades* agglutinin coated lectin beads were obtained from EY laboratories (San Mateo, CA), *Galanthus nivalis* lectin (GNL) and peanut agglutinin lectin beads from Vector Laboratories (Burlingame, CA). Bacteria strains were from ATCC (Manassas, VA). TOSOH TSK-GEL G4000 PWxl (30 cm x 7.8 mm) and G4000 SW (30 cm x 7.5 mm) HPLC columns were from Fisher Scientific (Pittsburgh, PA). Anion-exchange resin (Amberlite IRN-78 hydroxide-form, 80 mesh), cation-exchange resin (Dowex 50Wx8, 200 mesh), hydroquinone monomethyl ether (MEHQ), Orange II sodium salt, *N*-(2-hydroxyethyl) acrylamide (HEAA), and other chemicals, not noted above, were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). The chemical structures of some of key reagents used in this study are shown in Figure III-1.

Synthesis of disaccharides (see Scheme III-1)

Disaccharides employed in this study, namely β-D-galactopyranosyl-(1→6)-D-glucose (allolactose) and α-D-mannopyranosyl-(1→6)-D-glucose were prepared through a

modified Koenigs-Knorr reaction. As an example, 1.0 g of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose (23) (2.87 mmol) was dissolved in 10.0 mL dry CH_3CN. To the solution, 1.2 g of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (2.92 mmol) and 0.8 g mercury (II) cyanide (3.12 mmol) were added. The reaction was stirred for 16 h under an argon atmosphere at room temperature, at which time 2.0 mL of pyridine and acetic anhydride were added. The stirring continued for 5 min at 60°C, at which time the volume of the reaction mixture was reduced *in vacuo*. The syrupy residue was then redissolved in 200 mL of CH_2Cl_2, washed successively with water, saturated $NaHCO_3$, and brine, then dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by flash chromatography on a silica gel column (70-230 mesh, 60Å, 15mm x 50mm, ethyl acetate/hexane 1:1), yielding 1,2,3,4,2′,3′,4′,6′-octa-*O*-acetyl-β-allolactose (1.65 g, 85% yield) as a colorless syrup.

Under an atmosphere of dry nitrogen, per-*O*-acetyl-β-allolactose (1.0 g, 1.47 mmol) was then dissolved in 150 mL dry MeOH, to which was added, with rapid stirring, 1.5 mL of a sodium methoxide solution (25 wt. % in dry MeOH) over a 2 min period. The reaction was stirred for an additional 30 min at room temperature and stopped by the addition of cation-exchange resin (Dowex 50W×8, H^+ form). The resin was removed by filtration and the filtrate evaporated *in vacuo* to yield allolactose (0.49g, 97% yield).

Synthesis of 6-*O*-α-D-mannopyranosyl-D-glucose, prepared in a similar manner, yielded 0.56g (80% yield) of the disaccharide.

Synthesis of disaccharide lactones (see Scheme III-2)

Disaccharides melibiose, lactose, allolactose, and 6-*O*-α-D-mannopyranosyl-D-glucose, employed in this study, were first oxidized to their corresponding lactones according to Moore, et al. (24). As an example, 2.0 g of melibiose (5.84 mmol) were dissolved in 4.0 mL of water, diluted with 5.0 mL MeOH, and then added to 40 mL of MeOH containing 2.9 g of iodine at 40°C. With stirring, 70 mL of 4% (w/v) KOH in MeOH was then added dropwise over a period of 30 min until the iodine color completely disappeared. The mixture was then cooled on ice for 1 h. The precipitate of potassium melibionate was then filtered, rinsed with cold MeOH, recrystallized from 100 mL MeOH/water (9:1, v/v), then dried *in vacuo*. The resulting melibionic acid salt was then dissolved in 10 mL water and converted into its free acid form by passing the solution through a strongly acidic cation-exchange column (Dowex 50W×8, H^+ form) and then freeze-dried to yield approximately 2.0 g. This was then dissolved in a minimal amount of MeOH (~3.0 mL) to which absolute EtOH was added until the solution turned just cloudy, at which time the solvents were evaporated *in vacuo*. This was repeated 4 times to yield melibiono-1,5-lactone (1.94 g, 98% yield). Other disaccharides, oxidized in a similar manner, produced comparable yields (>95%).

Synthesis of glycomonomers

2-Gluconamidoethyl methacrylamide (GAEMA) and 2-lactobionamidoethyl methacrylamide (LAEMA)

2-Gluconamidoethyl methacrylamide (GAEMA) was prepared according to a published protocol (19) and 2-lactobionamidoethyl methacrylamide (LAEMA) was

prepared with some modifications. To the solution of 1.0 g of lactobiono-1,5-lactone (2.94 mmol) in 3.0 mL of MeOH, were added *N*-(2-aminoethyl) methacrylamide hydrochloride **(**AEMA· HCl, 0.58 g, 3.53 mmol) and 1.0 mg hydroquinone monomethyl ether (MEHQ, an inhibitor of self-polymerization) in 2.0 mL of MeOH and1.0 mL of triethylamine. The mixture was stirred at room temperature for 48 h, at which time 20 mL of water was added. Then, MeOH and triethylamine were removed from the mixture *in vacuo*. To remove any remaining lactobionate, the resulting aqueous solution was passed through an anion exchange column (Amberlite IRN-78, OH^- form, 10 mm x 20 mm) into a receiving beaker containing 1.0 mg of MEHQ. Residual triethylamine was then removed from the eluate *in vacuo*. Dowex 50W×8 (H^+) was added portionwise to the solution, until no ninhydrin reactive material remained. The mixture was then filtered and freeze-dried. Removal of MEHQ from the freeze-dried product was accomplished by dissolving the material in a minimum amount of MeOH, which was then added to ice cold acetone to precipitate LAEMA. Following filtration and drying, LAEMA (0.89 g, 65% yield) was obtained as white powder. The purity and structure of the final product were confirmed by ^{1}H NMR, ^{13}C NMR and mass spectrometry (Figure III-2, 3). Anal. Calcd for $C_{18}H_{32}N_2O_{12}$+3/2H_2O: C, 43.63; H, 7.12; N, 5.65. Found: C, 43.80; H, 7.00; N, 5.40%

2-Allolactobionamidoethyl methacrylamide (ALAEMA, see Scheme III-3)

To the solution of 1.0 g of allolactobionolactone (2.94 mmol) in 3.0 mL of MeOH, were added *N*-(2-aminoethyl) methacrylamide hydrochloride **(**AEMA, 0.58 g, 3.53 mmol) and hydroquinone monomethyl ether (MEHQ, 1.0 mg) in 2.0 mL of MeOH

and1.0 mL of triethylamine. The mixture was stirred at room temperature for 48 h, at which time 20 mL of water was added. Then, MeOH and triethylamine in the mixture were removed *in vacuo*. To remove any remaining allolactobionic acid, existing as its triethylamine salt, the resulting aqueous solution was passed through an anion exchange column (10 mm x 20 mm) into a receiving beaker containing 1.0 mg of MEHQ. Resulting triethylamine was then removed *in vacuo*. Cation exchange resin was added to the solution, stepwise, until no ninhydrin reactive material was left (by thin layer chromatography). The mixture was then filtered and freeze-dried. Removal of MEHQ in the freeze-dried product was accomplished by dissolving the material in a minimum amount of MeOH, which was then added to cold acetone to precipitate ALAEMA. Following filtration and drying, ALAEMA (0.82 g, 59% yield) was obtained. The purity and structure of the final product were confirmed by ^{1}H NMR, ^{13}C NMR and mass spectrometry (Figure III-4, 5). Anal. Calcd for $C_{18}H_{32}N_2O_{12}$+3/2H_2O: C, 43.63; H, 7.12; N, 5.65. Found: C, 43.54; H, 6.51; N, 5.43%.

6-α-D-Mannopyranosyl-D-gluconamidoethyl methacrylamide (MGAEMA)

To the solution of 1.0 g of 6-*O*-α-D-mannopyranosyl-D-gluconolactone (2.94 mmol) in 3.0 mL of MeOH, were added AEMA (0.58 g, 3.53 mmol) and 1.0 mg MEHQ in 2.0 mL of MeOH and1.0 mL of triethylamine. The mixture was stirred at room temperature for 48 h, at which time 20 mL of water was added. Then, MeOH and triethylamine in the mixture were removed *in vacuo*. To remove any remaining disaccharide acid, existing as its triethylamine salt, the resulting aqueous solution was passed through an anion exchange column (10 mm x 20 mm) into a receiving beaker

containing 1.0 mg of MEHQ. Resulting triethylamine was then removed *in vacuo*. Cation exchange resin was added to the solution, stepwise, until no ninhydrin reactive material was left (by thin layer chromatography). The mixture was then filtered and freeze-dried. Removal of MEHQ in the freeze-dried product was accomplished by dissolving the material in a minimum amount of MeOH, which was then added to cold acetone to precipitate MGAEMA. Following filtration and drying, MGAEMA (0.89 g, 64% yield) was obtained. The purity and structure of the final product were confirmed by ^{1}H NMR, ^{13}C NMR and mass spectrometry (Figure III-6, 7). Anal. Calcd for $C_{18}H_{32}N_2O_{12}+3/2H_2O$: C, 43.63; H, 7.12; N, 5.65. Found: C, 43.84; H, 7.03; N, 5.43%

2-Melibionamidoethyl methacrylamide (MAEMA)

To the solution of 1.0 g of melibionolactone (2.94 mmol) in 3.0 mL of MeOH, were added AEMA (0.58 g, 3.53 mmol) and 1.0 mg MEHQ in 2.0 mL of MeOH and1.0 mL of triethylamine. The mixture was stirred at room temperature for 48 h, at which time 20 mL of water was added. Then, MeOH and triethylamine in the mixture were removed *in vacuo*. To remove any remaining melibionic acid, existing as its triethylamine salt, the resulting aqueous solution was passed through an anion exchange column (10 mm x 20 mm) into a receiving beaker containing 1.0 mg of MEHQ. Resulting triethylamine was then removed *in vacuo*. Cation exchange resin was added to the solution, stepwise, until no ninhydrin reactive material was left (by thin layer chromatography). The mixture was then filtered and freeze-dried. Removal of MEHQ in the freeze-dried product was accomplished by dissolving the material in a minimum amount of MeOH, which was then added to cold acetone to precipitate MAEMA.

Following filtration and drying, MAEMA (0.94 g, 68% yield) was obtained. The purity and structure of the final product were confirmed by ^{1}H NMR, ^{13}C NMR and mass spectrometry (Figure III-8, 9). Anal. Calcd for $C_{18}H_{32}N_2O_{12}$+3/2H_2O: C, 43.63; H, 7.12; N, 5.65. Found: C, 43.19; H, 6.77; N, 5.37%

Kinetic experiment for the RAFT tri-component polymerization of GAEMA

To a 5 ml Schlenk tube equipped with a septum, was added 2.4 mL water containing 128.5 mg of GAEMA (419.5 μmol), 10.3 mg AEMA·HCl (62.9 μmol) and 166 μL of HEAA (1615.1 μmol) thus producing a monomers molar ratio of 20:3:77, respectively. To the solution were then sequentially added 0.3 mL dimethylformamide (DMF) containing 3.1 mg of (4-cyanopentanoic acid)-4-dithiobenzoate [11 μmol, chain transfer agent (CTA)], and 0.3 mL DMF containing 1.5 mg 4,4′-azobis (4-cyanovaleric acid) (5.4 μmol, initiator). The resulting molar ratio of $[M]_0$:[CTA]:[Initiator] was 380:2:1, where $[M]_0$ is the initial total monomers concentration. The solution was then degassed with 3 freeze–evacuate–thaw cycles and the Schlenk tube transferred to a water bath at 70°C. At each time point of the copolymerization (2, 4, 7, 11, 24, and 35 h), 200 μL of the solution were withdrawn with an argon-purged gas tight syringe and precipitated in 10 mL of cold acetone containing 1.0 mg of MEHQ. The precipitates were dried *in vacuo* and prepared for gel permeation chromatography (GPC) and NMR analysis, as noted below. The conversion rates of GAEMA were determined by comparing the proton signal changes from both H-2 (δ 4.30-4.40) and H-3 (δ 4.05-4.15) (Figure III-10).

One-step tri-component RAFT-based copolymerization (see Scheme III-4)

To a 1 ml Schlenk tube equipped with a septum, was added 0.4 mL water containing 21.4 mg of GAEMA or 32.8 mg of disaccharide monomers (70.0 μmol, Table III-1), 1.7 mg AEMA (10.5 μmol) and 27.5 μL HEAA (270 μmol), thus having a monomer molar ratio of 20:3:77, respectively. To the monomer solution were then sequentially added 50 μL DMF containing 0.53 mg of (4-cyanopentanoic acid)-4-dithiobenzoate (1.9 μmol) and another 50 μL DMF containing 250 μg of 4,4′-azobis-(4-cyanovaleric acid) (0.9 μmol) resulting in a molar ratio of $[M]_0$:[CTA]:[Initiator] to be 380:2:1. The mixture was degassed with 3 freeze–evacuate–thaw cycles and transferred to a water bath at 70°C for 24 h. At this time, an aliquot of the solution (100 μL) was treated with 20 mL of ice cold acetone and the precipitated polymers were analyzed by GPC, as described below. The remainder of the solutions were then dialyzed against deionized water (6 x 2L) over a period of 24 h (MWCO=3,500) and then lyophilized. The resultant statistical poly-methacrylamide/acrylamide (PMA) copolymers containing pendant glyconamides, 4-*O*-β-D-galactopyranosyl-D-gluconamide (lactobionamide), 6-*O*-β-D-galactopyranosyl-D-gluconamide (allolactobionamide), 6-*O*-α-D-galactopyranosyl-D-gluconamide (melibionamide) or 6-*O*-α-D-mannopyranosyl-D-gluconamide, were obtained. For ease of discussion, they are abbreviated as PMA-GAEMA, PMA-LAEMA, PMA-ALAEMA, PMA-MAEMA, and PMA-MGAEMA, respectively (Table III-1).

Synthesis of PMA-ALAEMA with different degree of polymerization (DP)

To test the ability of the tri-component RAFT reaction to produce varied copolymer lengths, reactions with varied concentrations of CTA and initiator were

performed (Table III-2). A parallel reaction without using any CTA was performed, as well. For all reactions, the amounts of monomers used and the reaction procedures were exactly the same as described in the section above. The synthesized PMA-ALAEMA glycopolymers with different DPs were characterized on GPC.

Post-modification of glycopolymers with fluorophores

Glycopolymers (5.0 mg) containing ~0.9 μmole of primary amine functional groups were individually dissolved in 0.9 mL phosphate buffered saline (PBS, 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5). To each solution, with rapid stirring, was slowly added 100 μL DMF containing 0.6 mg of carboxyfluorescein succinimidyl ester (1.3 μmol), or 1.1 mg of Texas Red-X succinimidyl ester (1.3 μmol). The reactions were gently stirred for 16 h in the dark and then dialyzed against 6 x 2 L distilled deionized water over a period of 24 h (MWCO=3,500). Following lyophilization, a colored flocculent product was obtained for each glycopolymer. In some experiments, to confirm the completeness of reaction between the primary amines and fluorophore succinimidyl esters, purified labeled glycopolymers were retreated with an additional fluorophore reagent and the products tested for any increase in fluorescence.

Binding tests of the synthetic glycopolymers with lectin coated agarose beads

The lectin-binding specificities of the synthesized glycopolymers were first probed by testing with plant lectin-coated agarose beads. As an example of the binding test protocol, a 50 μL suspension of GNL coated agarose beads, which binds α-mannose specifically, were washed three times with 1.5 mL PBS and resuspended in 0.5 mL PBS.

To the mixtures were added 3 µg of PMA-MGAEMA-Fluorescein or PMA-MAEMA-Fluorescein, respectively. Following a1h incubation in the dark at room temperature, the mixtures were each then washed three times with 1.5 mL PBS. In a parallel competitive binding test, the GNL agarose beads were pre-incubated with 1.0 mg of non-fluorescent PMA-MAEMA or PMA-MGAEMA for 20 min before the addition of 3 µg of PMA-MGAEMA-Fluorescein. The final beads pellets from the binding tests were each resuspended in 1.0 mL PBS, 4 µL aliquots were then subjected to fluorescence microscopy (Olympus BX43**,** FITC filter used), and 100 µL aliquots were taken for measurements of their fluorescence intensities (λex/λem = 490/520 nm, slit width=10 nm) on a microplate reader (BioTek Synergy Mx). Employing the same protocol, *Marasmius oreades* agglutinin-coated lectin beads were used to confirm the binding with α-galactoside containing copolymers, while peanut agglutinin lectin beads were used to probe for β-galactoside binding.

Lectin-mediated bacterial binding with fluorescent glycopolymers

S. aureus ATCC 25923 and *P. aeruginosa* ATCC 39018 were separately cultured on either mannitol salt or trypticase soy agar for 24 h. The colonies were then lifted and suspended in 20 mL of binding solution (155 mM NaCl, 1 mM $CaCl_2$, and 1% bovine serum albumin) to attain a bacterial suspension with an optical density of 1 at 600nm. For each binding experiment, 100 µg of fluorescent glycopolymers, dissolved in 100 µL sodium phosphate buffer (0.3 M, pH=7.4), were then added to 1.0 mL of a bacteria suspension to be tested, respectively, and incubated for 2 h in the dark at 35°C with gentle shaking. Specifically, *S. aureus* was treated with PMA-ALAEMA-Fluorescein,

while PMA-MAEMA-Texas Red was employed for binding to *P. aeruginosa*. In negative control experiments, either PMA-GAEMA-Fluorescein or PMA-GAEMA-Texas Red was used in the incubation. Following incubation, all bacteria-glycopolymer mixtures were then centrifuged (8,000 X g, 10 min) and the pellets were washed three times with 1.2 mL saline (155 mM NaCl containing 1 mM $CaCl_2$). The final pellets were then resuspended in 20 µL of saline, and 4 µL aliquots were taken for analysis by fluorescence microscopy. The green and red fluorescence-labeled bacteria in each photo representing one or several glycopolymer-binding *S. aureus* and *P. aeruginosa* respectively were quantified by the software cellSens Dimension (Olympus).

Employing the same protocol described above, *S. aureus* ATCC 25923 was also separately tested with 100 µg of fluorescein-labeled PMA-LAEMA, PMA-ALAEMA, PMA-MAEMA, and PMA-GAEMA in order to compare their carbohydrate-binding specificities. The bacteria pellets after three washings were resuspended in 100 µL PBS (pH=8), and the fluorescence intensities were measured on the microplate reader. All binding experiments were performed in triplicate.

Analysis

^{1}H and ^{13}C NMR spectra of samples dissolved in D_2O were recorded on a Bruker Avance 800 MHz spectrometer. ^{1}H and ^{13}C spectra were recorded at 800.14 and 201.19 MHz, respectively. Glycomonomers, dissolved in MeOH/water 50:50 (v/v) were subjected to direct electrospray mass spectrometry analyses, utilizing a Thermo Scientific LTQ Orbitrap XL hybrid Fourier transform mass spectrometer. Molecular weights (*M*w, *M*n) and the dispersity (*M*w/*M*n) of the glycopolymers were derived against a calibration

curve from polyethylene glycol standards (MW: 200-1,200,000 g/mol) using a Waters Alliance HPLC system equipped with a refractive index detector (Waters e2695, 2414) and TOSOH TSK-GEL G4000 PWxl and a TSK-GEL G4000 SW GPC columns using 0.1M Tris/0.1M sodium chloride buffer (pH=7) as eluent at a flow rate of 0.6 mL/min. The actual concentrations of primary amine functional groups within the glycopolymers were quantified with Orange II according to a published protocol (25). Total carbohydrate content of the synthesized glycopolymers was analyzed according to a published method (26). Elemental analysis was performed by Galbraith Laboratories, Knoxville, TN.

