

MANNOSE/TEMPO FUNCTIONALIZED PAMAM DENDRIMERS: THEIR
RELATIVE LOCATIONS AND COMPONENTS OF AFFINITY TOWARDS
CONCANAVALIN A

by

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ABSTRACT

Surface functionalized dendrimers are being used for several applications including the study of protein-carbohydrate interactions. Mannose-functionalized dendrimers with varying concentrations of saccharides on the dendrimer surface were synthesized. Spin labels (2,2,6,6-tetramethylpiperidine N-oxide) were incorporated onto the dendrimer's surface as well. Linebroadening effects in the EPR spectra of these compounds allowed us to determine the distance between spin labels (and thus between carbohydrates).

The mannose-spin labeled functionalized dendrimers were further studied to determine effects of the spin label in hemagglutination inhibition assays. Affinity chromatography was employed to separate any mixture of compounds based on their affinity towards Concanavalin A, a mannose specific protein. The spin label on these compounds was used to study the relative conformations of the different compounds obtained from the affinity column.

Synthesis of glucosamine functionalized dendrimers was undertaken unsuccessfully. Had the synthesis been a success, TEMPO residues would have been attached to the amino sugar. EPR studies would have been used to determine the relative locations of the TEMPO labeled carbohydrates directly.

CHAPTER ONE

INTRODUCTION

Dendrimers

Spherical macromolecules that contain an inner core followed by successive layers of branching are referred to as dendrimers. The different sizes are referred to as generation. As the dendrimer reaches higher generations, the size and number of terminal groups increases exponentially. Dendrimers were developed simultaneously in the laboratories of Newkome et. al¹ and Tomalia et. al²⁻⁴ in the mid 1980's. Since then, several applications for and modifications to dendrimers have emerged.⁵⁻¹¹

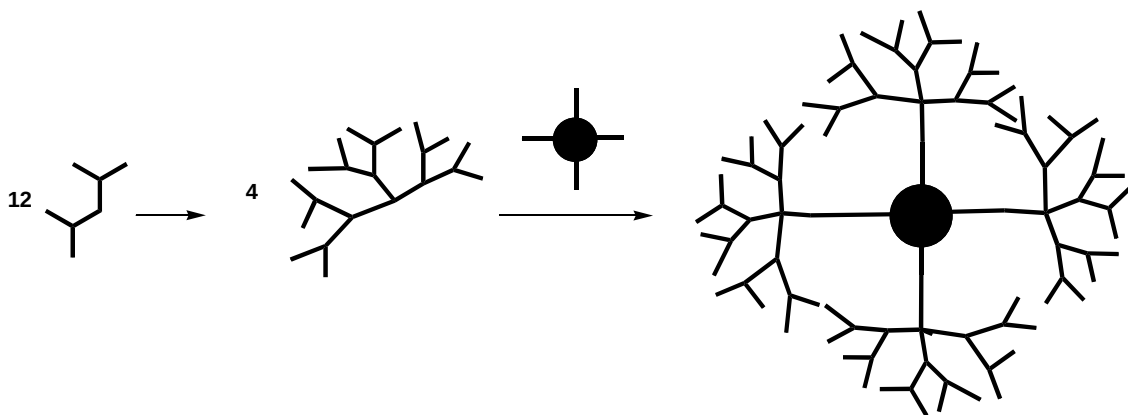


Figure 1.1 Schematic representation of convergent dendrimer synthesis

Dendrimers are prepared by two general paths; convergent¹² and divergent synthesis.¹¹ Convergent synthesis consists of synthesizing the “arms” or dendrons of the dendrimer first and reacting them together to form the final structure (Figure 1.1). Divergent synthesis begins at a central core and builds towards the periphery by adding successive layers to form higher generations (Figure 1.2). Divergent synthesis consists of

performing a few reactions iteratively to obtain higher generations. Due to the simplicity of the reactions, dendrimers that are synthesized divergently are easily accessible.

Convergent synthesis of dendrimers consists of a variety of different reactions and is more labor intensive, but generally produces more homogeneous molecules. By changing a few synthetic steps the core, middle or periphery of the dendron can be adjusted so that the final structure contains sections with different functionality.

Divergent synthesis is generally easier, but produces products with more imperfections.

Both convergently and divergently synthesized dendrimers have widespread applications.

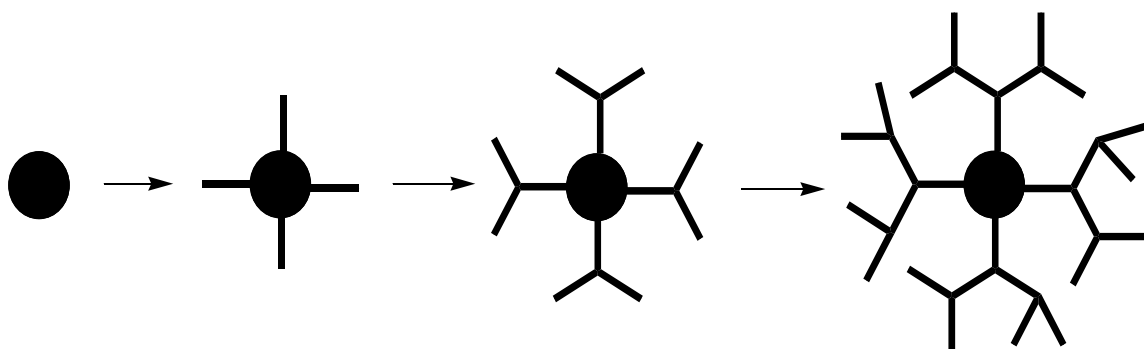


Figure 1.2 Schematic representation of divergent dendrimer synthesis

PAMAM Dendrimers

Poly(amidoamine) (PAMAM) dendrimers, called starburst dendrimers due to their shape, were developed by Tomalia et al. in 1985²⁻⁴ (Figure 1.3). PAMAM dendrimers contain an ethylene diamine inner core with amide and amine linkages alternating between carbon spacers along the branches and are usually terminated with

amines or esters. PAMAMs are attractive scaffolds because they are commercially available, robust and water soluble.