Results and discussion

Synthesis of glycomonomers

In order to provide for desired conformations of pendant sugars presented by glycopolymers to plant and bacterial lectins, we have chosen disaccharides lactose, melibiose, allolactose and α-D-mannopyranosyl-(1→6)-D-glucose as the specific glycoside sources. All disaccharides were then oxidized with iodine and dehydrated to their respective lactones. These disaccharide lactones, and gluconolactone, were then reacted with the primary amine functional group on AEMA to produce a group of glycomonomers, listed in Table III-1. While GAEMA and LAEMA have been previously reported (4,19), glycomonomers ALAEMA, MAEMA, and MGAEMA, are novel glycosynthons designed to provide α-galactoside, β-galactoside and α-mannoside, respectively, as the pendant carbohydrate residues. In contrast to the report on the preparation of LAEMA (19), we initially obtained varied amounts of the product which

was also of an unsatisfactory purity. A modified purification procedure was thus developed for the disaccharide lactone, which uses cation and anion-exchange resins, sequentially, to remove unreacted AEMA and disaccharide acids, respectively, resulting in less variable yields of 59% to 68% (Table III-1). Characterizations performed by NMR spectroscopy, mass spectrometry, and elemental analysis confirmed the high purities of the products. ^{1}H NMR spectroscopy of the glycomonomers also confirmed the correct conformations at the anomeric carbons in allolactose and α-D-mannopyranosyl-(1→6)-D-glucopyranose.

Kinetic experiment for the RAFT tri-component polymerization of GAEMA

GPC traces of the PMA-GAEMA copolymers at different time points indicate that the RAFT-based polymerization was well-controlled, as evidenced by the consecutive low dispersities (<1.3) of the products and of the steady evolution of the *M*n (Figure III-11). As expected, the examination of developing copolymers by ^{1}H NMR indicated that the consumption of GAEMA and HEAA monomers in the reaction proceeded at different rates with GAEMA being consumed somewhat faster. This observation is a focus of an ongoing investigation. Overall, > 95% of the conversion was completed in the first 24 h (Figure III-11b).

RAFT-based synthesis of glycopolymers

In this study, a facile one-step RAFT-mediated tri-component copolymerization strategy was employed. Compared to the bi-block or tri-block glycopolymers prepared through stepped RAFT-controlled polymerizations (15,19,27), this one-step reaction

should provide for a more random distribution of the glycomonomers in the products, as seen in naturally occurring glycoprotein, mucous glycoproteins and glycolipids. In this work, we prepared glycopolymers that contain 20 mol% of glycomonomer, HEAA (77 mol% on polymer) as spacer, and AEMA (3 mol%) as targets for post-modifications with fluorophores and then employed them in a lectin-binding study similar to that reported by Bovin in the use of early neoglycoconjugates (28). With the use of RAFT reactions, synthesized glycopolymers with different pendant sugars on the gluconamide linker (Table III-1) demonstrated low dispersities ($\leq$1.32), very similar GPC traces and *M*n (Figure III-12, Table III-3). As can be seen in Figure III-12, when plotted against the elution profiles of these glycopolymers, only PMA-GAEMA displayed a smaller *M*n due to the absence of a pendant pyranose. For all glycopolymers, these results demonstrate a high reproducibility of copolymerization and, following individual analysis of product components (i.e., carbohydrates and primary amine functional groups (Table III-3), it was determined that the ratio of incorporated monomers in the respective glycopolymers reflected the stoichiometric ratio of monomers employed in the initial RAFT reactions. In other words, the stoichiometry of components in RAFT reactions predictably defined the respective product composition. Structural confirmation of the resultant glycopolymers was performed by ^{1}H- and ^{13}C-NMR (Figure III-13-16).

That the pendant sugars are located on a gluconamide linker, is of special note, in that it results in formation of polymers that are more physiologically relevant because of the hydrophilic nature of the polysaccharides chains on many natural glycoproteins.

To test the ability of CTA to control the DPs and dispersity of the product copolymers, varied amounts of CTA (Table III-2) were employed in the polymerizations.

A class of PMA-ALAEMA polymers with determined DPs was prepared and they clearly showed that reactions with RAFT reagents produced polymers that possessed considerably lower dispersities than polymers prepared without any CTA in the reaction (Figure III-17).

Post-modification of glycopolymers with fluorophores

The reaction of primary amine functional groups with a variety of reactive fluorophores is a technique frequently employed in protein labeling. This versatility was incorporated into our investigation by post-modifying the amine functional groups in our purified glycopolymers by separately reacting them with either activated succinimidyl esters of carboxyfluorescein or Texas Red-X fluorophores.

After 16 h of reaction in PBS buffer, and 24 h dialysis against water, respective fluorescent polymers (i.e., PMA-MAEMA-Texas Red or PMA-GAEMA-Texas Red, and PMA-ALAEMA-Fluorescein or PMA-GAEMA-Fluorescein) were obtained, with no detectable degradation of the polymers, as determined by GPC analysis (data not shown). Compared to other labeling protocols applied to glycopolymers (2,19), this present method of post-modification is both simpler and amenable to choice of fluorescent label desired for different experimental designs. In our opinion, most of the water-soluble amine-reactive fluorophores, biotins or quantum dots (22), should be suitable for labeling of these synthesized glycopolymers.

Binding of synthetic glycopolymers with plant lectin coated agarose beads

In order to assess the lectin-binding specificities of the synthesized glycopolymers, each glycopolymer product was individually tested and confirmed with its corresponding lectin-coated agarose beads. As an example, the binding results with α-mannose-specific lectin GNL are presented here. Figure III-18a demonstrates the negligible binding of GNL beads with PMA-MAEMA-Fluorescein which contains α-galactose as the pendant sugar. In contrast, the α-mannose-containing polymer PMA-MGAEMA-Fluorescein showed very strong binding (Figure III-18b). To further validate the binding specificity with the synthesized glycopolymers, this binding was competitively inhibited when the GNL beads were pre-incubated with 1 mg of non-fluorescent PMA-MGAEMA (Figure III-18c), and, when pretreated with 1 mg of PMA-MAEMA, no such effects were observed (Figure III-18d). The fluorescence intensities measured on the microplate reader (Figure III-19) were consistent with the observation by microscopy. Similarly, the binding specificities of the three different galactose-containing glycopolymers were confirmed by employing agarose beads coated with either *Marasmius oreades* agglutinin or peanut agglutinin lectin and the respective competitive inhibition by the corresponding non-fluorescent glycopolymers (data not shown).

Lectin-mediated binding of bacteria with fluorescent glycopolymers

Having established the specific affinities of the synthetic glycopolymers towards plant lectin-coated agarose beads, application of these polymers with clinically relevant bacterial strains was performed. The well-studied α-galactose-binding *P. aeruginosa* lectin PA-IL, which plays a crucial role in their opportunistic infections (29,30), was first

studied. Ideal for this experiment, PMA-MAEMA-Texas Red, possessing α-galactose as the pendant sugar, was employed to test its binding ability with this organism. As a negative control, PMA-GAEMA-Texas Red, lacking the pendant α-galactose group, was used. In a parallel experiment, employing the same protocol, PMA-ALAEMA-Fluorescein possessing β-galactose residues was tested for the recognition of *S. aureus*, with PMA-GAEMA-Fluorescein used as a negative control. In three sets of repeated experiments, specific and dramatic binding of the respective glycopolymers between the bacteria and pendant sugars was observed by fluorescence microscopy (Figure III-20) when compared with the negative controls which demonstrated an occasional occurrence of non-specific binding (Figure III-21). The weak non-specific binding with the negative controls could be due to associations between a few bacteria and the glycopolymer fluorophores, or possibly some traces unreacted primary amine functional groups remaining on the glycopolymer. The latter hypothesis has been tested by subjecting the fluorescent glycopolymers to a second round of the fluorescent labeling. However, no further increase in fluorescence was noted, nor did this retreatment affect the small amount of the non-specific binding to the bacteria.

In another experiment where different fluorescein-labeled glycopolymers were used to test the carbohydrate-binding specificity of *S. aureus* ATCC 25923, PMA-ALAEMA and PMA-LAEMA, both possessing β-D-galactose as the pendant sugar, showed dramatically higher binding than either PMA-MAEMA, which possesses α-D-galactose, or the negative control PMA-GAEMA. These results suggest that this strain of *S. aureus* has a binding preference for β-D-galactose (Figure III-22).

Understanding that the outcome of synthesizing a group of tri-component polymers would produce statistical polymers, we anticipated the possibility of glycopolymer molecules that would not have incorporated any AEMA monomer and thus would not be fluorophore-labeled. Thus, the presence of these unlabeled glycopolymers would then potentially act as binding competitors. Although we did not attempt to determine the percentage of such species, considering the very low possibility of such polymers (4.7%, i.e., $0.97^{100} \approx 0.04755$), their competition effects in the present experiments were not considered to be significant. Also, based on statistical results, the majority of the synthetic copolymers (~87%) possess 1-5 AEMA monomers (shown in Table III-4), which is acceptable in the lectin-binding tests.

As the binding between lectins and monosaccharides is generally very weak, strong interactions most often require multivalency of the sugars (3,31). As the fluorescent-labeled glycopolymers produced in this investigation possess such multivalency (14-20 pendant carbohydrate residues per polymer), and strong associations are observed in our studies on both lectin beads and bacteria, these results suggest that these synthetic fluorescent glycopolymers could have application to many other carbohydrate-binding studies.

Some studies suggest that the length of the linker sugar may affect the binding affinities of the glycopolymers (32,33). In this present study, synthesis of disaccharides to be converted to lactones employed glucose as the only carbohydrate linker synthetic precursor. Hence, when the resulting disaccharide lactones were formed and reacted with AEMA, a gluconamide linker was produced. In light of these and earlier studies, our ongoing investigations include assessment of the effects of varying the length of the

linker, such as ribose (five-carbon sugar) or maltose (disaccharide), to which the pendant sugars are bound.

Lastly, interactions between lectins and carbohydrates often involve more than one glyco-epitope (34). Any disaccharides or oligosaccharides of interest, in theory, can be conjugated onto a linear polymer following this protocol. In addition, by varying the polymer length, the ratios of glycomonomers or labeling fluorophores (33,35), improved binding results might be achieved. Furthermore, with the combination of different fluorophores and sugars, it's now possible to accomplish the binding assays of multiple carbohydrates or crosslink different lectins all in one test. The flexibility of this method also allows for optimization of desired glycopolymer products for lectins, antibodies, or other carbohydrate-binding structures pertinent to particular glycobiology interests.

Conclusions

A class of tri-component statistical glycopolymers was successfully prepared using RAFT-based one-step polymerizations. Well-controlled reactions with good reproducibility yielded polymers with low dispersities and consistent compositions. Polymers with different degrees of polymerization and pendant sugars were synthesized. Each synthetic glycopolymer contained primary amine functional groups suitable for a variety of labels that can be attached via mild coupling chemistries, when needed. To demonstrate this, both fluorescein and Texas Red-X were used to convert the synthetic glycopolymers into excellent reporters of specific lectins immobilized on agarose beads or expressed on surface of two clinically relevant bacteria. The bacterial binding results illustrated our glycopolymers' potential for clinically relevant applications. Additional

investigations are merited to optimize the binding capacities of the desired synthetic glycopolymers for specific purposes, such as by varying the polymer length, monomer ratios, linker structures, and choice and content of labels. In conclusion, the method presented here offers a relatively straightforward and dependable way to prepare heterofunctionalized linear glycopolymers for a variety of different research aims involving carbohydrate-protein specific interactions.

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Table III-1. Synthesized glycomonomers, glycopolymers and their abbreviations.

Glycomonomer (yield)	Abbreviations	
	Glycomonomers	**Glycopolymers**
2-Gluconamidoethyl methacrylamide (98%)	GAEMA	PMA[a]- GAEMA
2-Lactobionamidoethyl methacrylamide (65%)	LAEMA	PMA-LAEMA
2-Allolactobionamidoethyl methacrylamide (59%)	ALAEMA	PMA-ALAEMA
2-Melibionamidoethyl methacrylamide (68%)	MAEMA	PMA-MAEMA
6-*O*-α-D-mannopyranosyl-D-gluconamidoethyl methacrylamide (64%)	MGAEMA	PMA-MGAEMA

[a] PMA stands for poly-methacrylamide/acrylamide

Table III-2. Parameters of reactions synthesizing PMA-ALAEMA with different DP

Copolymerizations	Amount of CTA used, /mg	Amount of initiator used, /mg	Target DP, $[M]_0/[CTA]$	Reaction yield after dialysis, %	Actual DPs [a]
#1	1.00	0.25	97	45	26
#2	0.53	0.25	190	78	101
#3	0.25	0.25	386	81	260
#4	0	2.00	NA	98	382

[a] calculated by $Mn/(0.77MW_{HEAA}+0.2MW_{ALAEMA}+0.03MW_{AEMA})$

Table III-3. Summary of the synthetic parameters and actual compositions of the RAFT glycopolymers

Glycopolymer	Pendant sugar(linkage)	*M*n, GPC g/mol	DP [a]	*M*w/*M*n	Actual content of glycomonomers, mol%	Actual content of primary amine, mol%	Reaction yield after dialysis, %
PMA-GAEMA	None	13696	89	1.32	NA	2.7	70
PMA-MAEMA	α-galactose (1→6)	17684	95	1.30	21	3.3	74
PMA-LAEMA	β-galactose (1→4)	18431	99	1.26	19	3.2	76
PMA-ALAEMA	β-galactose (1→6)	18943	101	1.21	18	3.0	78
PMA-MGAEMA	α-mannose (1→6)	16498	88	1.31	16	3.4	68

[a] calculated by Mn/(0.77MW_{HEAA}+0.2$MW_{glycomonomer}$+0.03MW_{AEMA}). Considering actual DP and contents of each monomer in the polymers, these statistical copolymers are denoted as P(GAEMA-*stat*-$AEMA_{2.4}$-*stat*-HEAA); P($MAEMA_{20.0}$-*stat*-$AEMA_{3.1}$-*stat*-$HEAA_{71.9}$); P($LAEMA_{19.0}$-*stat*-$AEMA_{3.2}$-*stat*-$HEAA_{76.8}$); P($ALAEMA_{18.0}$-*stat*-$AEMA_{3.0}$-*stat*-$HEAA_{80}$); P($MGAEMA_{14.1}$-*stat*-$AEMA_{3.0}$-*stat*-$HEAA_{70.9}$).

Table III-4. Statistical possibilities for copolymers to contain a certain number of AEMA

Number of AEMA in each copolymer (100mer)	Possibilities
0	4.7
1	14.7
2	22.5
3	22.75
4	17.1
5	10.1
6	5
7	2.06
8	0.74

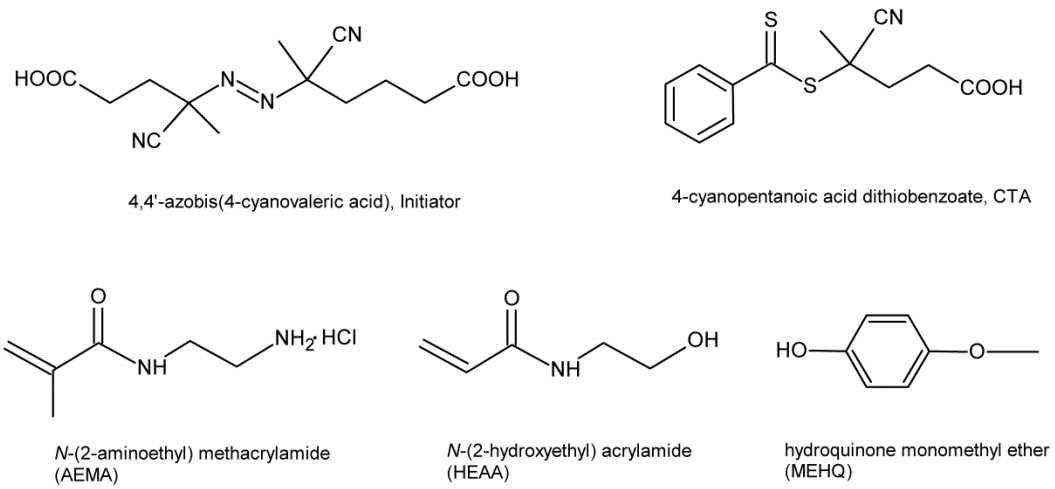


Figure III-1. Chemical structures of some key reagents used.

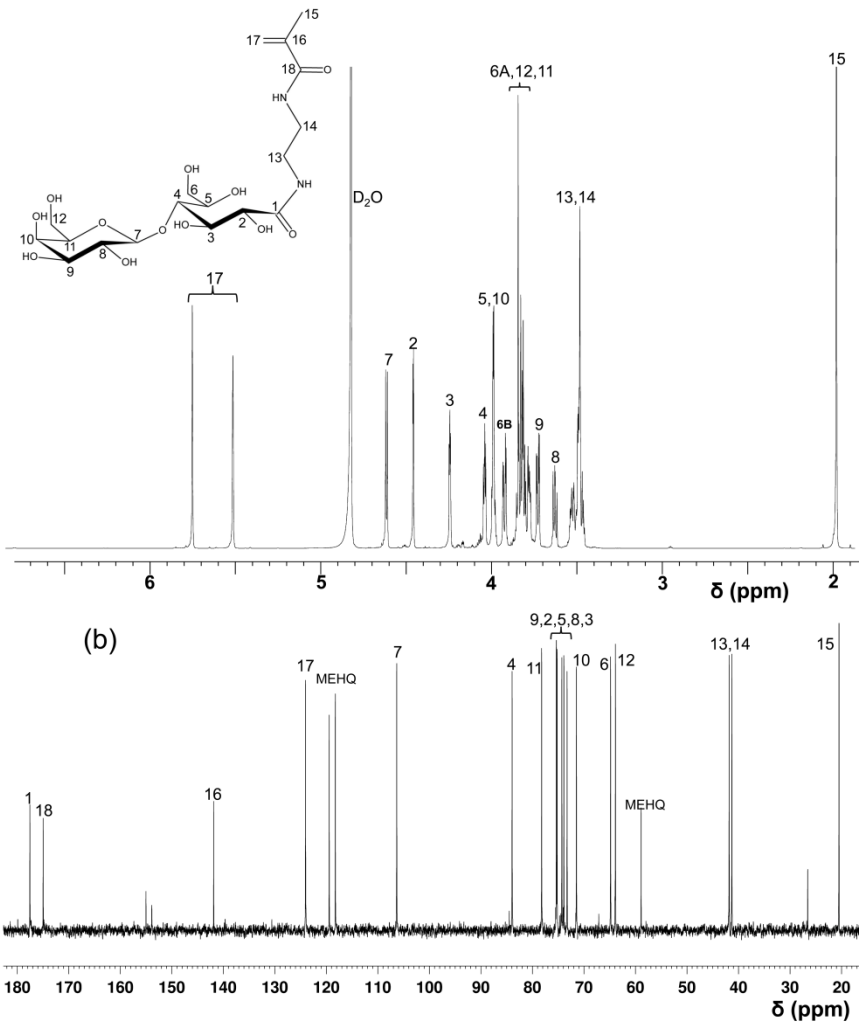


Figure III-2. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D_2O) for LAEMA.

^{1}H NMR (800 MHz, D_2O): δ (ppm) 5.70 (s, 1H, H-17A), 5.46 (s, 1H, H-17B), 4.56 (d, $J_{7,8}$ =7.8, 1H, H-7), 4.41 (d, $J_{2,3}$ =2.6, 1H, H-2), 4.19 (m, $J_{3,4}$ =4.2, 1H, H-3), 3.99 (m, $J_{4,5}$=6.4, 1H, H-4), 3.96-3.92 (m, 2H, H-5, H-10), 3.87 (m, 1H, H-6B), 3.77 (m, 1H, H-6A), 3.79-3.76 (m, 2H, H-12), 3.73 (m, 1H, H-11), 3.68 (dd, $J_{9,10}$ = 3.4, 1H, H-9), 3.58 (dd, $J_{8,9}$ = 9.9, 1H, H-8), 3.48-3.41 (m, 4H, H-13, H-14), 1.93 (s, 3H, H-15).
^{13}C NMR (201 MHz, D_2O): δ (ppm) 177.53 (C-1), 174.95 (C-18), 141.88 (C-16), 124.04 (C-17), 106.34 (C-7), 83.95 (C-4), 78.21 (C-11), 75.35 (C-9), 75.22 (C-2), 74.29 (C-5), 73.90 (C-8), 73.27 (C-3), 71.46 (C-10), 64.83 (C-6), 63.89 (C-12), 41.82 (C-13), 41.29 (C-14), 20.49 (C-15).

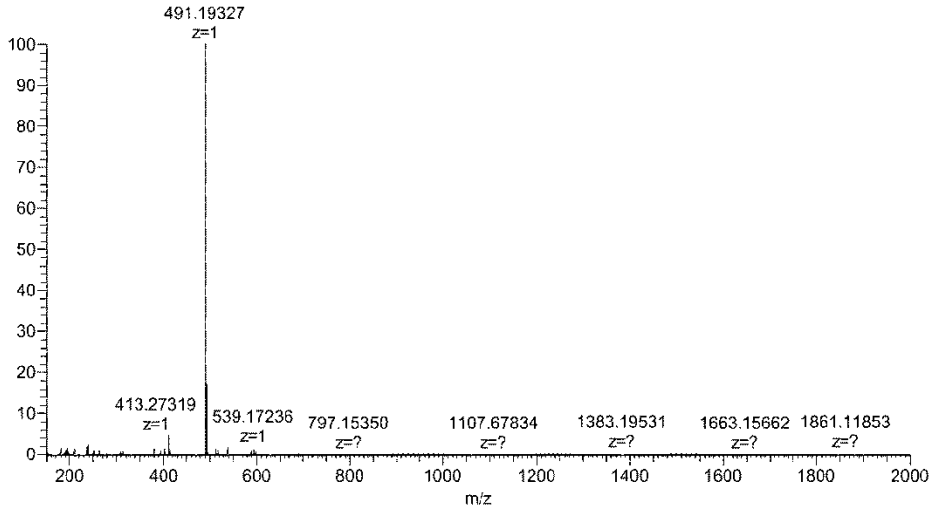


Figure III-3. Mass spectrum of LAEMA by ESI-MS (in methanol: water 50:50). Calculated *m/z* for $C_{18}H_{32}O_{12}N_2+Na^+$, 491.18475; found m/z 491.19327.

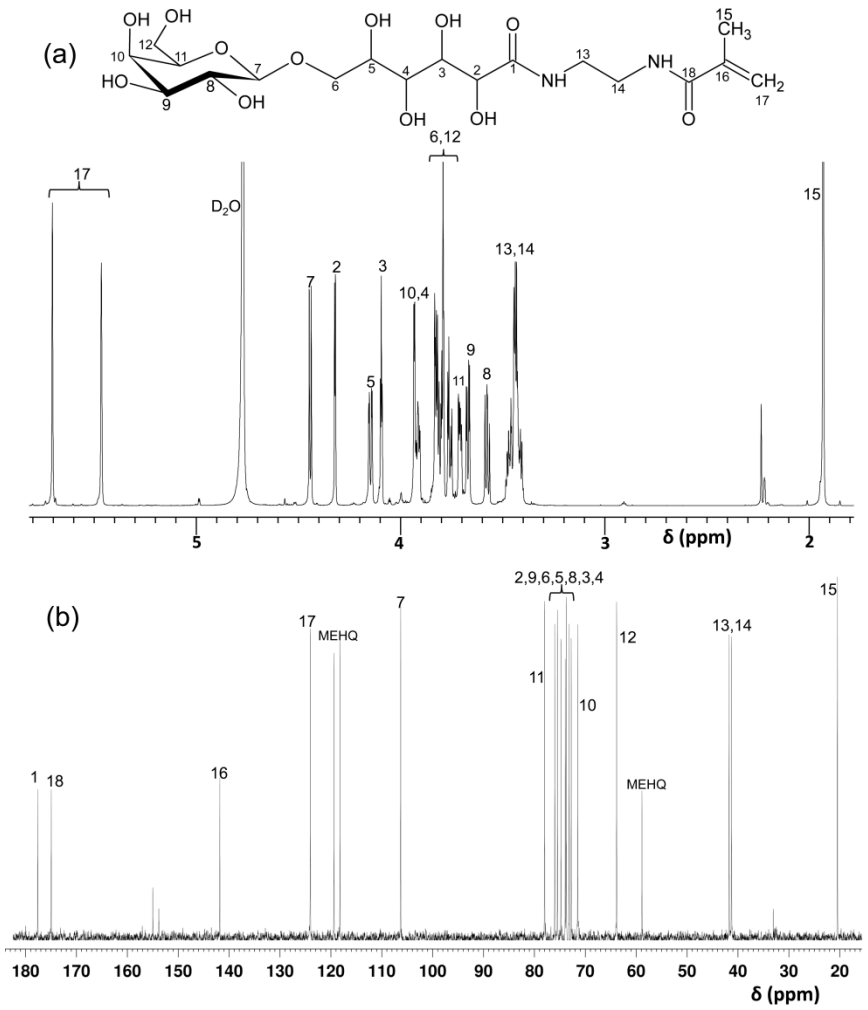


Figure III-4. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D_2O) for ALAEMA.

^{1}H NMR (800 MHz, D_2O): δ (ppm) 5.71 (s, 1H, H-17A), 5.47 (s, 1H, H-17B), 4.44 (d, $J_{7,8}$ =7.8, 1H, H-7), 4.32 (d, $J_{2,3}$ =3.5, 1H, H-2), 4.13-4.15 (m, $J_{5,6A}$ =11, $J_{5,6B}$ =2.6, 1H, H-5), 4.09 (m, $J_{3,4}$ =3.5, 1H, H-3), 3.93 (m, 1H, H-10), 3.92 (m, 1H, H-4), 3.83-3.81 (m, 2H, H-6), 3.80-3.75 (m, 2H, H-12), 3.71 (m, $J_{11,12A}$ =4.2, $J_{11,12B}$ =7.9, 1H, H-11), 3.67(dd, $J_{9,10}$ = 3.3, 1H, H-9), 3.58 (dd, $J_{8,9}$ = 9.9, 1H, H-8), 3.48-3.41 (m, 4H, H-13, H-14), 1.93 (s, 3H, H-15).
^{13}C NMR (201 MHz, D_2O): δ (ppm) 177.57 (C-1), 174.96 (C-18), 141.82 (C-16), 124.06 (C-17), 106.28 (C-7), 77.99 (C-11), 76.00 (C-2), 75.47 (C-9), 74.78 (C-6), 73.93 (C-5), 73.72 (C-8), 73.17 (C-3), 72.77 (C-4), 71.48 (C-10), 63.84 (C-12), 41.73 (C-13), 41.27 (C-14), 20.45 (C-15).

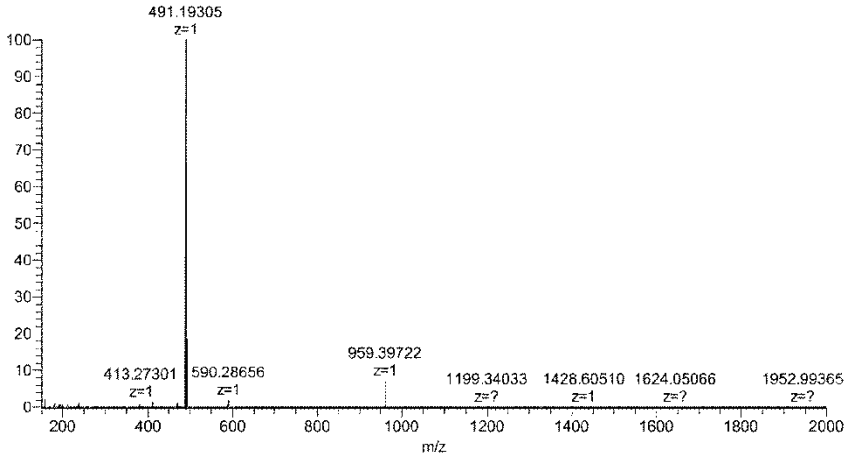


Figure III-5. Mass spectrum of ALAEMA by ESI-MS (in methanol: water 50:50). Calculated *m/z* for $C_{18}H_{32}O_{12}N_2$+Na^+, 491.18475; observed m/z 491.19305.

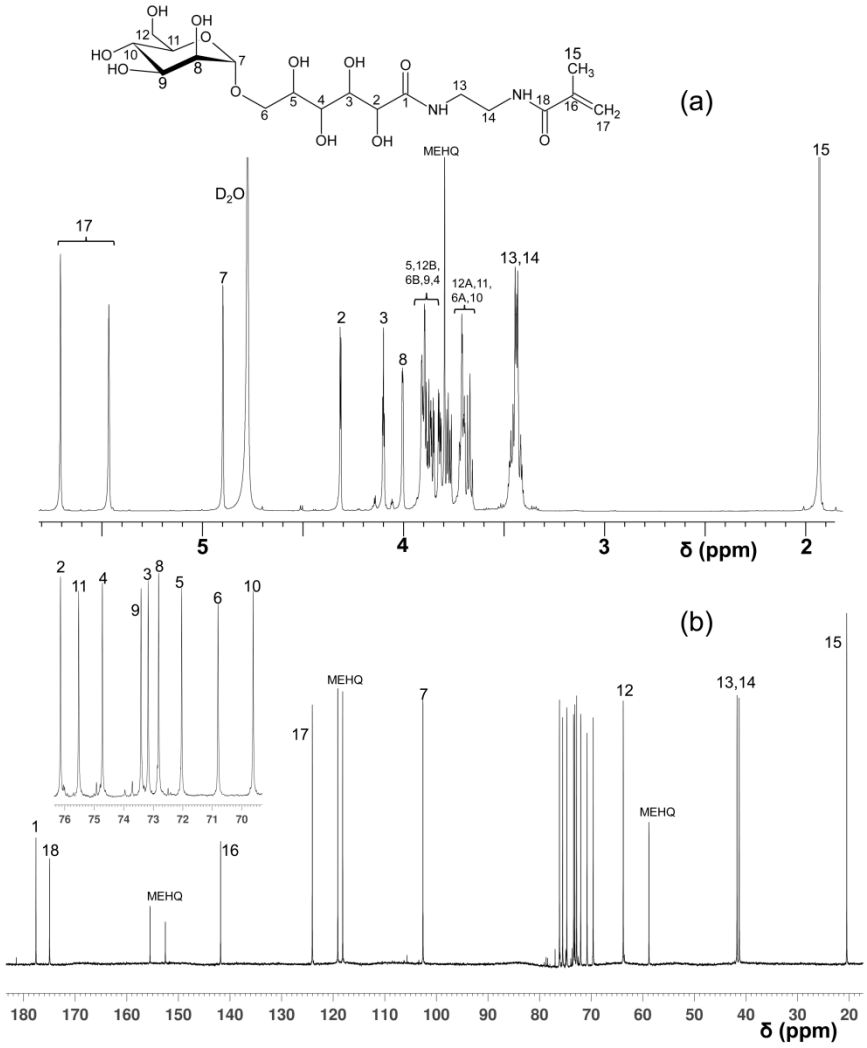


Figure III-6. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D_2O) for MGAEMA.

^{1}H NMR (800 MHz, D_2O): δ (ppm) 5.71 (s, 1H, H-17A), 5.47 (s, 1H, H-17B), 4.90 (d, $J_{7,8}$ =1.6, 1H, H-7), 4.31 (d, $J_{2,3}$ =3.5, 1H, H-2), 4.10 (m, $J_{3,4}$ =3.2, 1H, H-3), 4.00 (m, $J_{8,9}$ =3.3, 1H, H-8), 3.92-3.85 (m, 4H, H-5, H-12B, H-6B, H-9), 3.82 (dd, $J_{4,5}$ =7.7, 1H, H-4), 3.85-3.76 (m, 1H, H-12A), 3.73-3.69 (m, 2H, H-6A,H-11), 3.67 (m, $J_{10,11}$ =9.9, 1H, H-10), 3.47-3.42 (m, 4H, H-13, H-14), 1.93 (s, 3H, H-15).
^{13}C NMR (201 MHz, D_2O): δ (ppm) 177.59 (C-1), 174.86 (C-18), 141.83 (C-16), 124.05 (C-17), 102.61 (C-7), 76.15 (C-2), 75.53 (C-11), 74.73 (C-4), 73.42 (C-9), 73.17 (C-3), 72.82 (C-8), 72.04 (C-5), 70.81 (C-6), 69.62 (C-10), 63.79 (C-12), 41.74 (C-13), 41.31 (C-14), 20.47 (C-15).

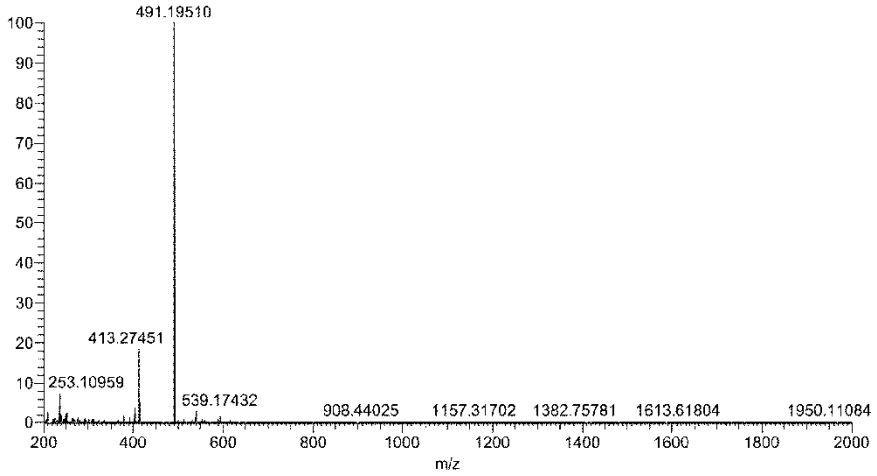


Figure III-7. Mass spectrum of MGAEMA by ESI-MS (in methanol: water 50:50). Calculated *m/z* for $C_{18}H_{32}O_{12}N_2$+Na^+, 491.18475; found m/z 491.19501.

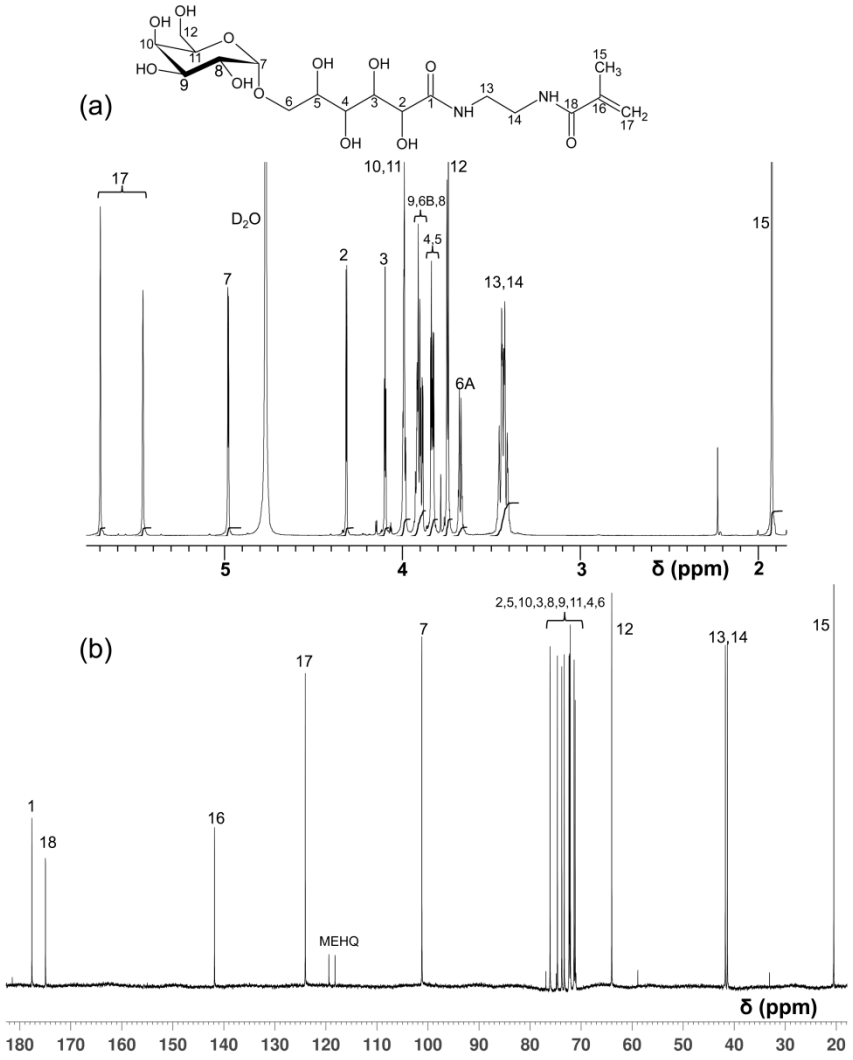


Figure III-8. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D_2O) for MAEMA.

^{1}H NMR (800 MHz, D_2O): δ (ppm) 5.70 (s, 1H, H-17A), 5.46 (s, 1H, H-17B), 4.98 (d, $J_{7,8}$ =3.6, 1H, H-7), 4.32 (d, $J_{2,3}$ =3.7, 1H, H-2), 4.10 (m, $J_{3,4}$ =3.4, 1H, H-3), 4.01-3.98 (m, 2H, H-10, H-11), 3.94-3.88 (m, 3H, H-9, H-6B, H-10), 3.85-3.81 (m, 2H, H-4, H-5), 3.76-3.73 (m, 2H, H-12), 3.68 (m, 1H, H-6A), 3.41-3.47 (m, 4H, H-13, H-14), 1.93 (s, 3H, H-15).
^{13}C NMR (201 MHz, D_2O): δ (ppm) 177.64 (C-1), 174.97 (C-18), 141.84 (C-16), 124.06 (C-17), 101.21 (C-7), 76.09 (C-2), 74.64 (C-5), 73.79 (C-10), 73.30 (C-3), 72.38 (C-8), 72.29 (C-9), 72.11 (C-11), 71.38 (C-4), 71.17 (C-6), 64.00 (C-12), 41.73 (C-13), 41.33 (C-14), 20.49 (C-15).