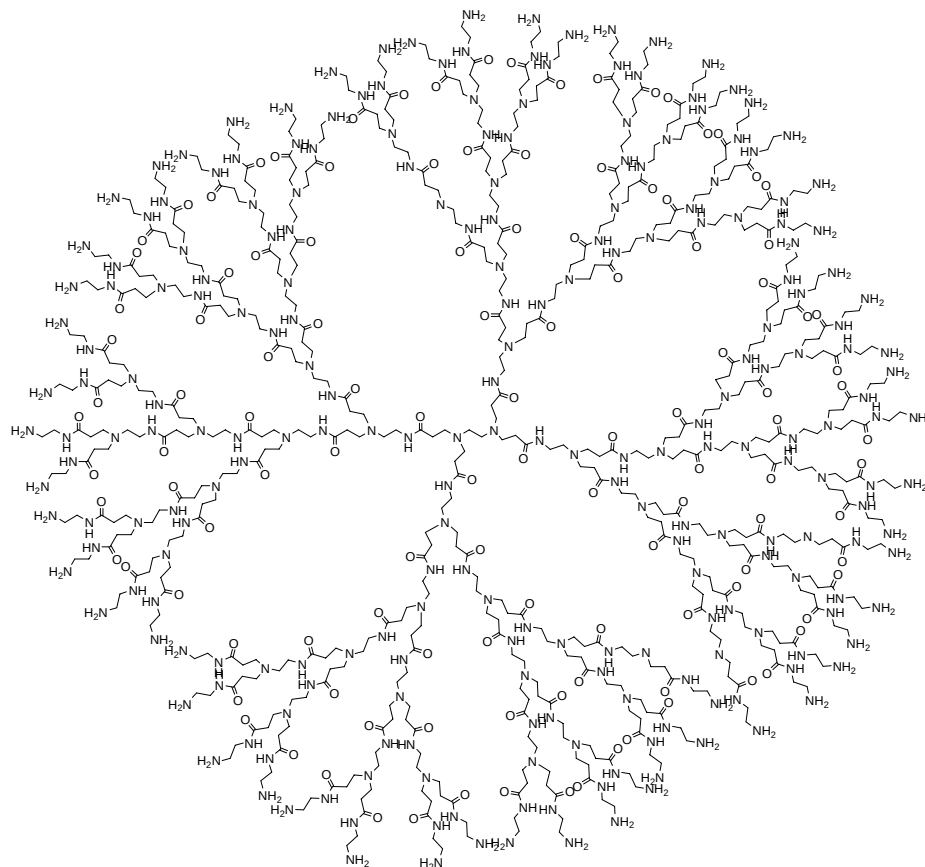


Figure 1.3 Fourth generation PAMAM dendrimer

The steps in PAMAM dendrimer synthesis are Michael addition of methyl acrylate to ethylene diamine followed by amidation of the resulting esters with ethylene diamine to give a symmetrical molecule with four terminal amines. The PAMAM dendrimer with 4 terminal amines is termed a generation 0 (G(0)) dendrimer. It should be noted that there is no universal notation for numbering dendrimer generations; consequently other dendrimers may differ in notation. Repeating the sequence of Michael addition followed by amidation using methyl acrylate and ethylene diamine

respectively gives a symmetric molecule with eight terminal amines (G1 PAMAM) (Figure 1.4).

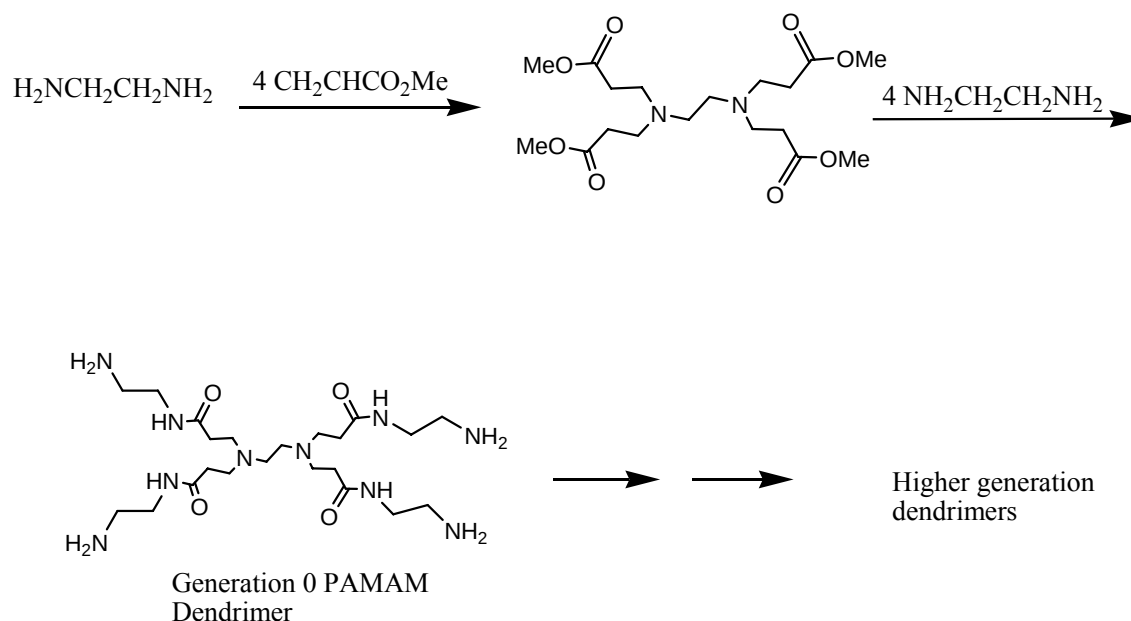


Figure 1.4 Synthesis of PAMAM dendrimers

Each repeated iteration of reactions creates a higher generation of dendrimer and theoretically doubles the number of terminal amines. As generations increase, the likelihood of an incomplete or a side reaction occurring on one or more of the terminal amines increases due to steric crowding.¹³ Reaction of the amines with only methyl acrylate leads to an ester terminated molecule called a half generation PAMAM dendrimer.

Incomplete reaction leads to imperfect structures at higher generations. For example, in G(4)-PAMAM dendrimer the theoretical mass is 14,164 a.m.u. which is consistent with 64 terminal amines. However using Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS), the molecular weight

varies from 13,125 to 13,500 (55-60 terminal amines) depending on the lot that was purchased. The shape of the spectrum is significantly broad.

At lower generations (G(1)-G(3)) the dendrimers contain sterically unencumbered endgroups. Around generation 4, the PAMAM dendrimers become more spherical to minimize steric crowding.¹⁴⁻¹⁵ The terminal amines have been experimentally shown to be on the periphery and not extensively backfolded into the interior of the structure,¹⁶ therefore the peripheral amines are readily available to be functionalized.

Surface Functionalized Dendrimers

Attaching a molecule to the periphery of the dendrimer forms a surface functionalized dendrimer.¹⁷ In homogenous functionalization, the entire periphery of the dendrimer is functionalized with the same functional group. Heterogeneous functionalization contains two or more different functional groups on the surface of the dendrimer. An example of a randomly heterogeneously functionalized dendrimer is shown in Figure 1.5.



Figure 1.5 Schematic representations of homogeneously and random heterogeneously functionalized dendrimers

Two ways in which heterogeneously functionalized dendrimers can be synthesized are by convergent dendrimer synthesis and the sub-stoichiometric method. In convergent dendrimer synthesis the dendrons contain different functional groups on the surface prior to dendrimer formation. One form of convergent dendrimer synthesis is done by functionalizing the dendrons differently prior to dendrimer formation.¹⁸ Each dendron can be functionalized with a different group, resulting in a dendrimer with regions of different peripheries (Figure 1.6).

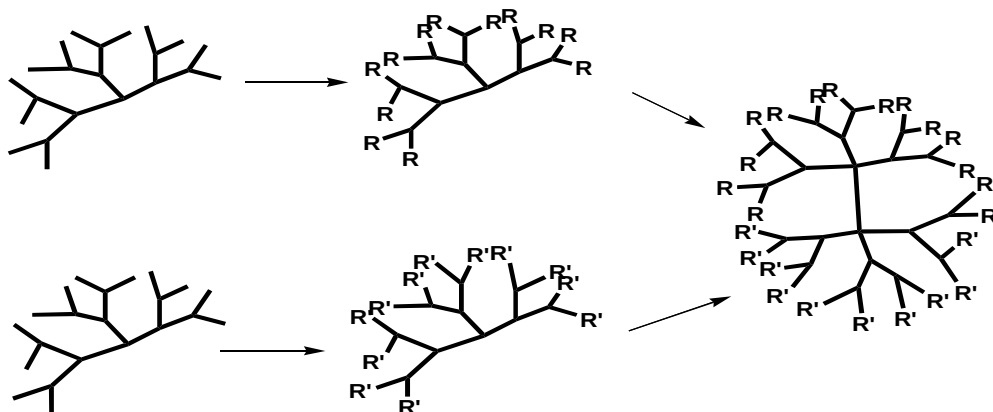


Figure 1.6 Schematic representation of heterogeneous functionalization of dendrimers through convergent synthesis of functionalized dendrons.

In another form of convergent dendrimer synthesis, the endgroups on each dendron are manipulated to create a dendrimer with two or more types of endgroups, which can then be functionalized separately (Figure 1.7).¹⁹ The result is a dendrimer with regions of different functional groups.