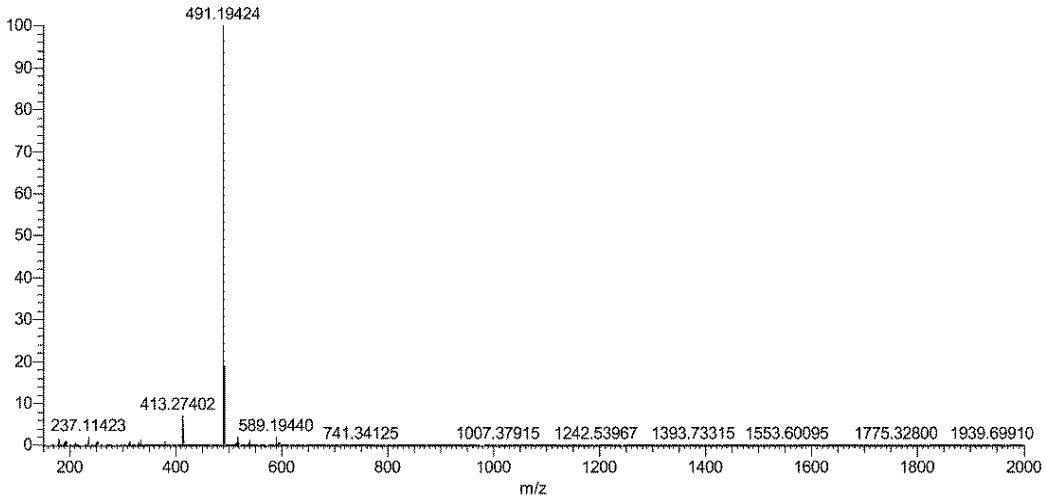


Figure III-9. Mass spectrum of MAEMA by ESI-MS (in methanol: water 50:50). Calculated *m/z* for $C_{18}H_{32}O_{12}N_2$+Na^+, 491.18475; found m/z 491.19424.

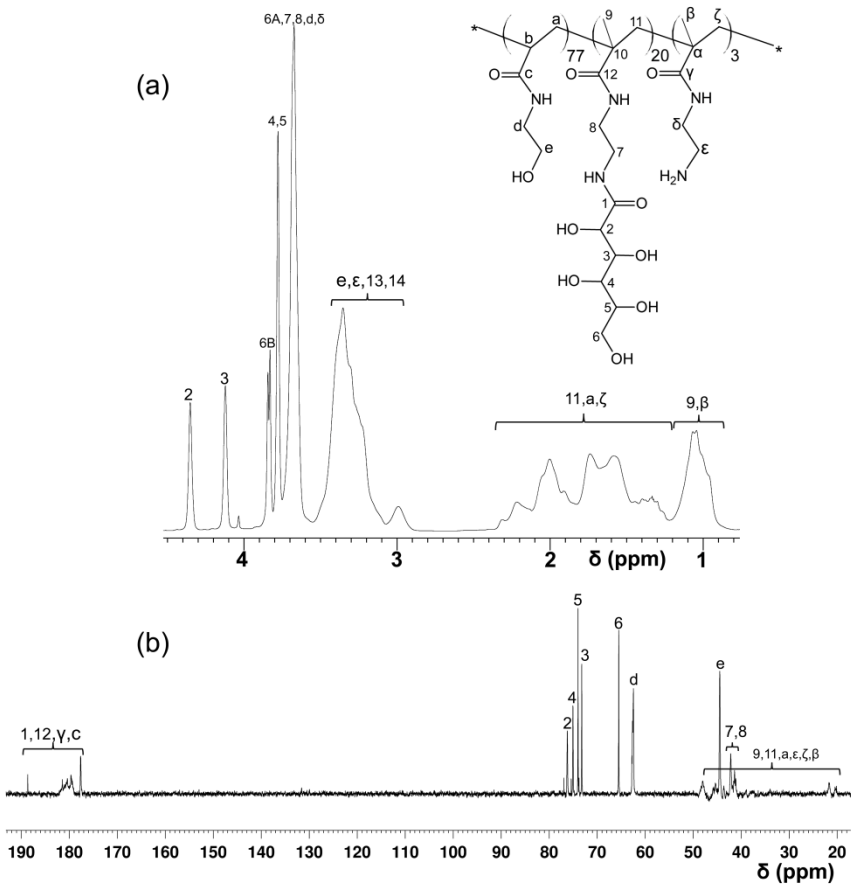


Figure III-10. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D_2O) for PMA-GAEMA glycopolymer.

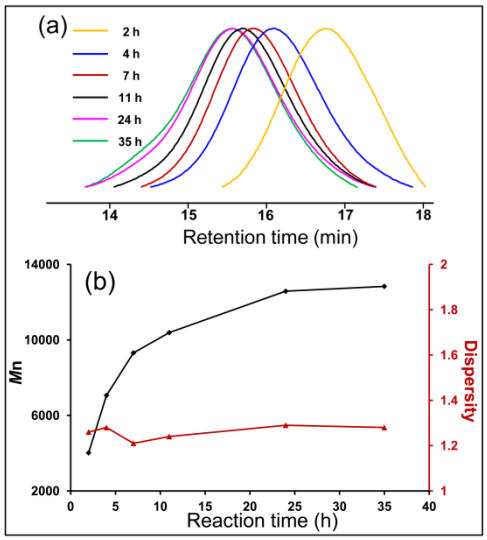


Figure III-11. Kinetic study for the RAFT polymerization of PMA-GAEMA. (a) Gel permeation chromatography traces of the copolymers indicating steady evolution of the copolymers with time. (b) *M*n dispersities of the glycopolymers over time. ($[M]_0$:[CTA]:[Initiator]=380:2:1; $[M]_0$=0.7 M; solvent, H_2O/DMF=4:1)

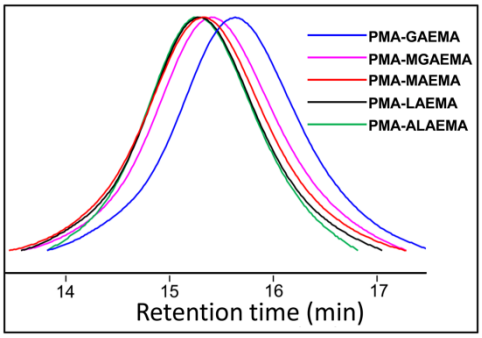


Figure III-12. Gel permeation chromatography traces of RAFT-based glycopolymers. ($[M]_0$:[CTA]:[Initiator]=380:2:1; $[M]_0$=0.7 M; solvent, H_2O/DMF=4:1**)**

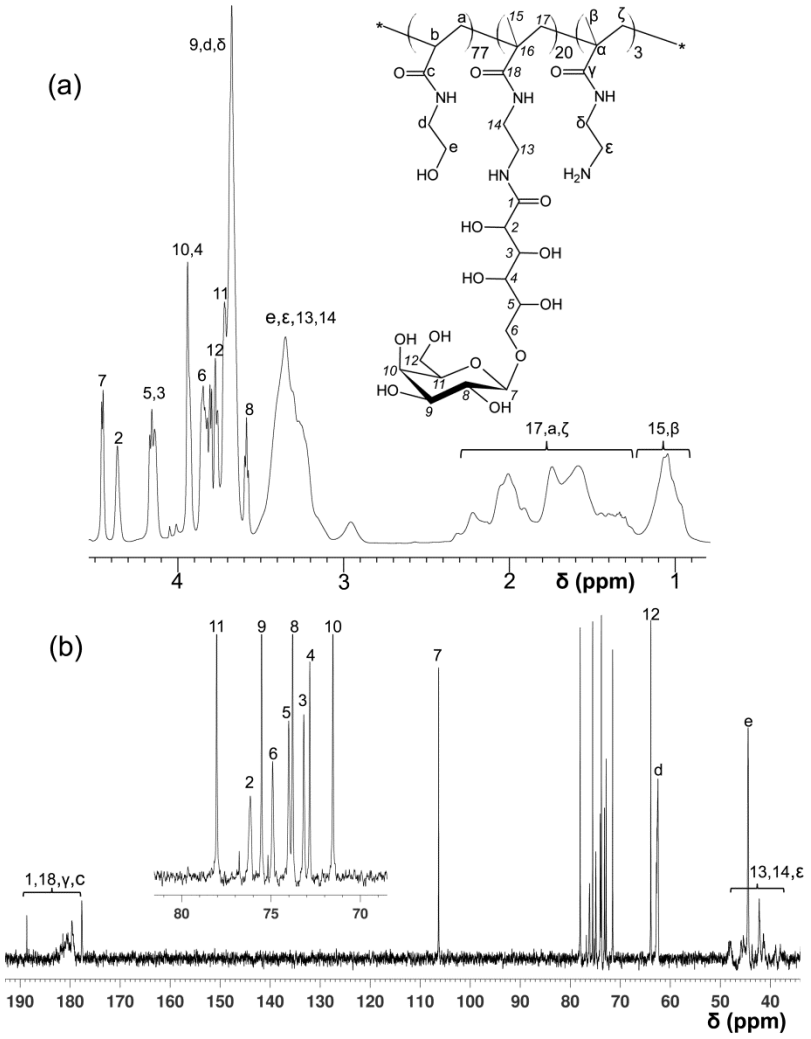


Figure III-13. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D_2O) for PMA-ALAEMA.

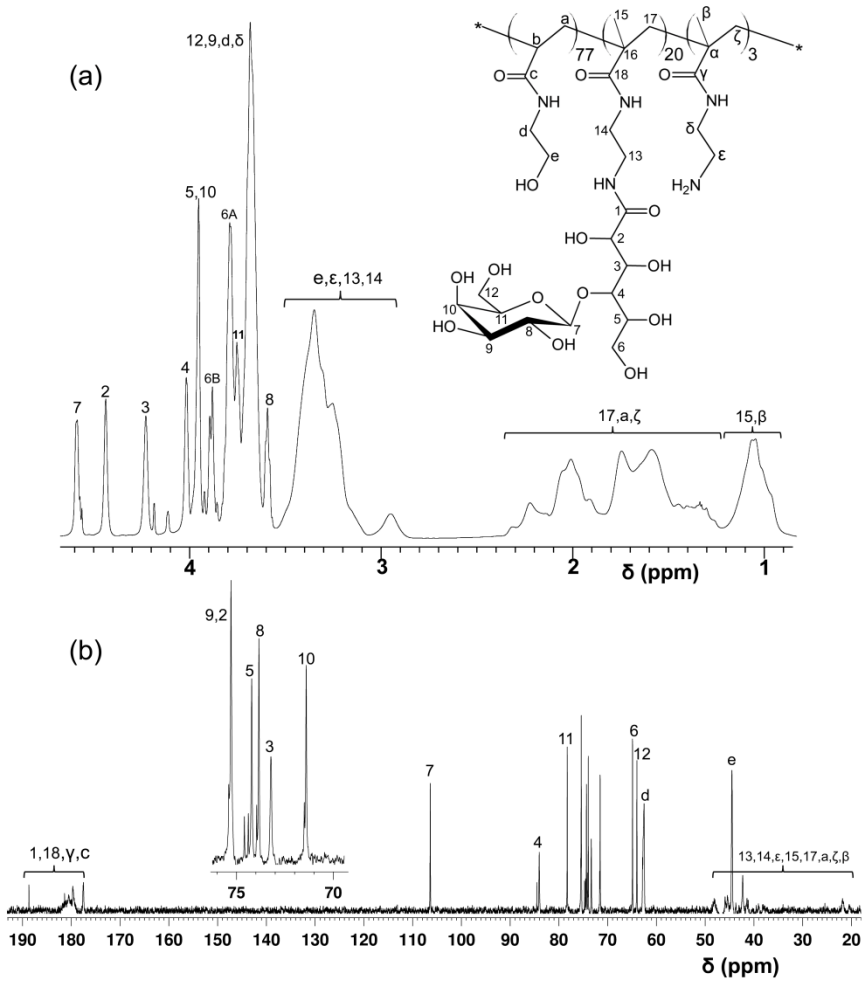


Figure III-14. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D_2O) for PMA-LAEMA glycopolymer.

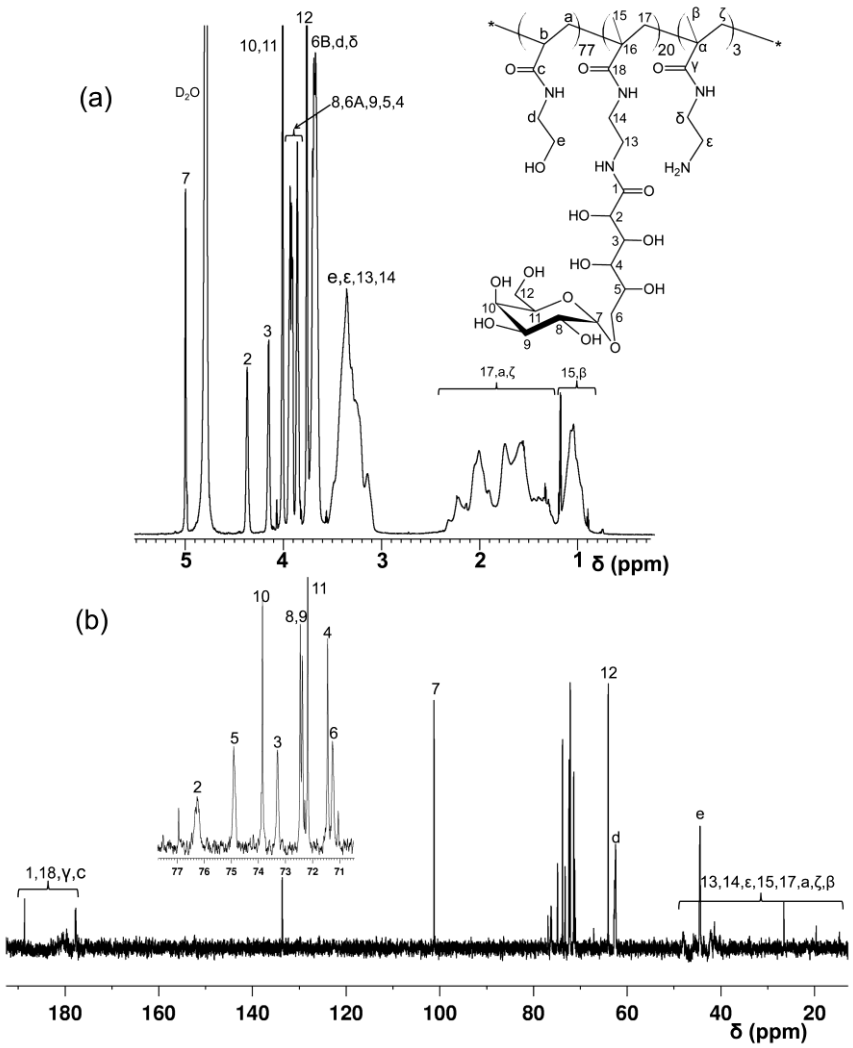


Figure III-15. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D_2O) for PMA-MAEMA glycopolymer.

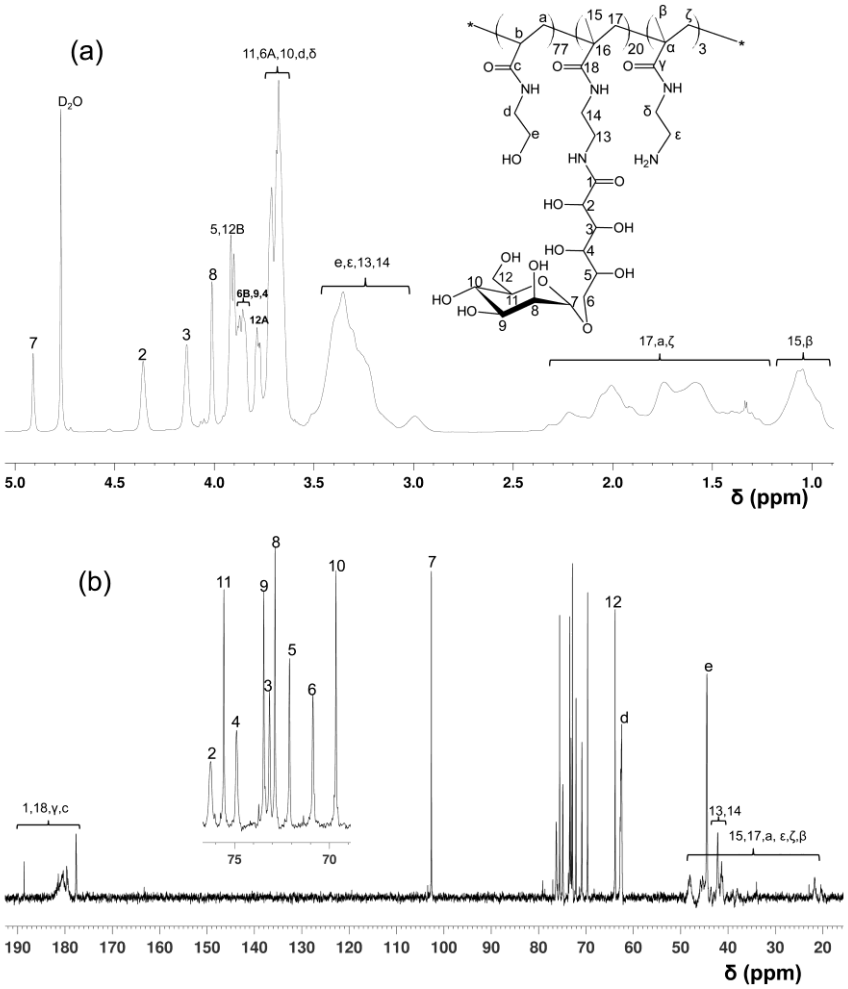


Figure III-16. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D_2O) for PMA-MGAEMA glycopolymer.

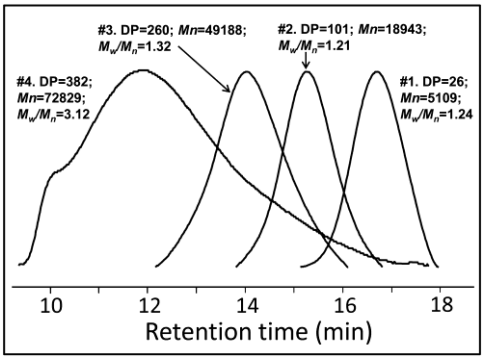


Figure III-17. Gel permeation chromatography traces of PMA-ALAEMA with different DPs from copolymerizations using different amounts of chain transfer agent ($[M]_0$=0.7 M; solvent, H_2O/DMF=4:1)

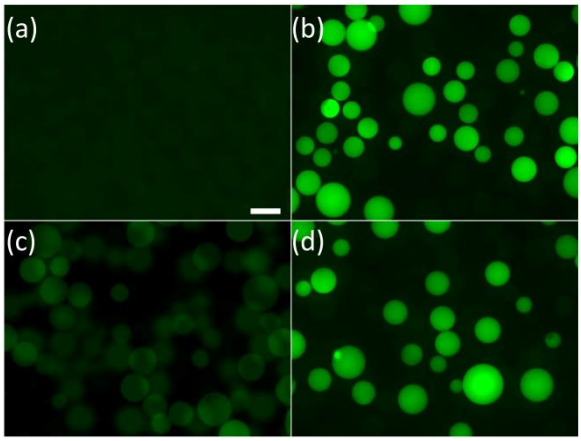


Figure III-18. *Galanthus Nivalis* lectin (GNL) coated agarose beads bind α-D-mannoside containing glycopolymers, but not those possessing α-D-galactoside. PMA-MAEMA-Fluorescein (3μg) showed only a weak non-specific binding with GNL (a), in contrast to the strong binding between PMA-MGAEMA-Fluorescein and the beads (b). Pre-incubation of the beads with 1.0 mg of non-fluorescent PMA-MGAEMA for 20 min before adding 3μg of PMA-MGAEMA-Fluorescein dramatically reduced the binding of the latter (c), but the pre-incubation with 1.0 mg of PMA-MAEMA didn't show any competitive inhibition effects (d). Scale bar = 100 μm.