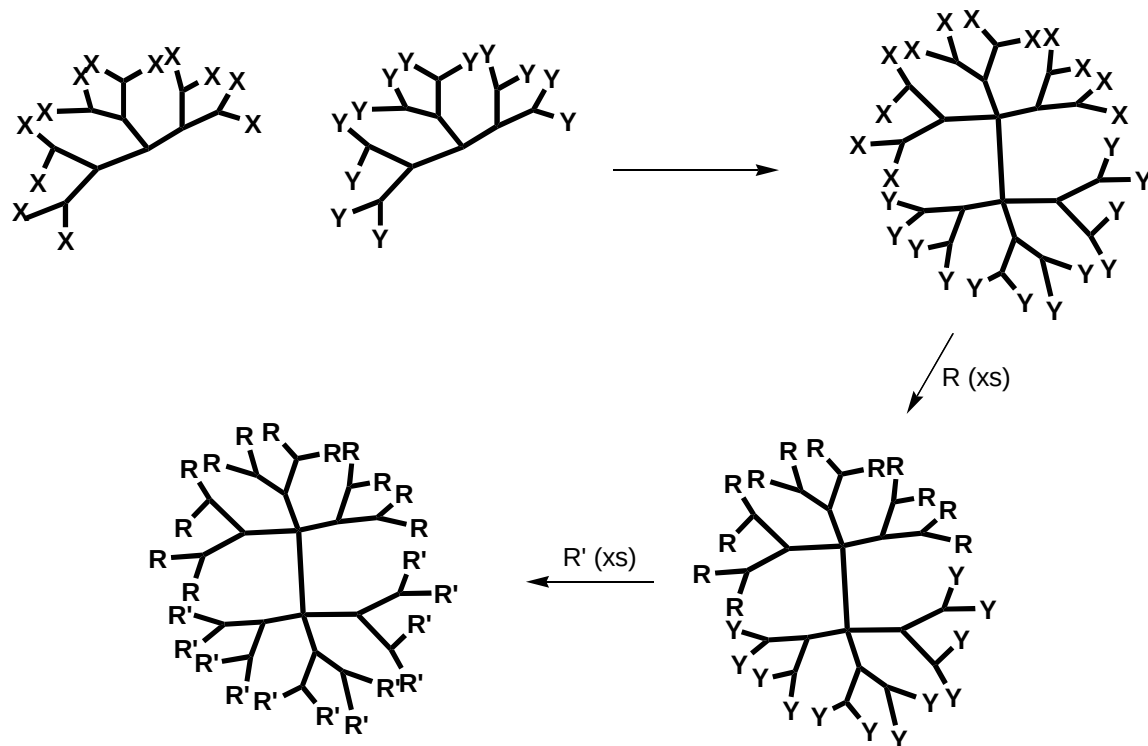


Figure 1.7 Schematic representation of heterogeneous functionalization of dendrimers by manipulating the endgroups during convergent synthesis.

The sub-stoichiometric method of dendrimer synthesis is done by controlling the equivalents of each functional group added to a whole dendrimer so that only a fraction of the endgroups are functionalized. This can be repeated to functionalize with multiple groups until all the surface terminal groups are functionalized. Dendrimers with varying amounts of two or more surface functional groups are easily constructed; however, there are no specific regions of a single functional group (Figure 1.8).

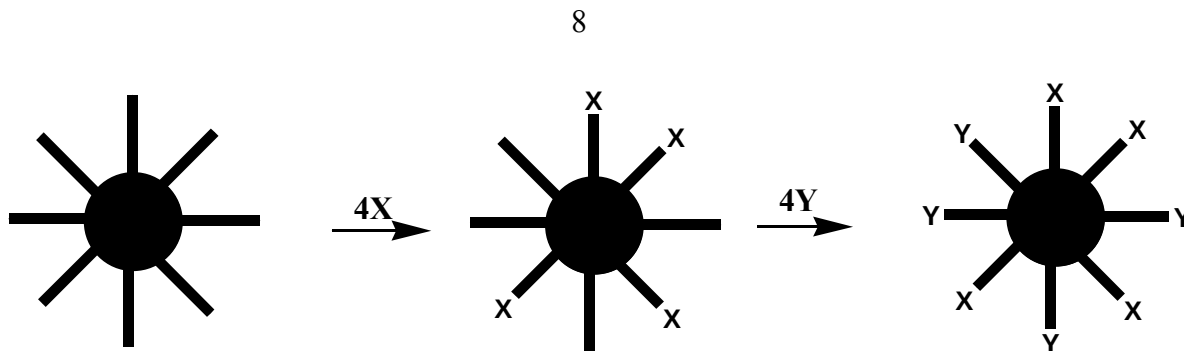


Figure 1.8 Schematic representation of heterogeneous functionalization of a dendrimer by controlling the equivalents of functional groups

In conclusion the convergent synthesis method to heterogeneously functionalized dendrimers is much more labor intensive. However, the locations of the functional groups are easily controlled. The sub-stoichiometric method of functionalization is much simpler but lacks control over the locations of the functional groups. Although both methods have advantages, for our purposes the sub-stoichiometric method was the best option.

Location of Terminal and Functional Groups Relative to the Dendrimer Core on PAMAM Dendrimers

Although PAMAM dendrimers are highly studied molecules, properties of their basic structure in solution are still debated. Information about size, shape and endgroup locations is difficult to determine. Several research groups have conducted molecular dynamic simulations on dendrimers to determine these properties. Most calculations agree that G(1) – G(3) PAMAM dendrimers are mostly unhindered and G(4) begins to become spherical while G(5) and higher generations are definitely spherical in structure.¹³⁻¹⁵

The studies on the locations of the endgroups are inconsistent as to whether the terminal groups are on the periphery or backfolded into the molecule.^{15-16,20} Given the dynamic structure of the PAMAM dendrimer, the lowest energy conformation can be difficult to determine by calculations. Experimental results indicate that the terminal groups are on the periphery.¹⁵

Several research groups have functionalized the peripheries of PAMAM dendrimers.¹⁶ This indicates that, regardless of endgroup backfolding into the dendrimer structure, the terminal groups are exposed enough to interact with other molecules and form covalent bonds and/or non-covalent interactions. In the Cloninger research labs, we have begun a study of the relative locations of dendrimer endgroups on heterogeneously functionalized dendrimers.

Spin Labels, EPR and Their Use with Macromolecules

Electron paramagnetic resonance (EPR) spectroscopy has emerged as a tool for studying conformations and relative locations of groups or sidechains on proteins and proteomimetic structures. Similar to nuclear magnetic resonance (NMR), EPR measures the energy required to invert the spin of an unpaired electron (instead of the spin of the nucleus) in the presence of a magnetic field. Therefore, to obtain EPR spectra, an unpaired electron must be present in the sample. Metals, triplet state molecules and stable free radicals are all used separately or in combination as unpaired electron tags in EPR spectroscopy.

Although NMR and EPR spectroscopy are very similar in theory, there are inherent differences in the spectra and techniques used to study them. EPR is a much more sensitive technique, allowing for its use with very dilute samples. EPR typically is tuned to one type of radical and therefore gives only one signal, unlike the multiple ^1H environments detected by ^1H NMR. Both NMR and EPR data are plotted as absorption spectra, however, EPR is plotted as the first derivative of absorption intensity with respect to field strength for ease of analysis (Figure 1.9). The first derivative shows a signal both above and below the baseline and makes the spectrum appear narrower while stressing changes from the baseline more than as the absorption spectrum.

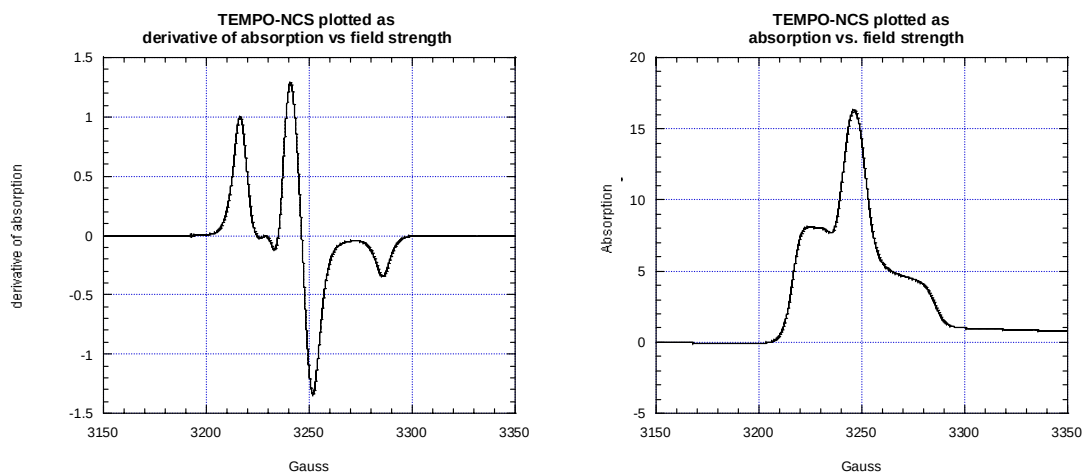


Figure 1.9 EPR spectra of 4-isothiocyanato-2,2,6,6-tetramethylpiperidine N-Oxide (12.5 mM in DMSO) plotted as the derivative of absorption (left) and as absorption (right).

Nitroxides are easily attached to two or more sites on a macromolecule.²¹⁻²² The structures of more rigid nitroxides such as 4-amino-2,2,6,6-tetramethylpiperidine N-Oxide (TEMPO-NH₂),¹ (Figure 1.10) are preferred because the interspin distance studies on flexible nitroxides could be due to the movement of the nitroxide rather than the macromolecule. The observed interspin changes are accepted as more accurate with

more rigid spin labels. Also, the methyl groups attached to the carbons alpha to the nitrogen stabilize the radical. In conducting a sequence of reactions or assays the stabilized radical can be maintained.

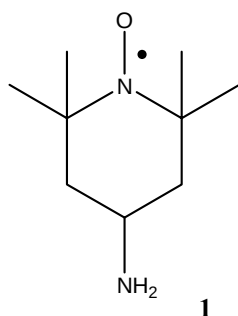


Figure 1.10 2,2,6,6-tetramethyl-4-aminopiperidine N-oxide (TEMPO-NH₂) (**1**)

EPR techniques can be used to determine several solution phase properties of the macromolecules such as conformation, orientation, accessibility to solvent and interspin distances. The technique known as Site Directed Spin Labeling (SDSL) is often used to determine the folding of proteins. For example, SDSL is capable of deciphering the difference between a continuous helix from a helix-loop-helix.²³

Linebroadening in the EPR spectrum occurs when two or more radicals are close in proximity to each other. This linebroadening can be quantized to determine intermolecular distances between spins. The technique of determining interspin distances through EPR linebroadening was developed for use on proteins²⁴⁻²⁸ and has been adapted by our collaborators for use with dendrimers. The relative locations of spin-labels on dendrimers are determined using this strategy. More details about this strategy will be given in CHAPTER TWO.

Protein-Carbohydrate Interactions

The interaction between proteins and carbohydrates is an important first step for many biological interactions at the cellular level.²⁹⁻³¹ Interactions between a lectin and a monomeric carbohydrate are weak. Therefore, interactions which involve more than one carbohydrate are accepted to play a significant role in protein-carbohydrate interactions. The proximity effect (statistical effect) occurs when an increase in binding affinity is seen due to several carbohydrates clustered around the binding sight. This usually occurs when the carbohydrates are covalently linked to each other. Therefore, when one sugar becomes unbound from the protein, another quickly replaces it by binding to the now vacant binding sight. Multivalent binding occurs in molecules with several saccharide residues that are large enough to span two binding sights on the protein at the same time (Figure 1.11). A significantly larger increase in binding affinity (relative to monosaccharide) is seen in multivalent binding than in the proximity effect. Multivalent binding is believed to play a more significant role in physiologically relevant protein-carbohydrate interactions.

Previously in Dr. Cloninger's research group, carbohydrate functionalized dendrimers have been used to study protein carbohydrate interactions.³²⁻³³ By varying the generation of the dendrimer, the size of the scaffold was varied. Homogeneous mannose-functionalized dendrimers (G(1)-G(6)) (Figure 1.12) were synthesized and studied by hemagglutination assays with Concanavalin A (Con A). G(1) and G(2)

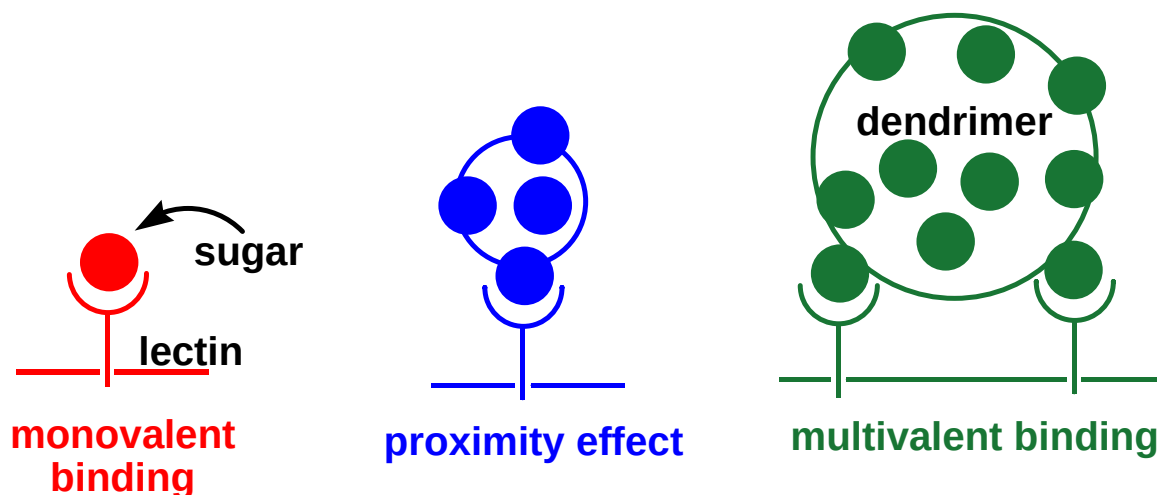


Figure 1.11 Schematic representation of: (a) monovalent binding (b) glycoside clustering (c) multivalent binding

mannose-functionalized dendrimers were found to have hemagglutination inhibition activities similar to methyl mannose. Generation 3 mannose-functionalized dendrimer was approximately one order of magnitude more active than methyl mannose whereas G(4)-G(6) mannose functionalized dendrimers were approximately two orders of magnitude higher in activity relative to methyl mannose. These results suggest that the

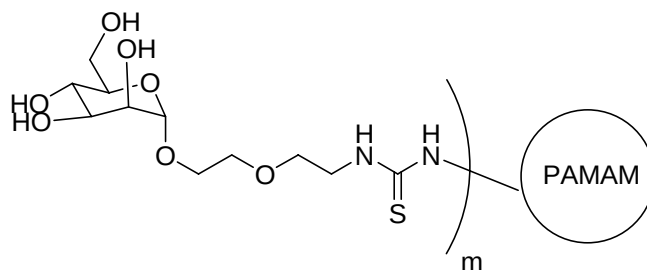


Figure 1.12 Homogeneous mannose functionalized dendrimer

proximity effect is occurring in G(3) mannose functionalized dendrimers and that multivalent binding is occurring in G(4) – G(6) mannose functionalized dendrimers.³²

Next, four generations of dendrimers with varying concentrations of carbohydrates were studied. Partially loaded G(4)-G(6) mannose-functionalized dendrimers were synthesized with the degree of functionalization ranging from a few saccharide residues to full loading with saccharide residues on the dendrimer's surface. The terminal amines to which carbohydrates were not added were functionalized with an ethoxyethanol spacer (Figure 1.13). The ethoxyethanol spacer does not interact with Con A. The partially loaded mannose functionalized dendrimers were studied via hemagglutination assays. The activity increased with increasing carbohydrate loading until about 50% mannose loading and then decreased as the carbohydrate loading surpassed 50%. Steric crowding of the large carbohydrates probably inhibited binding to Con A in the dendrimers with greater than 50% mannose loading.³³

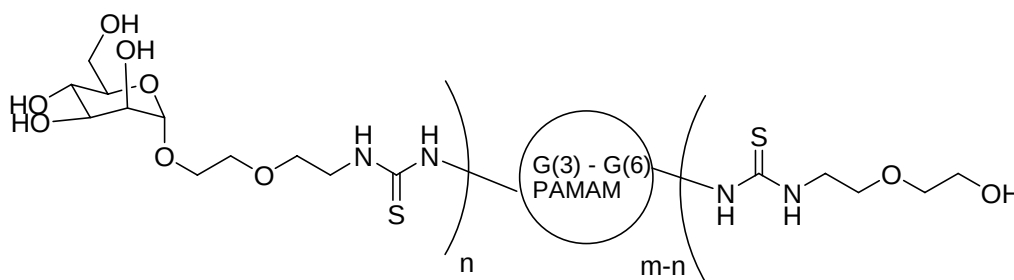


Figure 1.13 Heterogeneous functionalized dendrimers for hemagglutination studies.