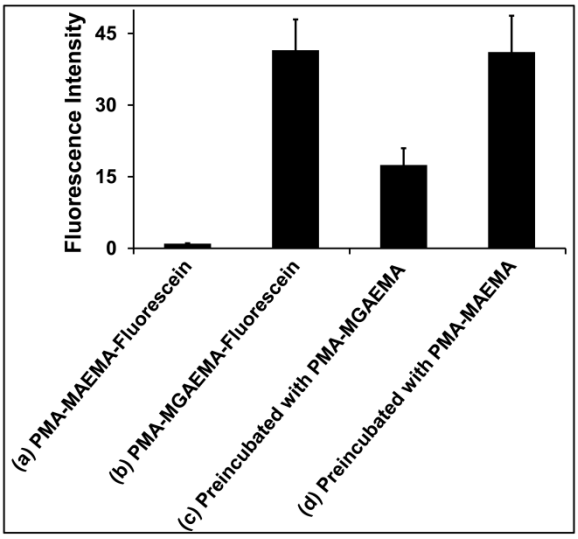


Figure III-19. *Galanthus Nivalis* lectin coated agarose beads bind with α-mannose containing glycopolymers, but not with those containing α-galactose. The measured fluorescence intensities (λex/λem = 490/520 nm, slit width=10 nm) of the samples a-d in Figure 5 were consistent with the observation by fluorescence microscopy.

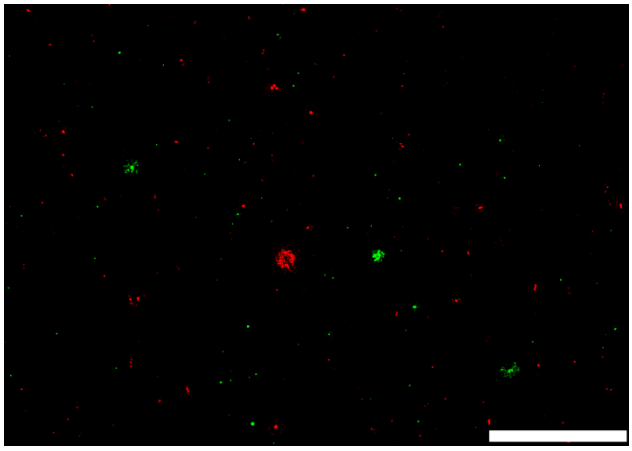


Figure III-20. Representative lectin-mediated binding of bacteria to synthetic fluorescent glycopolymers. Fluorescence microscopy of a mixture of the binding tests: *P. aeruginosa* with PMA-MAEMA-Texas Red (100 µg) and *S. aureus* with PMA-ALAEMA-Fluorescein (100 µg). Scale bar = 100 µm.

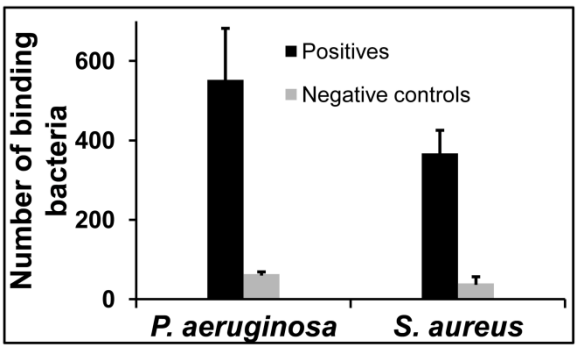


Figure III-21. Quantification of the binding bacteria observed in each sample using the software cellSens Dimension. The final bacteria suspensions from the binding tests were subject to fluorescence microscopy analysis with a magnification of 100X. The green and red fluorescence-labeled bacteria in each photograph representing one or several glycopolymer-binding *S. aureus* and *P. aeruginosa* respectively were analyzed and quantified separately. For both bacteria, glycopolymers containing corresponding pendant sugars (positives) showed dramatically stronger binding compared with fluorescent PMA-GAEMA (negative controls).

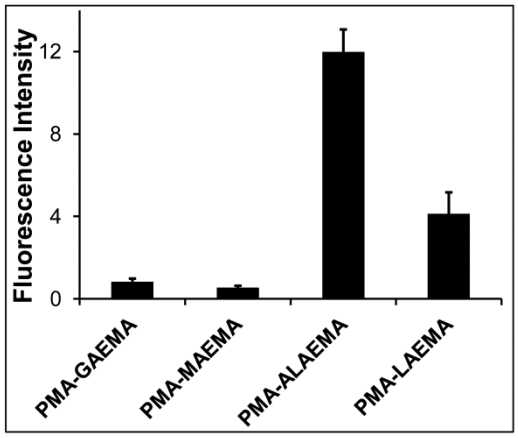
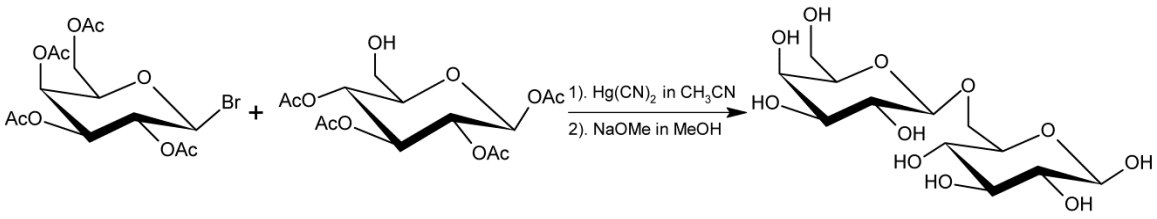
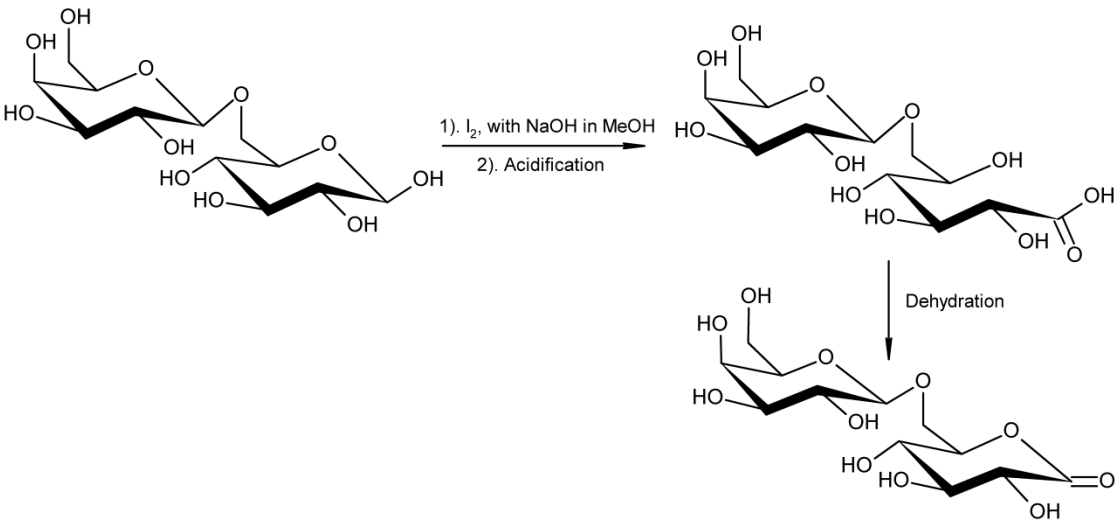


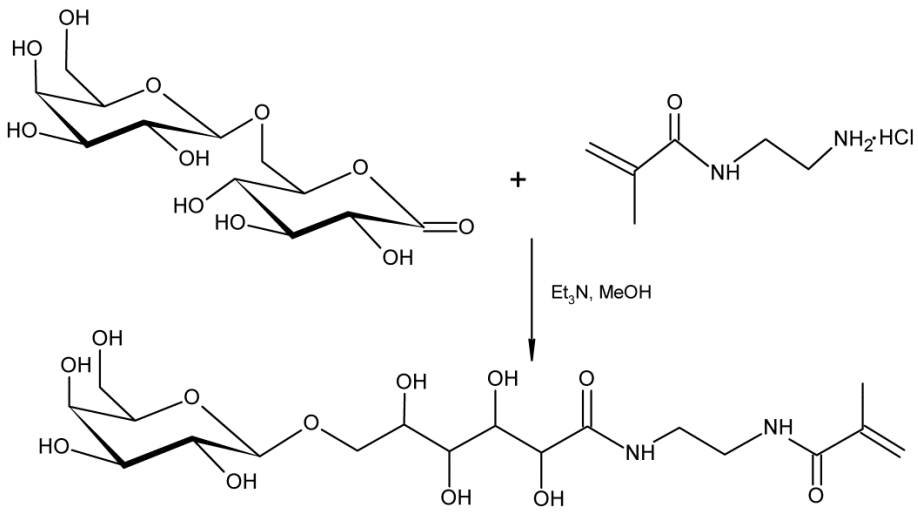
Figure III-22. *S. aureus* ATCC 25923 showed binding specificity towards glycopolymers containing β-galactose as the pendant sugar. Different fluorescent glycopolymers (100 µg) were used in corresponding binding test, and 100 µL of the final bacteria suspensions in PBS were used to measure their fluorescence intensities on the microplate reader (λex/λem = 490/520 nm, slit width=10 nm).



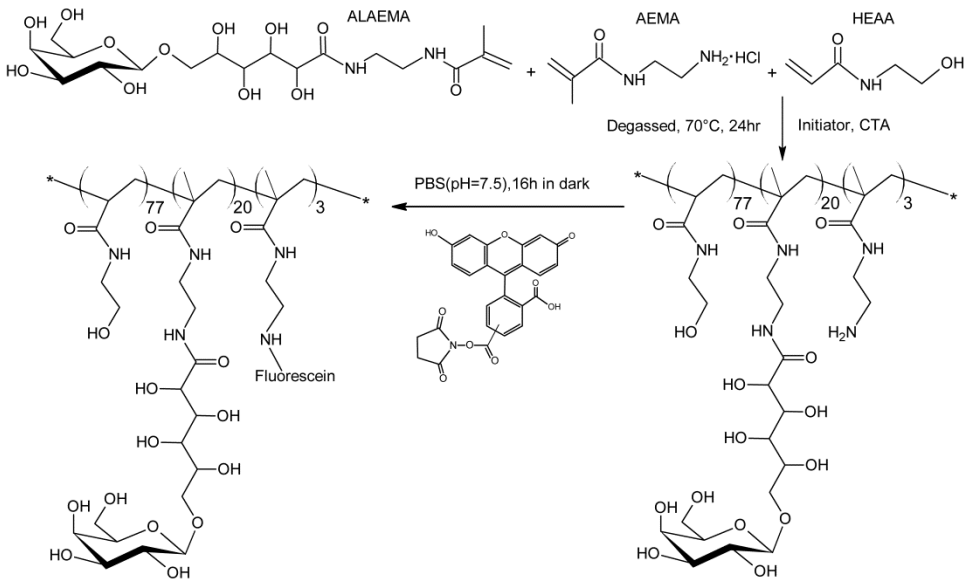
Schemes III-1. Synthesis of allolactose.



Schemes III-2. Synthesis of allolactonolactone.



Schemes III-3. Synthesis of ALAEMA.



Schemes III-4. Synthesis of fluorescent glycopolymers PMA-ALAEMA-Fluorescein containing β–galactoside as the pendant sugar.

Chapter IV. Monosaccharide-binding Profile of *Pseudomonas aeruginosa* Cystic Fibrosis Isolates Determined by testing with Multivalent Fluorescent Glycopolymers

Abstract

As a critical step of the persistent *Pseudomonas aeruginosa* infection in cystic fibrosis lungs, the bacteria use two lectins, LecA and LecB, together with other adhesins to bind specific carbohydrates on the surface of respiratory tract to start colonization. Blocking this binding with inhaled anti-adhesive carbohydrates offers a promising treatment for the infection. Due to the diverse living microenvironment of the bacteria in

the patients' lungs, *P. aeruginosa* isolated from cystic fibrosis often present varied colony morphologies and physiological phenotypes. Therefore, we asked whether *P. aeruginosa* with different morphologies also have different carbohydrate-binding profiles, and what monosaccharides can be bound by the bacteria on a cellular level. To answer these questions, a group of *P. aeruginosa* laboratory strains and clinical isolates with various morphologies were tested against a panel of fluorescent glycopolymers possessing distinct pendant monosaccharides. As revealed by fluorescence microscopy, fluorimetry, flow cytometry and transmission electron microscopy, among the eight monosaccharides tested, α-D-galactose, β-D-*N*-acetylgalactosamine, and β-D-galactose-3-sulfate demonstrated strong binding with all the fifteen clinical isolates and three laboratory stains, and α-D-*N*-acetylneuraminic acid also showed weak binding with some of the *P. aeruginosa* tested. However, within an isogenic bacterial population, only a small percentage (~1%) showed observable binding with the glycopolymers, revealing another phenotypic heterogeneity of the bacteria. Therefore, the phenotypic diversity regarding monosaccharide-binding was not observed among different colony morphologies, but within isogenic populations.

Introduction

P. aeruginosa is a ubiquitous Gram-negative bacillus and a major opportunistic pathogen which is responsible for the chronic respiratory infection in a majority of the cystic fibrosis (CF) patients (1). Within the respiratory tract of CF patients, *P. aeruginosa* normally first appear as motile and non-mucoid, which is the typical morphology of environmental isolates. In the lungs, over time, due to the environmental

stresses (2) and the formation of biofilms (3-5), the bacteria start to exhibit a wide range of phenotypic diversities. These would include, among others, the appearance of different colony morphologies, elaboration of various virulence factors, and numerous physiological heterogeneities (6,7). As a result, and with prolonged antibiotic usage, total eradication of the infection by any current antibiotics becomes very difficult (8). To improve the outcome of antibiotic treatment in CF, a number of new strategies have evolved (9-11). Among these, preliminary clinical trial with sugar inhalation therapy, designed to competitively inhibit the bacterial adhesion within the airway and possibly further eliminate the bacterial infection, has attracted much attention (12).

P. aeruginosa binding with certain carbohydrate components of the respiratory mucins and host cell surface glycoproteins or glycolipids is a crucial step in their bacterial adhesion and colonization (13,14). Therefore, inhibiting this binding by inhaled competitive carbohydrates offers a logical and possible therapeutic strategy. Compared with the chemotherapy approaches, this anti-adhesion therapy is considered to be safer, more economical, and less possible to produce drug-resistant bacteria (10,15,16). Administration of simple sugars or their glycosides as therapeutic agents has shown encouraging results in vitro (17), in ex vivo (18), and in animal models (19). A successful clinical trial and a case report using D-galactose and L-fucose in the inhalation therapy to treat CF or non-CF patients with *P. aeruginosa* infections have been published, as well (12,20). However, our knowledge regarding the *P. aeruginosa*'s specific binding with monosaccharides, still needs to be explored further.

P. aeruginosa express two soluble lectins, LecA and LecB (also known as PA-IL and PA-IIL), and several other adhesins, like pilin, flagellar cap protein, and flagellin

related to virulence factors of the bacteria (21-23). LecA and LecB bind to α-D-galactose and α-L-fucose respectively on glycans (24). Various disaccharides and oligosaccharides have also been identified as the binding targets of pilin and flagellar structures (22,25). Furthermore, a number of *P. aeruginosa* strains and clinical isolates have been demonstrated to bind mucin glycan components (13,26-28). To our knowledge, there hasn't been a thorough examination of the monosaccharide-binding profile of clinical *P. aeruginosa* isolates from CF patients to determine their primary binding targets on a cellular level.

To assess bacterial and isolated bacterial proteins' binding with carbohydrates, mucins and epithelial cells, a number of methods have been developed to include, amongst others, solid phase binding assays, glycoconjugate bindings tests, and glycan microarrays (29-31). Among these methods, synthetic fluorescent glycopolymers have been successfully used in various sugar-lectin interaction studies (32,33), including bacterial carbohydrate-binding tests. For instance, fluorescent glycopolymers containing Lewis antigens or blood group determinants showed different degrees of specific binding with several *P. aeruginosa* laboratory strains and clinical isolates (26). Recently, our group also reported the preparation of a set of glycopolymers and their applications in lectin-mediated bacterial binding tests (34).s

In this report, a panel of fluorescent polyacrylamide-based glycopolymers possessing multivalent monosaccharide side chains (PAA- Fluor-Sugars, structure shown in Figure IV-1) was chosen. The non-reducing end of respiratory mucin glycans plays major role in the bacterial binding. To represent these peripheral sugars that are mainly composed of L-fucose, D-galactose, D-*N*-acetylgalactosamine, and D-*N*-acetylneuraminic

acid, some of which are occasionally sulfated (35-37), nine different monosaccharides (Table I) were included in this study to test against a group of *P. aeruginosa* laboratory strains and clinical isolates with different colony morphologies (Table II). We aimed to answer two questions regarding the carbohydrate-binding of *P. aeruginosa* in CF lungs: what monosaccharides *P. aeruginosa* can bind specifically on a cellular level, and whether different morphologies also have different carbohydrate-binding profiles. Answers to these questions will improve our understanding of the pathogenesis of *P. aeruginosa* infection in CF lungs and provide new perspective for the sugar inhalation anti-adhesion therapy being tested in clinic to treat bacterial infection.

Materials and methods

Materials

Polyacrylamide glycopolymers possessing monosaccharide side chains (Table I) were purchased from GlycoTech (Gaithersburg, MD). M9 minimum media, bovine serum albumin (BSA), sodium dodecyl sulfate, α-L-fucosidase (bovine kidney) were obtained from Sigma-Aldrich (St. Louis, MO). Gold-labeled anti-FITC antibody was purchased from Electron Microscopy Sciences (Hatfield, PA). Agarose beads coated with *Griffonia Simplicifolia* lectin I, *Erythrina Cristagalli* lectin, soybean agglutinin, wheat germ agglutinin, and *Aleuria aurantia* lectin were purchased from Vector Laboratories (Burlingame, CA), and *Limulus polyphemus* lectin beads were from EY laboratories (San Mateo, CA).

Bacterial strains and growth conditions

The *P. aeruginosa* laboratory strains were obtained from ATCC (Manassas, VA), and the clinical isolates were collected from patients admitted to University Hospital at University of Missouri, Columbia. The bacterial colonies grown on tryptic soy agar with typical morphologies were selected and subcultured at 35°C in M9 minimum media for 35 h or in TSB for 16 h (depending on whether culturable in minimum media, Table IV-2). This study was approved by the University of Missouri's Health Sciences IRB.