Affinity Chromatography

The separation of mixtures of oligosaccharides (molecules that contain several sugar residues) is a difficult task. However, the study of protein-carbohydrate interactions has shown that different oligosaccharides have different affinities to a given protein, and a common way to separate the oligosaccharide mixtures takes advantage of this property.³⁴⁻³⁷ Affinity Columns are created by attaching a protein to a solid phase material (usually sepharose or agarose gel) and packing the solid phase into a column. A solution containing the oligosaccharides to be separated is loaded onto the column and fractions are taken. The oligosaccharide with the lowest binding affinity flows through the column the fastest while the oligosaccharide with the highest binding affinity is retained on the column (Figure 1.14) until elution with a concentrated monovalent carbohydrate solution. For example, a solution that contains oligosaccharides of mannose, which has a high affinity for Con A; glucose, which has a moderate affinity for Con A; and galactose, which has no affinity for Con A, can be separated using a Con A affinity column.³⁶

Goals and Brief Project Description

The first focus of the research presented in this thesis was the location of carbohydrates on the surface of heterogeneously functionalized dendrimers. Given that PAMAM dendrimers are extremely dynamic, it is conceivable that the functional groups on the surface of the dendrimer can rearrange to form ordered structures. Compounds such as carbohydrates have several hydroxyl groups that can take part in hydrogen

bonding. Therefore, we were concerned with possible clustering of carbohydrates on the surface due to potential hydrogen bonding interactions.

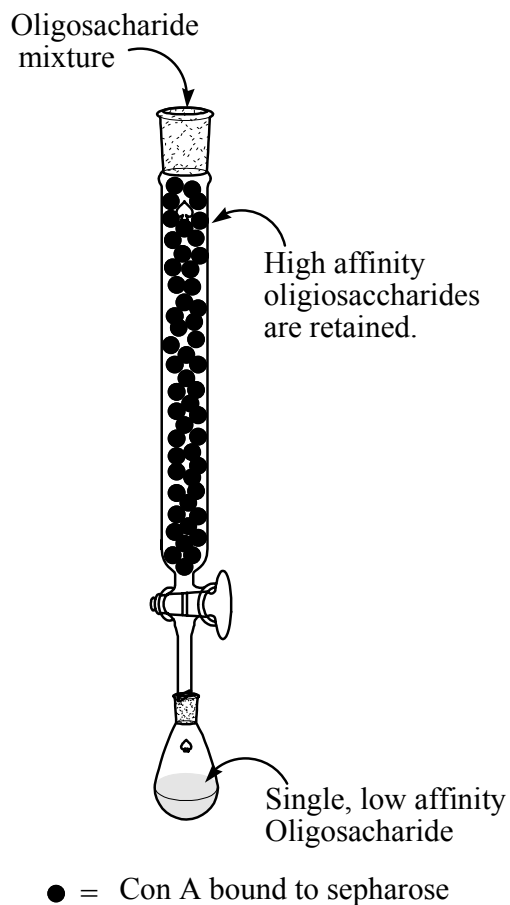


Figure 1.14 Pictorial representation of affinity chromatography

To study the potential clustering of the carbohydrate residues, we heterogeneously functionalized G(4)-PAMAM dendrimers with acetylated mannose residues and a spin label. The spin label allowed us to use EPR to determine the interspin distances and thus the relative locations of the spin labels. From the relative locations of the spin labels, the

relative locations of the non-EPR active group were inferred. The acetyl groups were removed from the mannose residues and the EPR studies were repeated. Comparison of the EPR results between the acetylated and deacetylated mannose residues enabled us to determine whether any changes in relative locations of the endgroups had occurred upon deprotection.

The second focus of this project relates to previous results of protein carbohydrate studies done in Dr. Cloninger's group³³ that indicate that multivalent binding occurs on G(4) and higher generation mannose/ethoxyethanol dendrimers. The main question to be answered was whether the results seen previously were given by the bulk of the material or by small idealized fraction of functionalized dendrimer.

The evaluation of this question employed the use of affinity chromatography. The EPR active spin label/mannose functionalized dendrimers were run through an affinity column that contained solid Con A bound to sepharose gel. The parts of the solution that are bound more strongly to Con A elute from the column later than the parts that are bound more weakly. The spin label allowed for the study of these molecules via EPR to determine the relative locations of endgroups in each fraction.

Summary of Results

EPR studies on the TEMPO/mannose functionalized dendrimers have shown that the spin labels are distributed randomly on the surface of the dendrimer for both the acetylated and non-acetylated mannose. Therefore, no clustering occurs due to hydrogen bonding. The hemagglutination assays reveal that the spin label does not interfere with

binding to Con A. Affinity chromatography revealed the material to all have the same affinity towards Con A; there is no mixture of material with different binding affinities. The synthesis of hydroxyl-protected, glucose functionalized dendrimers was completed, no studies were performed with these compounds due to the inability to remove the Cbz group.

Organization

First, a synthesis of mannose/TEMPO functionalized dendrimers is presented. A series of EPR experiments are reported next. They indicate that the spin labels were randomly distributed on the dendrimer's surface and therefore the mannose must also be randomly distributed.

To address the second area of research introduced in the section above, hemagglutinin inhibition assays were performed. The spin label does not affect the affinity of the mannose functionalized dendrimer; mannose/TEMPO and mannose/ethoxy-ethanol dendrimers have similar activity relative to methyl mannose. Affinity columns were completed on the mannose/TEMPO dendrimers. The water soluble dendrimers gave reasonable results; all the material was retained on the column until it was eluted with methyl mannose. The non-water soluble compounds were run through the affinity column in DMSO and the result was that the Con A was denatured. This was confirmed with a control study using a water soluble dendrimer in a column with DMSO.

TEMPO/mannose/ethoxyethanol dendrimers were synthesized to increase water solubility. The affinity chromatography results obtained were reasonable, all the material

was retained on the column until it was eluted with methyl mannose. The exception to this trend was the dendrimer that contained only 10% mannose, in which case the material was eluted immediately from the column. Hemagglutination inhibition assay results are consistent with the affinity chromatography results.

The synthesis of 1-O-(5-thiourea-3-oxapentyl)-3,4,6-tri-O-acetyl-2-(benzyloxycarbonylamino)-2-deoxy-D-glucoside functionalized dendrimers is reported last. However, since the benzyloxycarbonyl group could not be removed, further functionalization with TEMPO and EPR studies could not be done.

CHAPTER TWO

SYNTHESIS OF TEMPO/MANNOSE COATED DENDRIMERS AND EPR STUDIES

Background

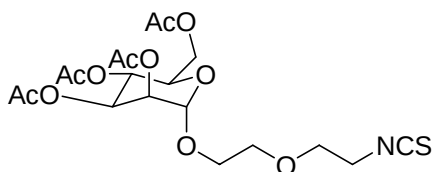
A major emphasis in the Cloninger research group is the use of carbohydrate functionalized dendrimers to study protein/carbohydrate interactions. Generations 1-6 of mannose functionalized dendrimers have been used to study interactions between mannose and Con A in the attempt to create a new system for the study of protein carbohydrate interactions. In many cases the dendrimers are heterogeneously functionalized.³³ For the protein-carbohydrate studies in the Cloninger research group, it is important to understand the relative locations of the carbohydrates on the dendrimer surface in order to accurately interpret the data. If the carbohydrates are clustered, the results of the various assays must be analyzed differently than if the carbohydrates are scattered.

In this chapter the synthesis of mannose/TEMPO functionalized dendrimers is described. The dendrimers are subsequently studied using EPR techniques developed in collaboration with the David Singel research group. The spin label, TEMPO, is EPR active and allows for the determination of distances between spin labels.²⁴⁻²⁵ The relative locations of the TEMPO residues are determined, which allow the relative locations of the carbohydrate residues to be determined by extrapolation. CHAPTER TWO discusses

the synthesis of mannose/TEMPO functionalized dendrimers, their characterization by MALDI-TOF MS and the EPR studies performed on the dendrimers.

Synthesis of 1-O-(5-isothiocyanato-3-oxapentyl)-2,3,4,6-tetra-O-acetyl- α -D-mannopyranoside (**2**)

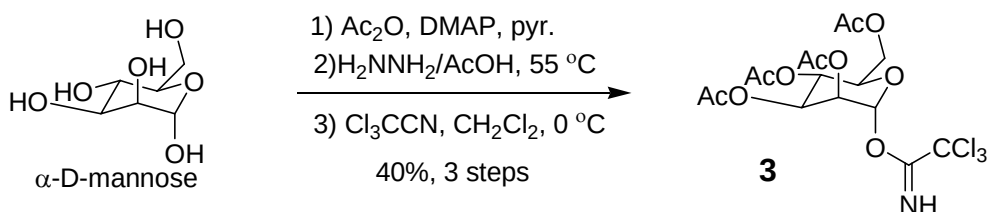
The synthesis of mannose/TEMPO functionalized dendrimers began with the synthesis of a derivative of mannose that could easily react with the endgroups of the dendrimer. The design was a mannose with ethoxyethoxyisothiocyanate at the anomeric position. The amine endgroup of the dendrimer reacts readily with the electrophilic isothiocyanate. After previous attempts in our group, it was found that 1-O-(5-isothiocyanato-3-oxapentyl)-2,3,4,6-tetra-O-acetyl- α -D-mannopyranoside (**2**) worked well.³⁸



2

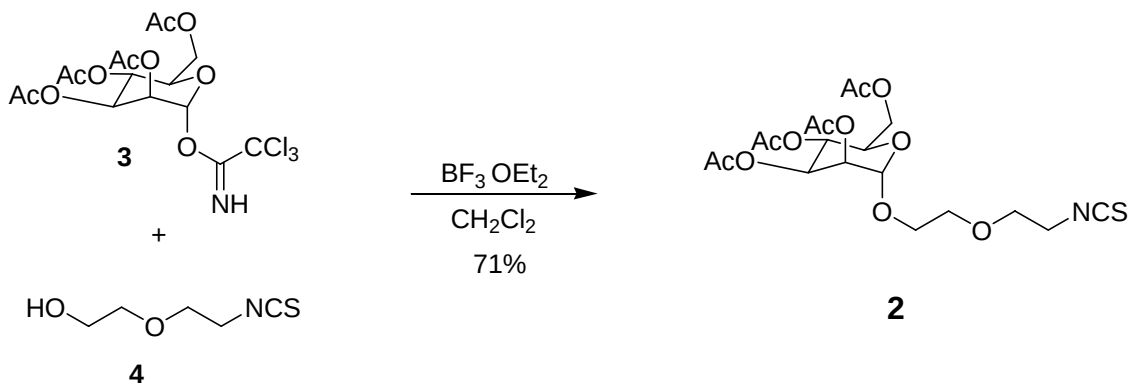
The conditions for synthesizing **2** have been previously worked out in the Cloninger research group.³³ As shown in Scheme 2.1, the hydroxyls on D-mannose were acetylated with acetic anhydride in pyridine in the presence of a catalytic amount of DMAP. Next, the anomeric position was selectively deprotected using hydrazine acetate in DMF at 55 °C for 30 min. The resulting hydroxyl was activated with a

trichloroacetimidate group by addition of trichloroacetonitrile in the presence of DBU in methylene chloride at 0 °C to form **3**. Product **3** was purified by column chromatography.



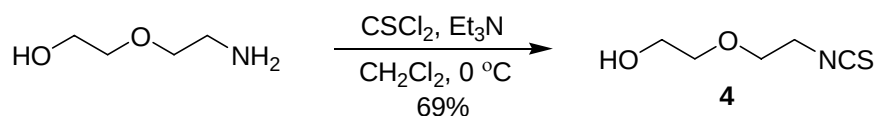
Scheme 2.1 Synthesis of 2,3,4,6-tetra-O-Acetyl- α -D-mannosyltrichloroacetimidate (**3**).

The trichloroacetimidate group activates the anomeric carbon towards nucleophilic attack by an alcohol. In the presence of a Lewis acid, 2-ethoxy-(2-isothiocyanato)ethanol (**4**) and **3** were used to form **2**. The coupling of **3** and **4** commenced in the presence of $\text{BF}_3 \cdot \text{OEt}_2$, dry methylene chloride and crushed 4 Å molecular sieves. This is shown in Scheme 2.2. After purification by column chromatography, mannose derivative **2** was ready to be used for dendrimer functionalization.



Scheme 2.2 Synthesis of 1-O-(5-isothiocyanato-3-oxapentyl)-2,3,4,6-tetra-O-Acetyl- α -D-mannopyranoside (**2**).

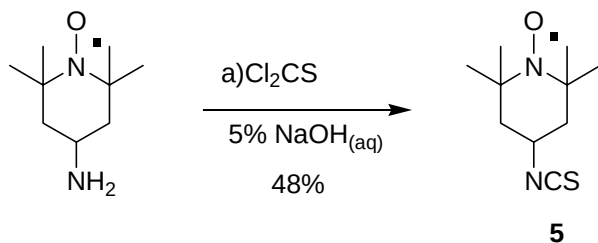
Isothiocyanate **4** was synthesized from 2-amino-(2-ethoxy)ethanol in CH_2Cl_2 buffered with Et_3N . Thiophosgene was added via syringe pump addition over the course of one hour (Scheme 2.3). The product was purified by column chromatography on silica gel (1:1 hexanes to ethyl acetate) followed by Kugelrohr distillation.



Scheme 2.3 Synthesis of 2-ethoxy-(2-isothiocyanato)ethanol, **4**.

Synthesis of TEMPO-NCS

The spin label 4-amino-2,2,6,6-tetramethyl-4-aminopiperidine N-oxide (TEMPO- NH_2) (**1**) is commercially available and stable in basic media. The only synthetic step that needs to be completed to obtain a suitable reagent for dendrimer functionalization is conversion of the amine to an isothiocyanate. Thiophosgene is added over 15 min. to a solution of TEMPO in 5% NaOH(aq) via syringe pump. The product is immediately filtered and washed with 5% NaOH to remove excess thiophosgene from the product. The product is dried under vacuum with phosphorous pentoxide to give TEMPO-NCS **5** (Scheme 2.4). EPR activity of **5** is confirmed by comparing the EPR spectrum of **5** with that of the starting material (**1**). This ensures that the free radical was not quenched in the course of the reaction. After assuring no decrease in EPR activity, **5** is used without further purification.