Binding tests of glycopolymers with lectin coated agarose beads

To verify the binding specificities of the glycopolymers, a method previously published was employed herein (34). As an example of this protocol, a slurry of agarose beads (100 µL) coated with soybean agglutinin, a GalNAc-binding lectin, was washed with PBS (3 x 1 mL), and then the pellets were resuspended in 0.5 mL PBS (with 1 mM $CaCl_2$), to which were added 20 µL of sodium phosphate buffer (0.3 M, pH=7.4) containing 10 µg of PAA-α-GalNAc-Fluor. The mixtures were incubated at 35°C for 1 h with gentle shaking, and then went through two repeated sets of centrifugation (4°C, 2, 000 g for 3 min) and washing (1 mL PBS). The final pellets were resuspended in 200 µL of PBS and observed by fluorescence microscopy.

Bacterial binding tests

The bacterial cultures were centrifuged at 4°C 9,000 x g for 15 min and the pellets were resuspended with filter-sterilized binding solution (155 mM NaCl, 1 mM $CaCl_2$, 0.5% BSA and 0.05% sodium dodecyl sulfate in distilled H_2O) to attain suspensions with

an optical density of 1.0 at 600 nm. The exact numbers of bacteria in the suspensions for each strain and isolate were determined by serial dilutions and plating of the suspensions. For each bacterial binding test, 18 μg of glycopolymers dissolved in 42 μL of sodium phosphate buffer (0.3 M, pH=7.4) were added to 1.0 mL of bacterial suspension. Bacterial suspensions without any glycopolymers and with conjugates containing no pendant sugars were used as negative control. The mixtures were then incubated at 35°C in the dark for 2 h with gentle shaking, followed by two repeated sets of centrifugation (4°C, 9,000 x g, 15 min) and washing (1.2 mL of saline, 155 mM NaCl containing 1 mM $CaCl_2$). The resulting bacterial pellets were resuspended in 100 μL of PBS (pH=8). The fluorescence intensities (λex/λem = 490/520 nm, slit width=10 nm) and optical densities at 600 nm of the suspensions were measured on a microplate reader (Synergy Mx, BioTek). The fluorescence signals of the samples were normalized with their optical densities and the fluorescence intensities of the corresponding glycopolymers. The bacterial suspensions were then subjected to fluorescence microscopy (10 x objective lens, Olympus BX43) analysis using the software cellSens Dimension (v1.7.1). The fluorescence-labeled bacteria were detected and enumerated. All bacterial binding experiments were performed in triplicate.

Preparation of Reversible addition−fragmentation chain-transfer (RAFT) polymerization based PAA-α-Fuc-Fluor

Synthesis of α-L-fucopyranosyl-(1→6)-D-glucose

α-L-Fucopyranosyl-(1→6)-D-glucose was prepared according to a published method with some modifications (47). Briefly, to a stirred solution of L-fucose (2.0 g)

and triethylamine (8.8 mL) in 60 mL of dry dimethylformamide (DMF), 8 mL of chlorotrimethylsilane were added at 0°C and the reaction was stirred at room temperature for 4 h. Pentane (100 mL) and ice (40 mL) were then added and mixed; the organic layer was washed with cold water (40 mL x 3), dried over magnesium sulfate and evaporated to get per-*O*-trimethylsilyl-L-fucose (4.4 g, 80% yield).

To a stirred solution of per-*O*-trimethylsilyl-L-fucose (2.7 g) in 20 mL of dry methylene chloride was added 0.86 mL of iodotrimethylsilane. Following 20 min of stirring at room temperature, the mixture was added to a solution of 2.3 g of 6-hydroxy-tetraacetyl-β-D-glucose (48) and 2, 6-di-tert-bytylpyridine (1.34 mL) in dry methylene chloride (40 mL). After 5 h of stirring at room temperature, 60 mL of MeOH were added to the reaction and stirred for 20 min to remove the trimethylsilyl groups. The mixture was then neutralized with ion exchange resin, filtered, and concentrated. The partially acetylated products (1.61 g, 54% yield) were obtained by column chromatography ($CHCl_3$: MeOH 8:1). To 1.0 g of the partially acetylated disaccharide dissolved in 2 mL of CH_2Cl_2 were added 50 mL of MeOH and 1.0 mL of sodium methoxide solution (25 wt. % in methanol). The mixture was stirred at room temperature for 20 min, neutralized with cation exchange resin, filtered, and evaporated to give the disaccharide α-L-fucopyranosyl-(1→6)-D-glucose with a quantitative yield.

Synthesis of fucose-glucono-1,5-lactone

To a solution of α-L-fucopyranosyl-(1→6)-D-glucose (0.4 g) dissolved in 1.0 mL of water and 2.0 mL MeOH, were added an iodine solution in MeOH (0.6 g in 8 mL) at 40°C. A solution of 4% KOH in MeOH (15 mL, w/v) was then added dropwise for ~15

min, and the mixture was kept stirring until the iodine color completely disappeared. The mixture was then cooled on ice and the precipitation was filtered. The pellet was rinsed with small amount of cold MeOH and redissolved in 0.5 mL of water and re-precipitated in 25 mL of -20°C methanol. The potassium salt was converted to the free acid form by passing the aqueous solution through a column containing 1 mL of cation-exchange resin. Lactone was formed by concentrating and drying the acid with repeated (5 times) evaporation of methanol and ethanol solution (0.33 g, 85% yield).

Synthesis of glycomonomer

To a solution of the disaccharide lactone (330 mg) in 2.0 mL of methanol were added 2.0 mL of methanol containing *N*-(2-aminoethyl) methacrylamide hydrochloride (197 mg) and hydroquinone monomethyl ether (MEHQ, 0.5 mg), and 0.3 mL of triethylamine. Following 16 h of stirring at room temperature, 10 mL of water were added, and the methanol and triethylamine in the mixture were then removed from the mixture under vacuum. The aqueous solution was passed through an anion exchange column into a receiving beaker containing 0.5 mg of MEHQ. After the newly released triethylamine was removed *in vacuo*, the solution was passed through a cation exchange resin column and freeze-dried to give the glycomonomer: 6-*O*-α-L-fucopyranosyl-D-gluconamidoethyl methacrylamide (424 mg, 92% yield). ESI-MS (in methanol: water 50:50): calculated *m/z* for $C_{18}H_{32}O_{11}N_2+Na^+$, 475.18983; observed m/z 475.19363. ^{1}H NMR (800 MHz, D_2O): δ (ppm) 5.70 (s, 1H, H-17A), 5.46 (s, 1H, H-17B), 4.92 (d, $J_{7,8}$ =3.2, 1H, H-7), 4.30 (d, $J_{2,3}$ =3.2, 1H, H-2), 4.11 (m, 1H, H-10), 4.08 (m, 1H, H-4), 3.94 (m, 1H, H-3), 3.90 (dd, 1H, H-8), 3.83 (m, 1H, H-5), 3.82-3.79 (m, 2H, H-6), 3.80-

3.75 (m, 2H, H-12), 3.79 (m, 1H, H-11), 3.60 (dd, 1H, H-9), , 3.47-3.40 (m, 4H, H-13, H-14), 1.92 (s, 3H, H-15), 1.22 (d, 3H, H-12). ^{13}C NMR (200 MHz, D_2O): δ (ppm) 177.59 (C-1), 175.02 (C-18), 141.89 (C-16), 124.08 (C-17), 101.82 (C-7), 76.13 (C-2), 74.73 (C-5), 73.24 (C-4), 72.85 (C-3), 73.44 (C-8), 71.87 (C-9), 77.15 (C-11), 69.59 (C-10), 58.86 (C-6), 41.79 (C-13), 41.36 (C-14), 20.52 (C-15), 18.17 (C-12).

Synthesis of glycopolymer PAA-α-Fuc

To a 1 mL Schlenk tube was added 0.4 mL of water containing 31.6 mg of 6-*O*-α-L-fucopyranosyl-D-gluconamidoethyl methacrylamide, 1.7 mg of *N*-(2-aminoethyl) methacrylamide and 27.5 μL of *N*-(2-hydroxyethyl) acrylamide. To the mixture were then added 50 μL of DMF containing 0.53 mg of (4-cyanopentanoic acid)-4-dithiobenzoate, and another 50 μL of DMF containing 250 μg of 4,4′-azobis-(4-cyanovaleric acid) sequentially. The solution was degassed with 3 freeze–evacuate–thaw cycles and kept in a water bath at 70°C for 24 h. The solutions were then dialyzed against deionized water (6 x 2 L) for 24 h (*MWCO*=3,500) and lyophilized to attain the PAA-α-Fuc (61 mg, 93.8% yield). The glycopolymers' dispersity (1.28) and degree of polymerization (95mer) were determined by GPC. ^{1}H and ^{13}C NMR spectra of the glycopolymer were shown in Figure IV-11, 12. The post-modification of the synthesized glycopolymers and their characterizations were performed as described in Chapter III.

Bacterial binding analysis by flow cytometry

Further verification of the bacterial glycopolymer-binding employed a Beckman Coulter MoFlo XDP flow cytometer and Summit software (v5.3.1.12636) for data

acquisition and analyses. For each bacterial sample, ~15,000 events were collected (debris and doublets excluded).

Transmission electron microscopy

To visualize the binding of *P. aeruginosa* with fluorescent glycopolymers, transmission electron microscopy was utilized. Formvar carbon-coated nickel grids were floated on 25 µL of the resultant bacterial suspension from the binding tests for 10 min, air-dried, fixed with 4.0% paraformaldehyde in PBS for 30 min at room temperature. The grids were then washed on 40 µL of PBS (2 x 5 min) and once on PBS containing 0.05% Tween-20 (v/v) and 1% BSA (PBS-Tween-BSA). Grids were floated on a droplet of gold-labeled anti-FITC antibody at 1:20 in PBS-Tween-BSA at 4°C for 16 h, washed with PBS-Tween-BSA (6 x 5 min) and PBS (3 x 5 min). Following post-fixation with 2% glutaraldehyde in PBS (v/v) for 5 min, and washing on PBS (2 x 5 min) and distilled H_2O (5 x 2 min), the grids were then examined with a JEOL 1400 transmission electron microscope without staining.

Results and discussion

Comparative binding of different fluorescent glycopolymers to *P. aeruginosa* isolates and strains

The fluorescent glycopolymers (PAA-Sugars-Fluor) used in this study are listed in Table IV-1. The lectin-binding specificities and capabilities of some of these polymers were confirmed by testing with corresponding lectin-coated agarose beads (Table IV-3). Those containing no pendant sugar but only aminoglucitol (spacer molecule) were used

as negative control. Fifteen *P. aeruginosa* clinical isolates and three laboratory strains were tested, including five mucoid, four non-mucoid and nonmotile, and nine motile (see Table II). After the incubation with glycopolymers and the following washings, the bacterial pellets were resuspended and subject to tests with fluorescence microscopy and fluorimetry. BSA and sodium dodecyl sulfate were used in the binding tests to prevent non-specific bacterial binding with the polymers.

Analysis by fluorescence microscopy revealed that among the eight monosaccharides, α-Gal, β-GalNAc and β-SO4-Gal showed relatively strong binding with all strains and isolates tested, and weaker binding was observed from α-Neu5Ac with some of the *P. aeruginosa* (Figure IV-2). Under microscope, the difference between positive and negative binding bacterial samples was obvious (Figure IV-3). With a higher magnification, it can be observed that the bacteria that demonstrate strong binding with the glycopolymers, in fact, only accounted for ~1% of the total population (Figure IV-4), suggesting a heterogeneous expression of lectins within an isogenic bacterial group. To determine whether the low bacterial binding ratio was due to the washing and centrifugation during the tests, an aliquot of the bacterial suspensions after the incubation with glycopolymers was placed onto a microscope slide without washing and air-dried. After the unbound materials were gently rinsed off with phosphate buffered saline (PBS), the samples were analyzed by fluorescence microscopy. As expected, a higher binding ratio (~2.5%) was observed, but the majority of the bacteria still didn't show any observable binding (Figure IV-5). This result demonstrates that some of the bacterial binding with glycopolymers was washed off during the two rounds of centrifugation and washing, but it doesn't change the fact that a very small portion of the bacteria showed

much stronger binding than the rest of the population. In another experiment where soybean agglutinin-coated agarose beads were tested with PAA-β-GalNAc-Fluor, more intense centrifugations (9,000 x g, 2 x 15 min) didn't show a decreased binding compared with a less intense centrifugation (2,000 x g, 2 x 3 min) (data not shown), suggesting the lectin binding with the glycopolymer was able to stand the centrifugation at such a speed. Considering that only a very small percentage of the fluorescent glycopolymers remained in the sample after the binding tests (~0.1%, based on fluorescence intensities), this low binding ratio was very unlikely because the amount of polymers used in the tests was too low.

Of the three binding monosaccharides, α-Gal is the binding target of LecA (24), and β-GalNAc and α-Neu5Ac have been suggested to be involved in the *P. aeruginosa* binding process as well (25,26). Therefore, as the first report of profiling monosaccharide-binding of clinical *P. aeruginosa* isolates, results observed in this study regarding these three monosaccharides were consistent with the previous findings. However, β-Gal, which was considered to be involved in the binding of *P. aeruginosa* (13,38), didn't show any noticeable binding in our tests. This suggests that β-Gal might not be involved in the lectin binding, even though it's at the non-reducing end of the oligosaccharides that were implicated in the binding of *P. aeruginosa.* But it's still possible that β-Gal can be recognized by LecA when it's conjugated to other structures, especially these with high hydrophobicity (39).

As the binding target of LecB of *P. aeruginosa*, α-L-fucose didn't show strong binding with the bacteria in various binding solutions. To confirm this finding, we synthesized fluorescent polyacrylamide-based glycopolymers containing α-L-fucoside,

which showed stronger binding with *Aleuria aurantia* lectin than the commercial PAA-α-Fuc-Fluor (data not shown). When the synthesized glycopolymers were used in the *P. aeruginosa*'s binding tests, no strong binding was detected either. This surprising negative binding results can be explained by the observation reported by Jaeger et al. that LecB was actually released from the outer membrane of the bacteria by the treatment with L-fucose or 4-nitrophenyl α-L-fucose (37,40). It's currently under investigation in our laboratory whether LecB was released from the outer membrane in the binding tests with PAA-α-Fuc-Fluor.

Also of particular importance is the fact that the sulfation of the monosaccharides dramatically changed their binding affinities with the bacteria. Compared to β-Gal, β-D-galactose-3-sulfate exhibited much stronger binding with *P. aeruginosa*; while β-D-GalNAc-3-sulfate could not be bound by the bacteria, β-GalNAc was one of the three strongest binding-monosaccharides. Taking into consideration that the sulfation level of mucin glycans is changed in CF patients (41), the alteration of the bacterial binding with sulfated carbohydrates may have implications in the pathogenesis of *P. aeruginosa* infection in CF patients.

There's no obvious difference when the monosaccharide-binding results between mucoid *P. aeruginosa* with non-mucoid, and motile with nonmotile were compared. Some isolates cultured in tryptic soy broth (TSB), or cultured in M9 media but harvested at the exponential phase (16 h) were also tested with the glycopolymers using the same protocol, and a similar binding pattern was observed (Figure IV-6). When the bacteria were subject to a doubled amount of polymers, both α-Gal and SO_4-β-Gal showed stronger binding results, but not β-GalNAc, the bacterial binding sites of which might be

saturated with the routine amount of PAA-β-GalNAc-Fluor (18 μg) used already. As an illustration, the binding results from a clinical isolate CF 3435 was shown here in Figure IV-6.

Besides the observation and analysis by fluorescence microscopy, the fluorescence intensities of the bacterial suspensions were also measured on a microplate reader and the results were consistent with the microscopy data (Figure IV-7). Similar to the results shown in Figure IV-2, the fluorescence intensities of the different strains and isolates also varied noticeably. This could be partially caused by different sizes and shapes of the bacteria (colony-forming unit for each strain and isolates varies from 1.9–5.7 x 10^9/mL, as shown in Table II), and also possibly the varied levels of lectin-expression.

Visualization of monosaccharide-binding of *P. aeruginosa*

The binding of glycopolymers by *P. aeruginosa* ATCC BAA-47 and CF 8314-1 were visualized by transmission electron microscopy. The bacteria binding fluorescent glycopolymers were incubated with gold-labeled anti-fluorescein isothiocyanate (FITC) antibodies. Heavily gold-labeled bacteria were detected from the binding tests with α-Gal, β-GalNAc and β-SO4-Gal, where dramatic uneven distributions of labeling were observed. For instance, in the ATCC BAA-47 binding test with PAA-α-Gal-Fluor, only ~1% of the bacteria were heavily labeled with gold granules (Figure IV-8a), the distribution of which was very scarce on the rest of the bacterial population. In the negative-binding tests, the labeling was almost negligible (Figure IV-9). For CF 8314-1, a nonmotile non-mucoid CF isolate, a similar labeling pattern was observed (Figure IV-

8b). The high heterogeneity of gold-labeling within the bacterial population agreed with the observation that only a very small portion of the bacteria showed observable binding with fluorescence microscopy (Figure IV-4).

Flow cytometry results

Following the glycopolymer-binding tests, some of the resultant bacterial suspensions were analyzed on a Beckman Coulter MoFlo XDP cell sorter. As an example, the results from a clinical isolate CF 3435 were presented in Figure IV-10. The three binding monosaccharides, α-Gal, β-GalNAc, and β-SO4-Gal, were well-differentiated from the other sugars by a small subpopulation of the bacteria (<1.5%) showing strong fluorescence, which was consistent with the observations from microscopy and transmission electron microscopy. During the analysis, some of the bacteria that were binding PAA-α-Gal-Fluor were isolated by the cell sorter, subcultured and retested with PAA-α-Gal-Fluor using the same protocol. Very similar binding results were obtained (data not shown), suggesting the sugar-binding heterogeneity observed in the bacterial population was not inheritable.

In a study published by Scharfman et al.(26), flow cytometry was employed to investigate the *P. aeruginosa*'s binding with PAA glycopolymers. Instead of two peaks in the histograms as presented in Figure IV-10, a shift along the abscissa (fluorescence intensity) was reported, suggesting that a majority of the bacteria bound the glycopolymers to some extent. This may be because, after incubation with the polymers, the bacteria were analyzed directly by flow cytometry therein without washing, and the fluorescence intensity shift can be in part due to non-specific binding. In contrast, in our

study the bacteria were washed stringently to eliminate the non-specific binding, and thus presented the two heterogeneous subgroups in the positive-binding tests.

Growing in the CF lungs, *P. aeruginosa* developed diverse phenotypes to adapt the local microenvironment (2,7). Even within an isogenic population, remarkable phenotypic variations can be observed (6). The heterogeneity in their carbohydrate-binding behaviors observed in this study, which reflects varied expression levels of lectins, is another phenotypic diversity of the bacteria.

As a regular procedure performed in many binding tests to further prove their specificities, competitive binding experiment was carried out in our study as well. Since there is no appropriate non-fluorescent glycopolymers available to be used as the competitor, monovalent glycosides, such as methyl α-D-galactoside (45 mg, 2,500-folder excess of the glycopolymers used), were tested as the competitive inhibitor. But no apparent inhibition effects were observed (data not shown). This can be explained by the much higher binding affinities possessed by the multivalent glycopolymers than that of any monovalent glycosides (42). And it's another target of our ongoing research to synthesize glycopolymers with similar structures as the polymers employed herein but without fluorophores, to be used as competitive inhibitors in bacterial binding studies.