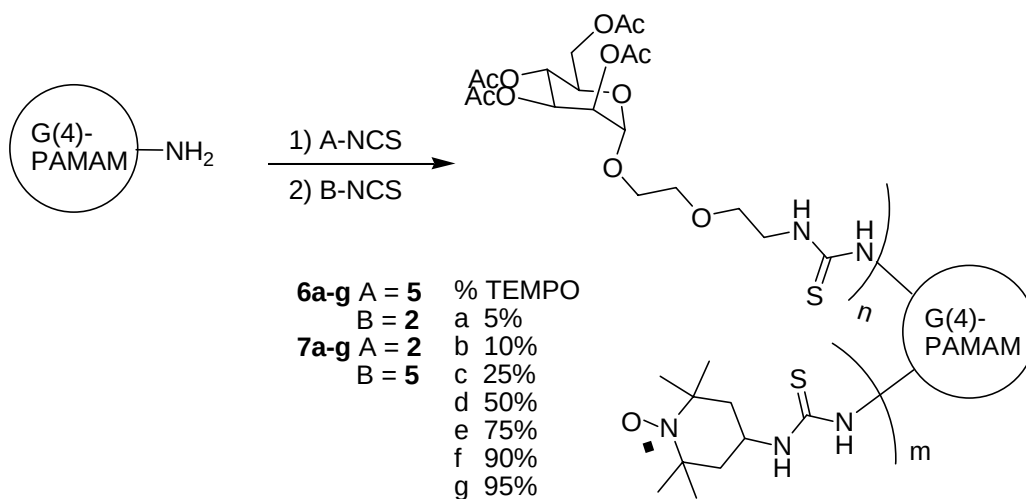
Scheme 2.4 Synthesis of 2,2,6,6-tetramethyl-4-isothiocyanatopiperidine N-Oxide (**5**).

Synthesis of Spin-Labeled Heterogeneously Functionalized Dendrimers

G(4)-PAMAM dendrimers containing two functional groups were synthesized by the sub-stoichiometric method. Surface functionalization with mannose derivative **2** and TEMPO-NCS **5** was completed to coat the exterior of the dendrimer. The functionalization was completed with different percent loadings of **2** and **5** on the dendrimer surface. The reactions were done in two sequences; one was accomplished by adding **5** first to give compounds **6a-g** and the other was completed by adding **2** first to give compounds **7a-g** (Scheme 2.5). MALDI-TOF MS and EPR were used to study **6a-g** and **7a-g** to determine whether there were any differences due to the order of addition. The two isothiocyanates were not added to the dendrimer at the same time because of differences in reactivity; isothiocyanate **2** is primary while **5** is secondary.

Since the theoretical number of endgroups and the actual number of endgroups are different, MALDI-TOF MS of the G(4)-PAMAM dendrimer was used to determine the average number of terminal amines. In most cases the average mass of the dendrimer was approximately 13,125 g/mol, which suggests an average of 55 terminal

amines on the dendrimer's surface. Dendrimers with an average mass of up to 13,500 (60 endgroups) were used.



Scheme 2.5 Synthesis of heterogeneous thiourea-based 4-thiourea-2,2,6,6-tetramethylpiperidine N-Oxide and 1-O-(5-thiourea-3-oxapentyl)-2,3,4,6-tetra-O-acetyl- α -D-mannopyranoside G(4)-PAMAM functionalized dendrimers (**6a-g** and **7a-g**).

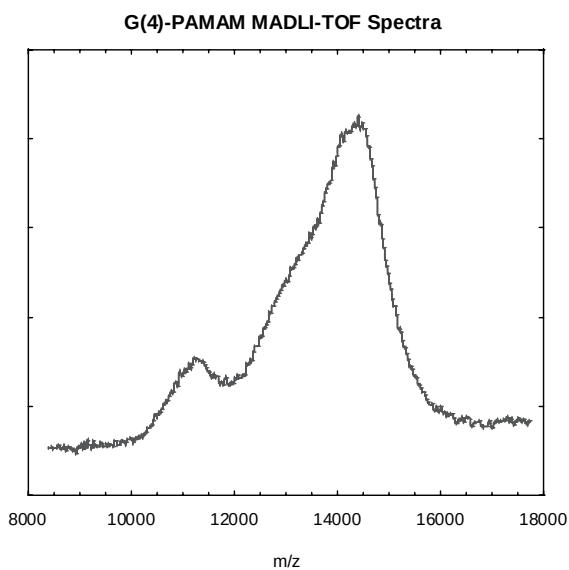


Figure 2.1 MALDI-TOF MS of G(4)-PAMAM dendrimer.

Functionalization reactions were done by sequential addition of **2** and **5** to a G(4)-PAMAM stock solution. Aliquots of **2** (25 mM) and **5** (25 mM) in DMSO were added sequentially to a stock solution of G(4)-PAMAM dendrimer in DMSO (25 mM in endgroups). MALDI-TOF MS was obtained before addition of the second reactant to ensure complete functionalization with the first reactant (usually 48 h). Once the reaction was complete, the second reactant was added and allowed to react until complete (typically 48 h). MALDI-TOF MS was used to determine completion of the reaction. Since the total volume of **2** and **5** was equal to the initial volume of the G(4)-PAMAM stock solution, no endgroups were left unreacted. Reactions were completed so that **2** and **5** were added in different concentrations to the dendrimer's surface. Table 2.1 shows the amounts used and percent loadings of a typical reaction using 500 μL of the G(4)-PAMAM stock solution in each reaction.

Table 2.1 Amounts of **2** and **5** used and percent loadings of **2** and **5** on G(4)-PAMAM dendrimer (**6a-g** and **7a-g**).

Product	μL Mannose ^a	% Mannose	μL TEMPO ^a	% TEMPO
A	475	95	25	5
B	450	90	50	10
C	375	75	125	25
D	250	50	250	50
E	125	25	375	75
F	50	10	450	90

G		25	5	475	95
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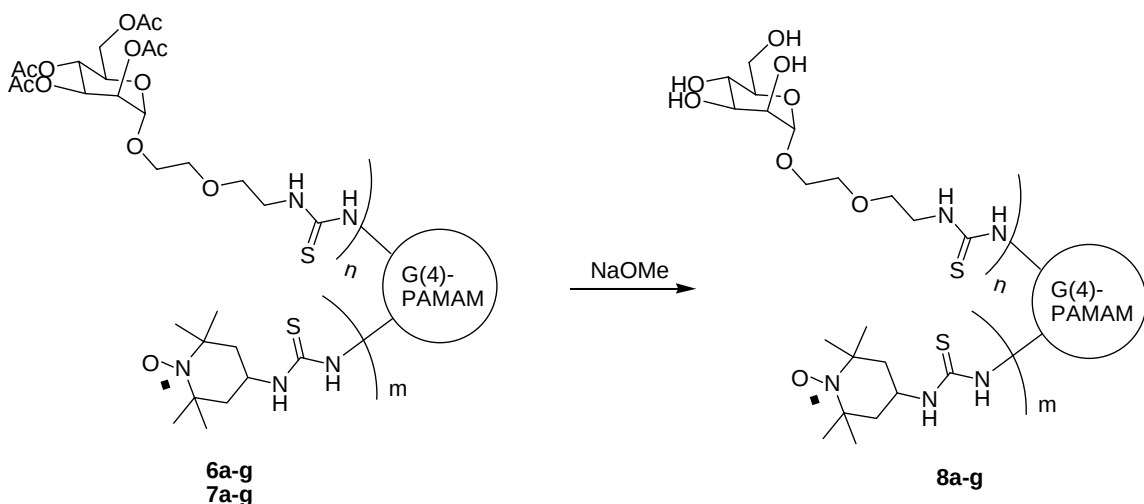
^aThe amounts of **2** and **5** are based on 500 μ L of G(4)-PAMAM stock solution.

The reactions were completed in two series so that both isothiocyanate **2** and isothiocyanate **5** was added as the first reactant. The addition order was found to be inconsequential to the product formed as shown by both MALDI-TOF MS and EPR spectroscopy. This will be discussed in more detail in the MALDI-TOF MS and EPR sections. Due to differences in reactivity of a secondary isothiocyanate and a primary isothiocyanate, **2** and **5** were never added to the dendrimer at the same time. The primary isothiocyanate (**2**) reacts faster than the secondary isothiocyanate (**5**). Reacting both isothiocyanates with G(4)-PAMAM dendrimer at the same time could result in non-uniform functionlization. The solutions of product would contain dendrimers with more variation of percent loadings. Also, by the sequential addition method of functionalization, it was possible to determine the amount of each functional group on the dendrimer's surface by analyzing the MALDI-TOF MS of the unfunctionalized, partially functionalized and fully functionalized dendrimers. Dendrimers **6a-g** and **7a-g** were characterized by MALDI-TOF MS and by EPR. A final deacetylation step in the synthesis was completed to unmask the hydroxyls on the mannose residues of **6a-g** and **7a-g**, forming **8a-g**.