Using D-galactose and L-fucose in the sugar inhalation therapy has been effective to treat *P. aeruginosa* infections in CF (12) or other immune compromised patients (20). Taking our findings into account, simple α-galactosides, such as methyl α-D-galactose and melibiose, would have stronger anti-adhesive effects than D-galactose. Also, it may be beneficial to add GalNAc, β-SO4-Gal and Neu5Ac into the inhalation therapy. Even though binding between lectins and carbohydrates often involves more than one

monosaccharide, using simple glycosides in the sugar inhalation treatment is still a sensible option, considering the difficulties of acquiring many of the oligosaccharides and the potential by-effects of inhaling high-molecular-weight molecules.

The carbohydrate-binding specificities of *P. aeruginosa* lectins LecA and LecB have been extensively studied (43), but the binding profiles of other adhesins of the bacteria, like flagellin, pillin, or LPS are still not very clear. Using the protocols presented here, the monosaccharide-binding profiles of the bacteria were well-established on a cellular level. Our strategy holds promise for applications to other human pathogens, like *Staphylococcus aureus, Helicobacter pylori, Escherichia coli,* to name a few, whose carbohydrate-binding behaviors are all of great interest (44,45).

Conclusion

A protocol using fluorescent glycopolymers together with a panel of analytical instruments was established to investigate the *P. aeruginosa* binding with monosaccharides. Among the eight monosaccharides tested, α-Gal, β-GalNAc, and β-SO4-Gal showed strong specific binding by all the tested *P. aeruginosa* laboratory strains and clinical isolates, as revealed by fluorescence microscopy, fluorimetry, transmission electron microscopy and flow cytometry. Within an isogenic population, only a small percentage (<1.5%) of the bacteria showed strong binding with the glycopolymers, revealing an intriguing phenotypic heterogeneity of the bacteria. Taking these findings into consideration, the sugar combinations in anti-adhesion therapy for *P. aeruginosa* infection in CF should be reconsidered. The treatment might be improved by adding GalNAc, β-SO4-Gal and Neu5Ac, and if possible, replacing D-galactose with methyl α-

D-galactose or melibiose, both containing α-D-galactose residue. Our results provide new perspectives for the pathogenesis of *P. aeruginosa* infection in CF lungs. Also, using this protocol, the carbohydrate-binding patterns of other bacterial pathogens can be determined as well.

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Table IV-1. Glycopolymers and their pendant monosaccharides used in this study

Abbreviations for glycopolymers	Pendant monosaccharides
PAA-α-Gal-Fluor	α-D-Galactose
PAA-β-Gal-Fluor	β-D-Galactose
PAA-α-GalNAc-Fluor	α-D-N-Acetylgalactosamine
PAA-β-GalNAc-Fluor	β-D-N-Acetylgalactosamine
PAA-β-GlcNAc-Fluor	β-D-N-Acetylglucosamine
PAA-β-SO4-Gal-Fluor	β-D-Galactose-3-sulfate
PAA-α-SO4-GalNAc-Fluor	β-D-N-Acetylgalactosamine-3-sulfate
PAA-α-Neu5Ac-Fluor	α-D-N-Acetylneuraminic acid
PAA-α-Fuc-Fluor	α-L-Fucose

Table VI-2. *P. aeruginosa* clinical isolates and lab strains tested in this study

Clinical Isolates and Lab Strains	Description
CF 8314-1	Nonmotile, nonmucoid, CF sputum, green diffusible pigment
CF 8314-2	Mucoid, CF sputum, from the same patient as the previous isolate
CF 8981-1	Nonmotile, nonmucoid, CF sputum, restricted growth in M9 media, cultured in TSB[a]
CF 8981-2	Mucoid, CF sputum, from the same patient as the previous isolate, brown diffusible pigment
CF 3318	Motile, CF sputum, restricted growth in M9 media, cultured in TSB
CF 3247	Motile, CF sputum
CF 3396	Mucoid, CF sputum
CF 3443	Small colony variant, CF sputum
CF 3371	Nonmotile, nonmucoid, dry-looking, CF throat, aggregated in M9 media, cultured in TSB
CF 3372	Mucoid, CF throat, from the same patient as the previous isolate
CF 3435	Motile, CF throat
CF 3437	Motile, CF throat, restricted growth in M9 media, cultured in TSB
CF 3446	Motile, CF throat
3380	Motile, non-CF hip infection
3391	Motile, non-CF sputum
ATCC BAA-47	PAO1, motile, green diffusible pigment
ATCC 15442	Motile
ATCC 33468	Mucoid

[a] Tryptic soy broth

Table VI-3. Lectins used to validate the binding specificities of the glycopolymers and their corresponding target carbohydrates

Lectins	Target carbohydrates
Griffonia Simplicifolia lectin I	α-Gal
Erythrina Cristagalli lectin	β-Gal
Soybean agglutinin	GalNAc
Limulus polyphemus lectin	α-Neu5Ac
Wheat germ agglutinin	β-GlcNAc
Aleuria aurantia lectin	α-Fuc

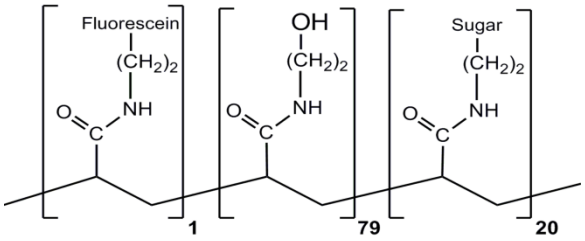

Figure IV-1. The schematic structure of the fluorescent glycopolymers used in this study.

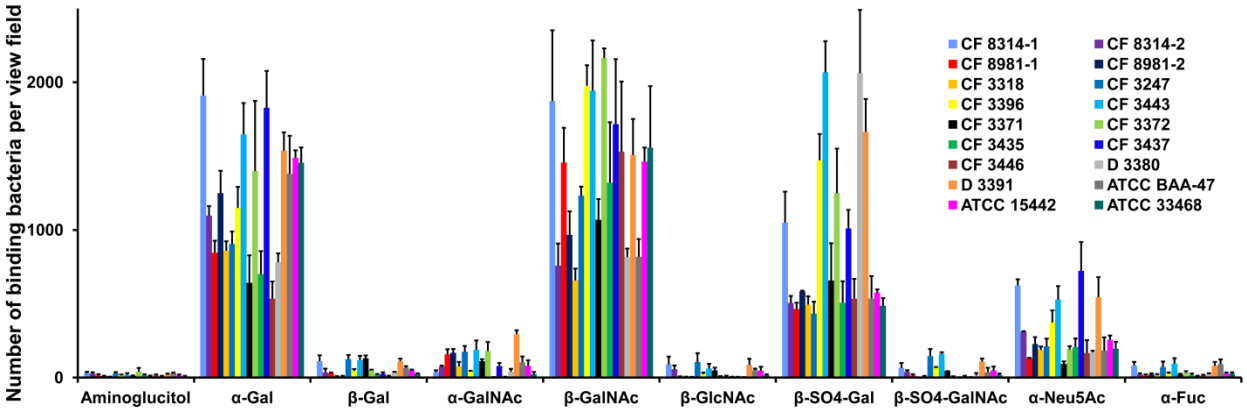

Figure IV-2. The number of binding bacteria in each test by fluorescence microscopy. The resulting bacterial suspensions following binding testes were analyzed by fluorescence microscopy. Three photos were taken randomly for each sample, where the bacteria binding with the glycoconjugates were enumerated by the software cellSens Dimension. α-Gal, β-GalNAc and β-SO_4-Gal demonstrated high numbers in all of the *P. aeruginosa* strains and isolates tested, and α-Neu5Ac showed weak binding with some of the isolates. The average numbers from triplicate experiments were used here, and error bars stand for standard deviations.

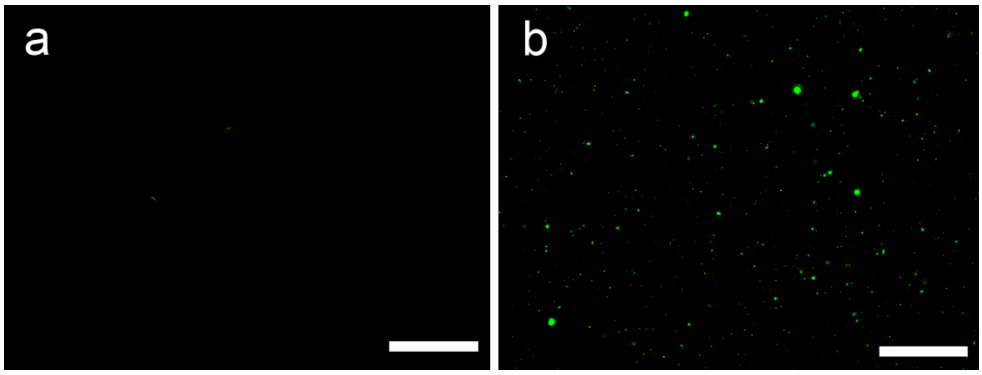

Figure IV-3. Representative results of bacterial binding test with fluorescent glycoconjugates by fluorescence microscopy. A motile *P. aeruginosa* isolate CF 3435 was tested with 18 µg of PAA-α-GalNAc-Fluor and PAA-β-GalNAc-Fluor separately. Compared with the very weak binding with PAA-α-GalNAc-Fluor (a), strong binding was observed with PAA-β-GalNAc-Fluor (b). Scale bar=100 µm.

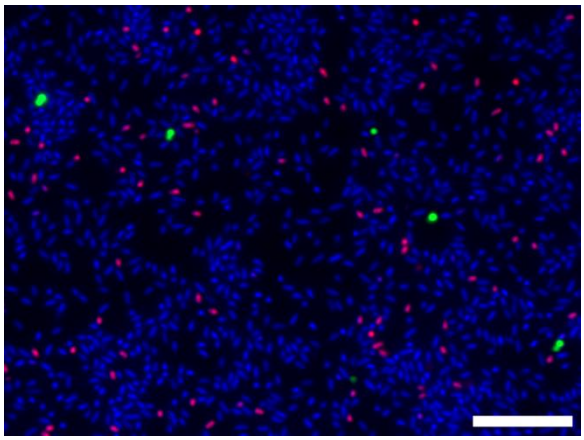

Figure IV-4. Only a small portion of the *P. aeruginosa* ATCC BAA-47 population showed strong binding with PAA-α-Gal-Fluor. Following the binding tests, the bacterial suspension in 1 mL PBS was labeled with 4',6-diamidino-2-phenylindole (DAPI, 0.5 nmol) and propidium iodide (20 pmol) for 20 min in the dark, centrifuged, resuspended in 1 mL PBS (pH=8), and then analyzed by fluorescence microscopy. Only ~1% of the bacteria in this view (in green) were binding with the glycoconjugates. Live bacteria cells were labeled with DAPI (in blue), and dead with propidium iodide (in red). Scale bar=10 μm.

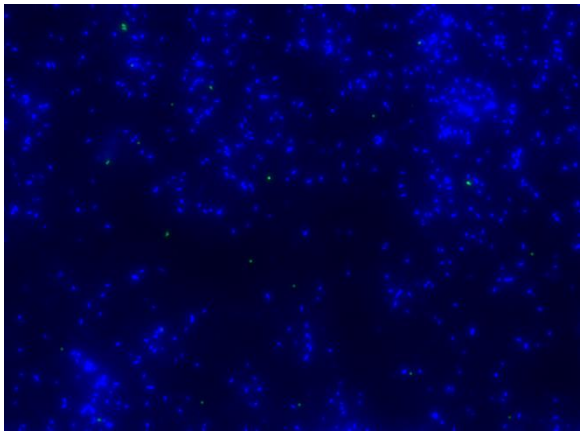

Figure IV-5. A higher binding ratio was observed when the bacteria were not washed extensively. An aliquot of the bacterial suspensions after the 2 h incubation with glycoconjugates was dropped on a glass slide and air-dried. After the unbound materials were rinsed off with PBS, the samples were subject to fluorescence microscopy analysis. A higher binding ratio (~2.5%) was observed, but the majority of the bacteria still didn't show any strong binding.

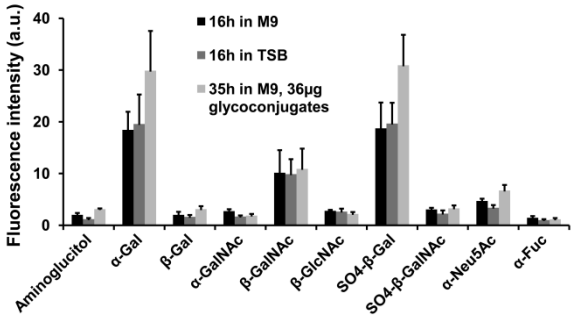

Figure IV-6. Monosaccharide-binding pattern of *P. aeruginosa* CF 3435 cultured in different media harvested at different time or tested with double amount of glycoconjugates. The culture of CF 3435 in M9 minimum media or TSB were harvested at 16 h and tested with fluorescent glycoconjugates using the standard procedure. A similar sugar-binding pattern was observed with the culture in M9 media harvested at 35 h. When tested with double amount of glycoconjugates, α-Gal and SO_4-β-Gal showed higher binding signals, but not β-GalNAc.

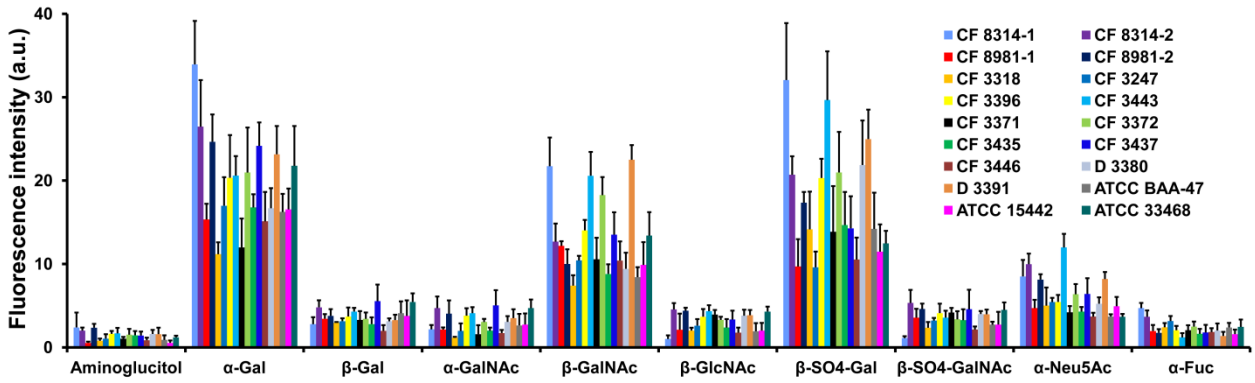

Figure IV-7. Normalized fluorescence intensities of the bacterial suspensions from the binding tests. In all the strains and isolates of *P. aeruginosa* tested, the fluorescence intensities of the three sugars: α-Gal, β-GalNAc, and β-SO4-Gal, were dramatically higher than those from the rest sugars, and α-Neu5Ac bound weakly to some of the isolates tested.

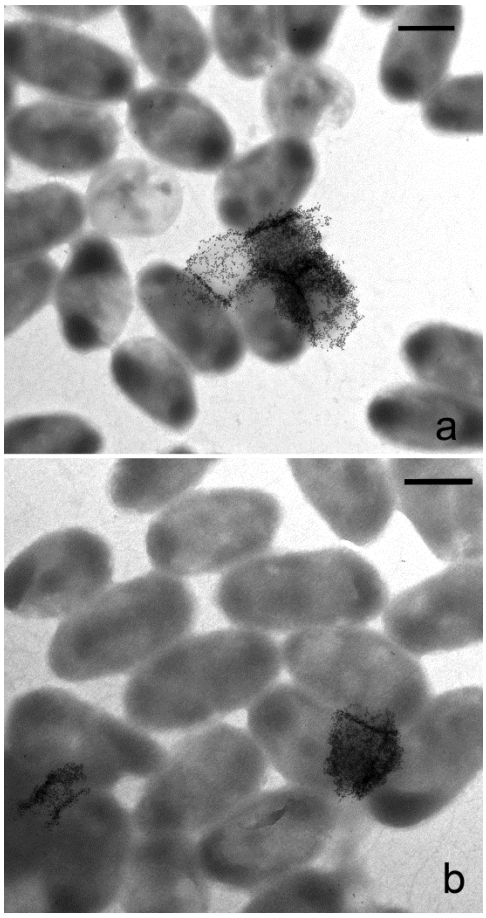

Figure IV-8. Transmission electron micrographs of a *P. aeruginosa* laboratory strain ATCC BAA-47 (a) and a clinical isolate CF 8314-1 (b) binding with PAA-α-Gal-Fluor. Following binding tests, the bacterial suspensions deposited on Formvar carbon-coated nickel grids were incubated with gold-labeled anti-FITC antibody. Heavy labeling was observed in both strains' tests with α-Gal, but only ~1% of the bacteria were heavily labeled. Scale bar=0.5 μm.

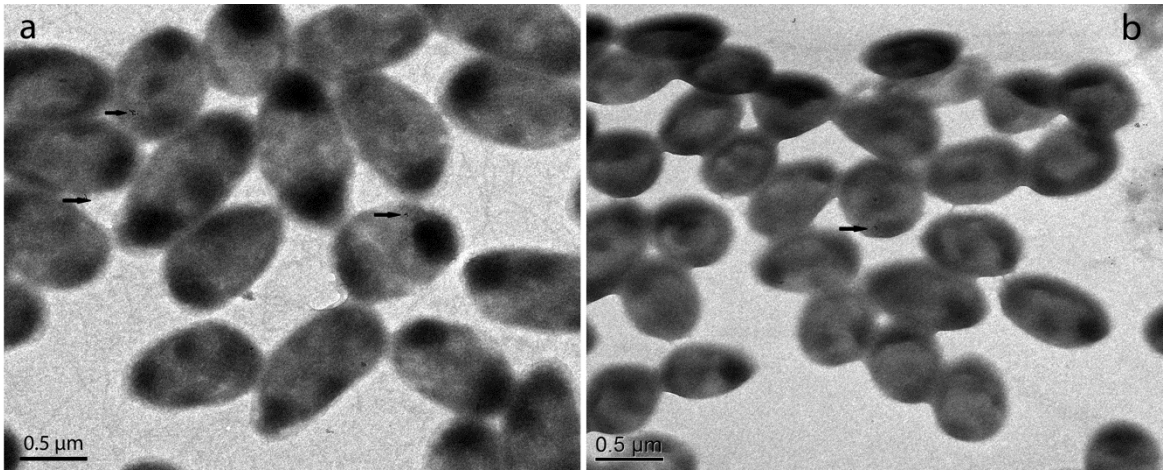

Figure IV-9. Transmission electron micrographs of a *P. aeruginosa* lab strain ATCC BAA47 (a) and a clinical isolate CF 8314-1 (b) binding with PAA-β-Gal-Fluor. Following binding tests, the bacterial suspensions deposited on Formvar carbon-coated nickel grids were incubated with gold labeled anti-fluorescein isothiocyanate antibody. Only scarce labeling was observed in both strains' tests with β-Gal (arrowhead).

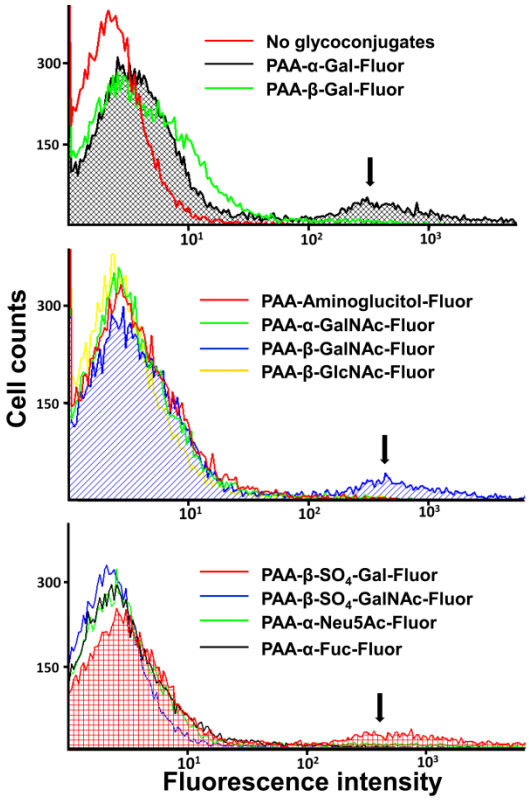

Figure IV-10. Flow cytometry data of *P. aeruginosa* isolate CF 3435 binding with different glycoconjugates. A small portion of bacteria with strong fluorescence (~1.5%, indicated with arrows) well-differentiate α-Gal, β-GalNAc, and β-SO4-Gal from the other sugars.

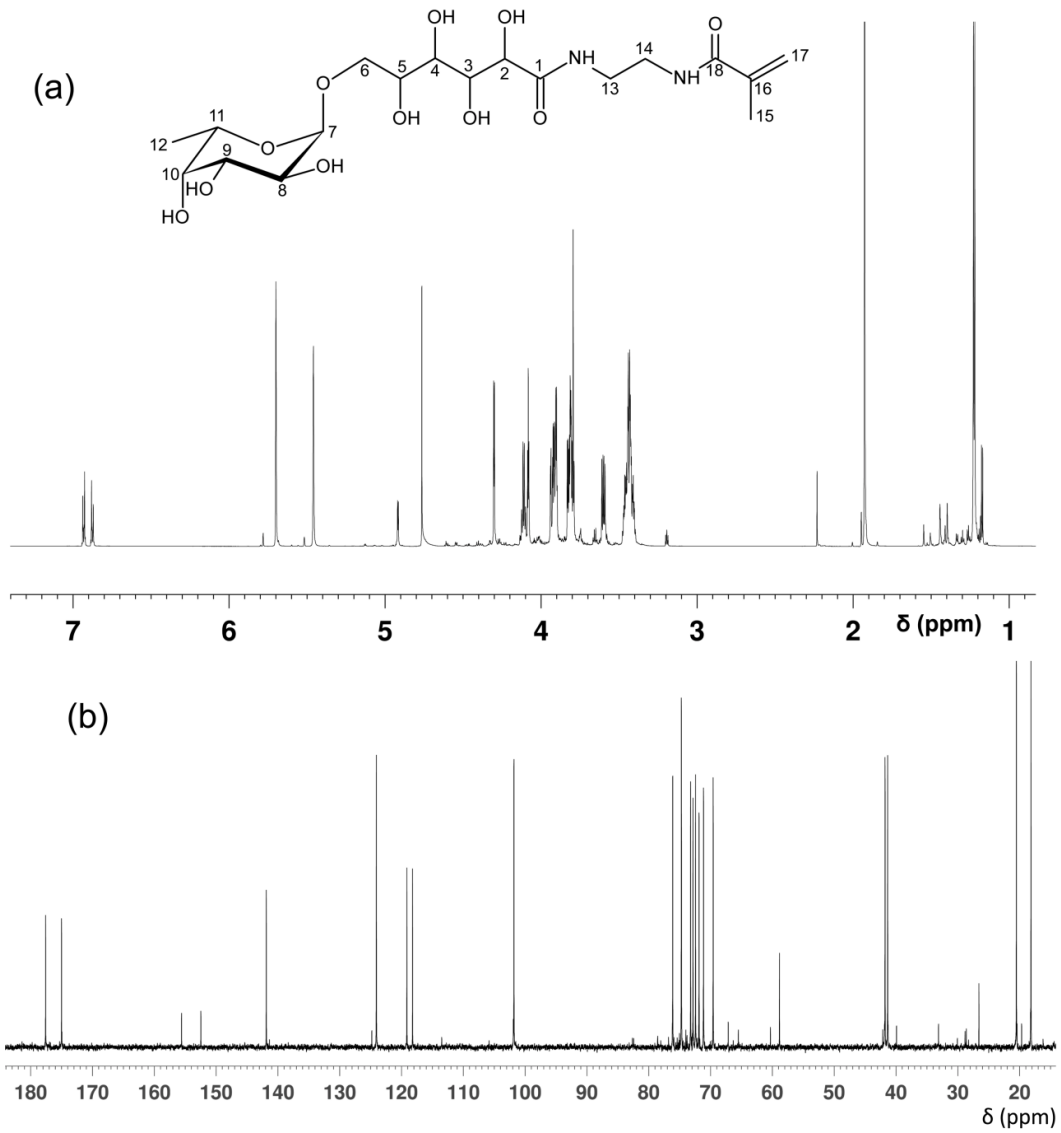

Figure IV-11. ^{1}H- (a) and ^{13}C-NMR (b) spectra (D2O) of 6-*O*-α-L-fucopyranosyl-D-gluconamidoethyl methacrylamide.

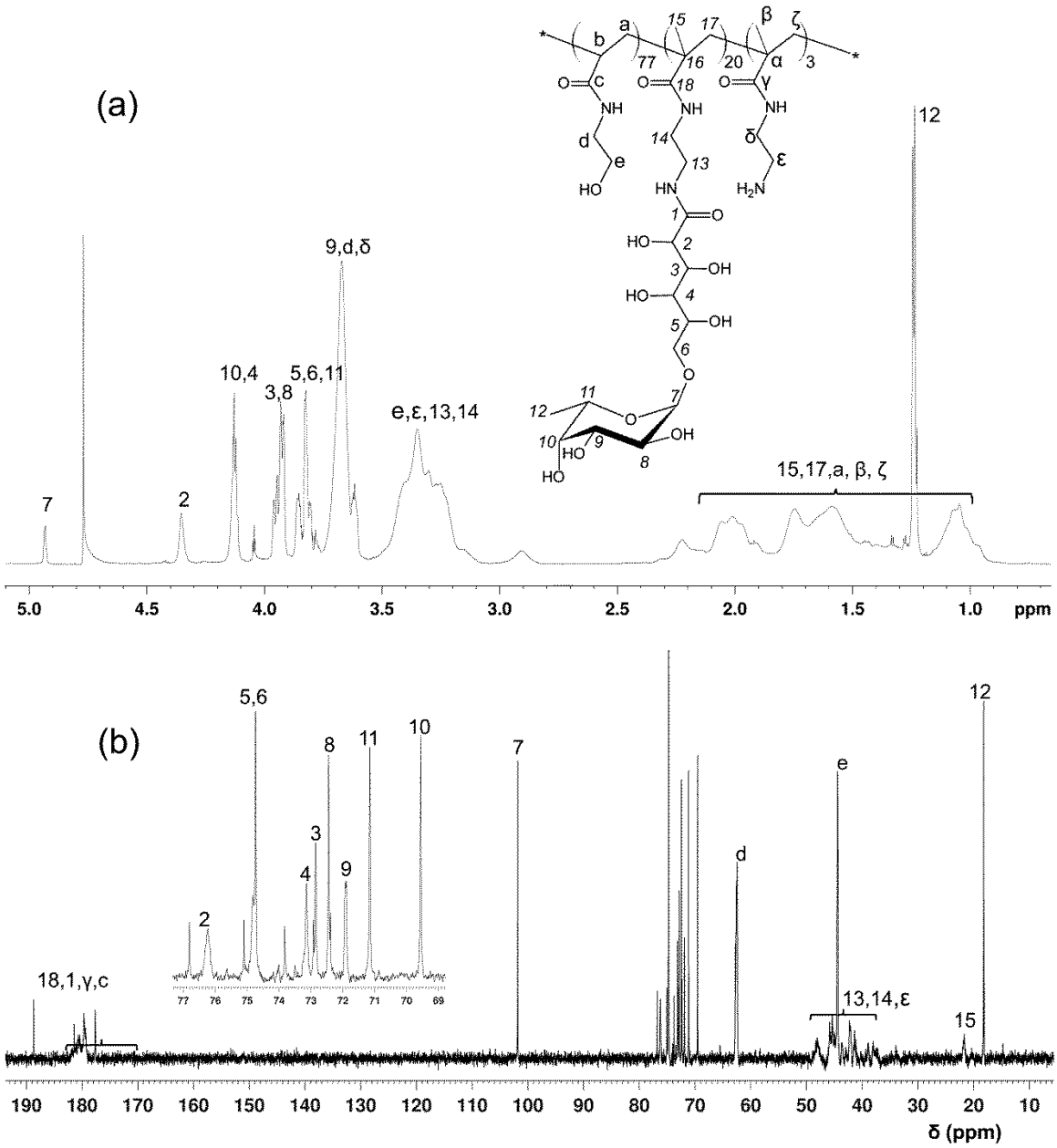

Figure IV-12. Assigned ^{1}H- (a) and ^{13}C-NMR (b) spectra (D2O) of PAA-α-Fuc.

Chapter V. Discussions and Future Directions

Among different glycoconjugate structures, linear statistical glycopolymers were chosen in this work for their structural comparability with mucins and functional versatilities in lectin-binding studies. They are compatible with a variety of labeling tags including different fluorophores, heavy metals and biotin (1,2); they can also be conjugated with gold nanoparticles or plastic beads for biocapture assays, or onto a chip for glyco-microarray (3); linking with phospholipids makes them suitable to insert into liposome as anchored vectors (4), or into cell membrane to generate a pseudo-glycocalyx (5). A few of these versatilities were exhibited in Chapters II-IV.

Chapters II and III present our efforts to synthesize and utilize linear glycopolymers, where two different synthesis strategies were explored. Chapter IV demonstrate the use of commercial fluorescent glycopolymers in our pursuit to the comprehension of the monosaccharide-binding behaviors of morphologically distinct *P. aeruginosa* cystic fibrosis isolates. The glycopolymers that were synthesized in Chapter III were not adopted in Chapter IV for two reasons. First, the technical difficulties of synthesizing some of the glycopolymers, especially these containing sulfated sugars would require an inordinate amount of time. Second, the synthesized glycopolymers still possess lower bacterial-binding avidities than the commercial polymers, which give us a target for future research.

To optimize the performance of the synthesized glycopolymers in lectin-binding tests, a number of structural factors need to be considered. The distribution of different monomers in the heterogeneous glycopolymers might affect their binding capacities. In the syntheses described in Chapter III, *N*-(2-hydroxyethyl) acrylamide (HEAA) was used as the spacer to interspace glycomonomers in the copolymer. Due to the inherent structural difference between this spacer monomer and the glycomonomer, their polymerization rates are different, causing uneven distributions of the two monomers. Taking that into account, 2-gluconamidoethyl methacrylamide (GAEMA), which is structurally more comparable with the glycopolymers than *N*-(2-hydroxyethyl) acrylamide, might be a better option as the spacer monomer. Theoretically, this will lead to a more uniform distribution of different monomers. The structures of HEAA, GAEMA and a representative glycomonomer ALAEMA are shown in Figure V-1.

Other structural factors of the glycopolymers influencing their lectin-binding avidities include the length of the polymers, the linker molecule between the polymer backbone and the pendant sugar, and the density of pendant sugars on the polymer. Generally, increasing the glycopolymer length improves binding avidity (1), however, a longer polymer also causes more cross-linking of the binding targets, which is unfavorable in some cases. Therefore, an optimized range of polymer length needs to be determined for bacterial lectin-binding studies. Also, a longer linker between the pendant sugar and polymer backbone normally enhance their binding (6,7). As discussed in Chapter III, by using a larger acceptor sugars (maltose, for example) during the glycosylation step in the preparation of the glycopolymers, a longer linker molecule will be formed. Furthermore, instead of using *N*-(2-aminoethyl) methacrylamide (AEMA) in the syntheses of glycomonomers, other acrylamide derivatives with a longer chain can be tested as well. The preparation of one of these possible alternatives is proposed in Scheme V-1 as an illustration, which is inspired by syntheses procedures from two literatures (8,9).

To date, the carbohydrate-binding specificities of the two important adhesins of *P. aeruginosa*—pili and flagella are still not fully characterized. One interesting finding in our research is that the mucoid *P. aeruginosa* isolates, which don't possess flagella, showed similar binding pattern with the bacteria having flagella. This suggests that the binding profile of the flagellar components was probably not depicted in this work, possibly because the vigorous processing during the binding tests disengaged some of the flagella from the bacteria. To investigate the sugar-binding of these adhesins, examining glycopolymer-binding bacteria that are gently processed and specifically stained for pili

and flagella (phosphotungstic acid, for example) by transmission electron microscopy is a possible method. Additionally, a protocol using latex beads coated with isolated *P. aeruginosa* pili or flagella to inspect their binding was reported recently (10). Combining the use of fluorescent glycopolymers with this method offers another possibility to investigate the binding specificities of these adhesins.

One of our discoveries in the *P. aeruginosa* binding tests is the heterogeneity of carbohydrate-binding phenotypes within an isogenic bacterial population. This might be a bet-hedging strategy taken by the bacteria to maximize their survival. In evolutionary biology, bet hedging is defined as a risk-spreading strategy displayed by isogenic populations that evolved in unpredictably changing environments favoring different phenotypes at different times (11). In the case of CF lung *P. aeruginosa* infection, lectin-mediated adhesion to the airway tract is a key step for the pathogen, however, the expression of lectins and some other virulence factors are down-regulated in the subsequent biofilms (12-14). This suggests that different lectin expression levels are favored by the bacteria at different infection stages. Considering the constantly changing microenvironment for the bacteria in CF lungs, it's safe to state that the phenotypic heterogeneity of the bacterial sugar-binding observed in this work is a bet-hedging strategy.

As noted in Chapter IV, a great number of bacterial pathogens have been studied of their carbohydrate-binding behaviors. The methodology developed in this work can be applied to many other bacteria species, among which *S. aureus* is the next target of our research, because of their prevalence and significance in CF lung infections (15), and the presence of adhesins on their surface (16).

One of the reasons to study the bacterial carbohydrate-binding is to provide references for the anti-adhesion therapy. As discussed in Chapter IV, based on the findings in this work, the formula of the anti-adhesion therapy for CF patients with *P. aeruginosa* infection should be reconsidered. Improved treatment might be achieved by adding GalNAc and replacing D-galactose with melibiose. L-Fucose has been shown *in vitro* to restore the compromised ciliary movement caused by LecB (17). Therefore, even though α-L-Fuc didn't show observable binding with *P. aeruginosa* in our tests, it should be still beneficial to include it in the nebulizer when treating the infection.

Several reported unsuccessful clinical trials using inhibitor for one particular lectin to prevent or treat bacterial infections (18,19) imply the importance of a thorough characterization of the carbohydrate-binding profile of the pathogen, where a cocktail of competitive carbohydrates might be more effective. Employing the protocol presented herein, it's possible to fully characterize the monosaccharide-binding profiles of various pathogens, which may promote the efficacies of anti-adhesion therapies.

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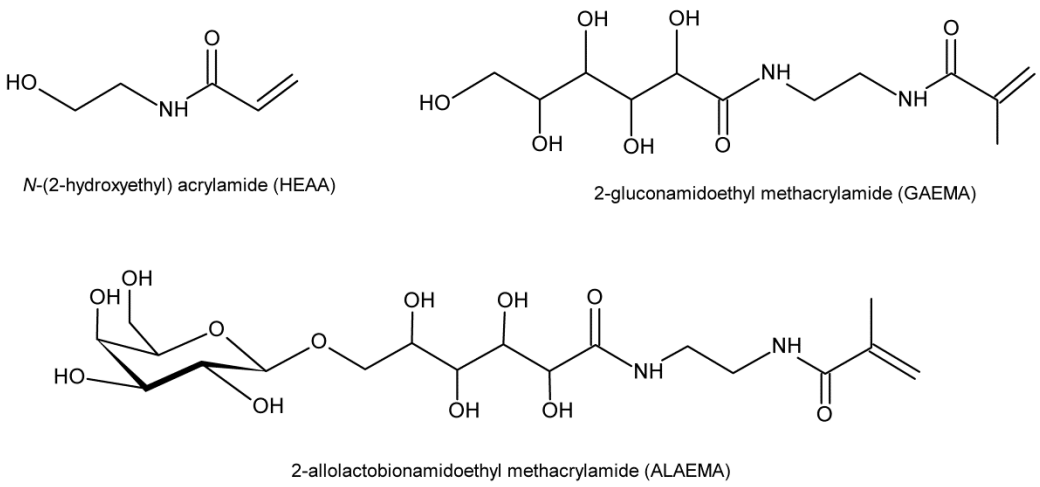
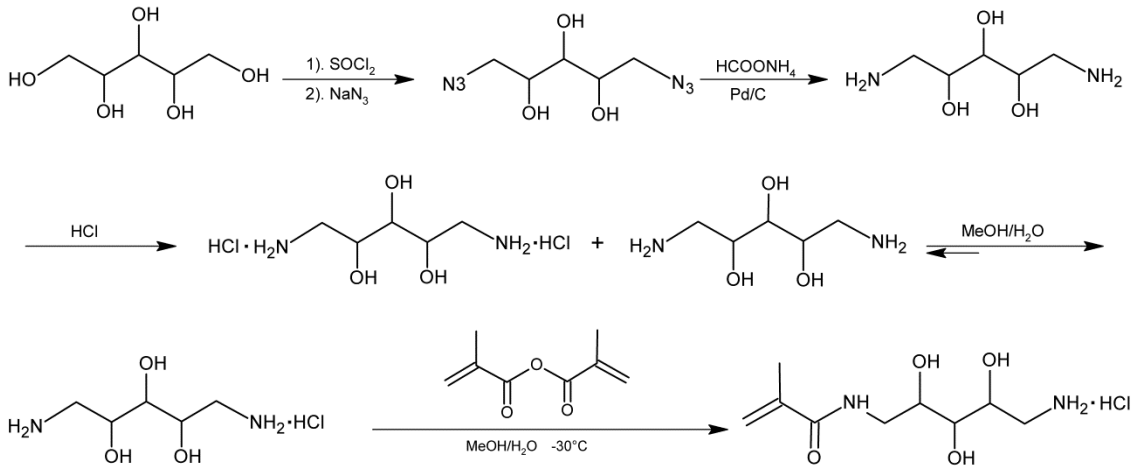


Figure V-1. Chemical structure of HEAA, GAEMA and ALAEMA



Schemes V-1. Synthesis of *N*-(5-aminoxylitolyl) methacrylamide hydrochloride—a possible alternative for AEMA in the preparation of glycomonomers

VITA

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