Removal of Acetyl Groups on the Mannose/TEMPO Functionalized Dendrimers

The final step of the synthesis consisted of removing the acetyl groups on the mannose to form 1-O-(5-thiourea-3-oxapentyl)- α -D-mannopyranoside and 4-thiourea-

2,2,6,6-tetramethylpiperidine N-Oxide heterogeneously functionalized G(4)-PAMAM dendrimers (**8a-g**) as shown in Scheme 2.6. Prior to the deprotection step, NaOMe was added to 100% TEMPO loaded dendrimers; EPR results showed that none of the spin labels were quenched. The deprotection was accomplished with 1.0-1.5 equivalents of sodium methoxide in methanol per saccharide residue. The sodium methoxide solution was added to a dendrimer solution 12.5 mM in endgroups in DMSO for dendrimers **6a-g** and **7a-g**. Dendrimers **8a-g** were characterized by MALDI-TOF MS and EPR.



Scheme 2.6 Synthesis of 1-O-(5-thiourea-3-oxapentyl)- α -D-mannopyranoside and 4-thiourea-2,2,6,6-tetramethylpiperidine N-Oxide heterogeneously functionalized G(4)-PAMAM dendrimers (**8a-g**).

General MALDI-TOF Characterization of Heterogeneously Functionalized Dendrimers

The functionalized dendrimers were characterized by MALDI-TOF MS to ensure complete reaction. As noted earlier, the number of terminal groups on the unfunctionalized G(4)-PAMAM dendrimer was first calculated. Usually, the dendrimer

used contained an average of 55 endgroups. The number of endgroups varies with the lot of dendrimer purchased. The percent loading of the first functional group on the dendrimer was determined by comparing the MALDI-TOF spectrum of the partially functionalized dendrimer with that of the unfunctionalized dendrimer. After the dendrimer was fully functionalized, a MALDI-TOF MS was obtained and compared with the unfunctionalized dendrimer spectrum as well as with the partially functionalized dendrimer spectrum to determine the amount of the second isothiocyanate that added to the dendrimer. The analysis by MALDI-TOF MS is discussed in more detail in the individual characterization sections.

Dendrimers **6a-g** (**5** first), **7a-g** (**2** first) and **8a-g** (deacylated mannose) were characterized separately. Each product was synthesized and characterized by MALDI-TOF MS a minimum of three times prior to other studies. This ensured that the functionalization was consistent.

The MALDI-TOF spectra were not sharp peaks as normally found in mass spectrometry, but broad peaks. The broad peaks are due to defects in the dendrimers as discussed in CHAPTER ONE. In order to determine the extent of loading, the weight averaged molecular weights (M_w) of the unfunctionalized, partially functionalized and fully functionalized dendrimers were found using XTOF version 5.1.1 software on a Bruker Biflex III instrument. Given the broad mass range of the dendrimers the concern of uniform functionalization arises. For example a dendrimer that is thought to be 50% loading of A and B may contain some populations that are 40% A and 60% B and other populations that are 60% A and 40% B. The MALDI-TOF MS peaks become broader

with functionalization. These non-uniform populations could contribute to that increase in broadness. Some increase in broadness occurs due to the non-uniform number of endgroups in the dendrimer being functionalized; however, the peaks do not become significantly broader, which suggests that there is very little non-uniform functionalization occurring. The XTOF software allows for the analysis of a continuous polymer peak. The continuous option under the polymer module of the XTOF program the operator is allowed to choose a high and low mass in which the program calculates the M_w , M_n and polydispersity. The range of the peak was obtained by selection the theoretical molecular weight of the compound as one endpoint and a point of comparable intensity on the other side of the peak as the second endpoint. Further studies on the loading distribution of similar heterogeneously functionalized dendrimers by Eric Walter in the Singel Research Group indicate the functionalization is uniform.³⁹

The average number of functional groups per dendrimer on the partially functionalized dendrimer was found by subtracting the mass of the partially functionalized dendrimer from that of the unfunctionalized dendrimer. This gave the mass contribution from the functional group. In order to determine the number of functional groups, the mass was divided by the molecular weight of the functional group. The average percent loading of the partially functionalized dendrimers was determined by dividing the number of functional groups by the total number of endgroups and multiplying by 100%. An example calculation of dendrimer **6c** is shown below. The M_w of **6c** was 16,506 g/mol and the M_w of the starting dendrimer was 13,500 g/mol. The number of TEMPO-NCS on the average dendrimer was determined by: (16,506 g/mol –

13,500 g/mol)/213g/mol to give 14.1 TEMPO-NCS residues attached to the dendrimer. Given that the dendrimer has 55 endgroups on average the % loading of the **5** was calculated to be 25.6% ($14.1/55 \times 100\%$).

The percent loading of the second functional group was determined by subtracting the M_w of the partially functionalized dendrimer from that of the fully functionalized dendrimer and dividing by the M_w of the second functional group. For example, **6c** had a final M_w of 35,478 Daltons so the number of **2** attached was 39.8 $((35,478\text{g/mol} - 16,506\text{g/mol})/477\text{g/mol})$. This is a 72.3% loading on the surface of the dendrimer ($39.8/55 \times 100\%$).

Characterization of Dendrimers Where TEMPO-NCS Was Added First

Dendrimers **6a-g** were characterized by MALDI-TOF MS and analyzed by the method discussed in the previous section. Shown in Tables 2.2 and 2.3 are the data for partial and full loading of dendrimers **6a-g** respectively. The actual % loadings are slightly different than the theoretical, but within reason given that the dendrimers themselves vary in the number of endgroups. All data was taken in triplicate and analyzed by the same methods. Shown in Tables 2.2 and 2.3 is the average of the three or more trials obtained. There are little or no discrepancies in the molecular weight data over three or more trials.

Table 2.2 MALDI-TOF MS data of partially functionalized dendrimers **6a-g**.

Theoretical % 5	M _w	M _N	Polydispersity	Number of 5	Actual % 5
5	14,590 ± 317	14,267 ± 473	1.0167	2.9 ± 1.5	5.3 ± 0.9
10	15,276 ± 646	15,016 ± 614	1.0158	5.3 ± 3.0	9.6 ± 13.9
25	16,795 ± 425	16,492 ± 477	1.0186	14.1 ± 2.0	25.6 ± 1.5
50	18,959 ± 657	18,674 ± 681	1.0192	25.7 ± 3.1	46.7 ± 1.3
75	22,000 ± 349	21,620 ± 490	1.0126	40.7 ± 1.6	74.0 ± 3.5
90	23,426 ± 929	23,043 ± 955	1.0198	49.2 ± 4.4	89.5 ± 7.5
95	24,297 ± 215	23,817 ± 232	1.0193	52.6 ± 1.0	95.6 ± 1.9

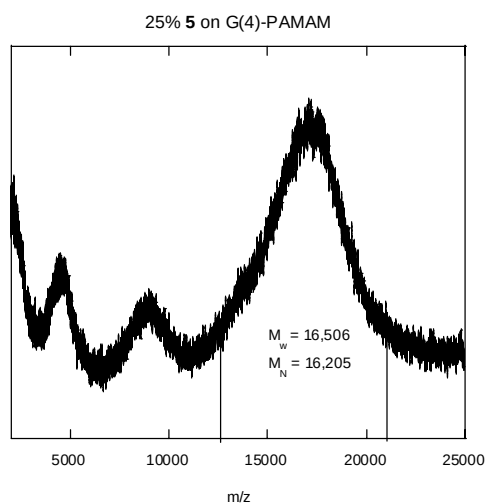
Table 2.3 MALDI-TOF MS data of fully functionalized dendrimers **6a-g**.

Theoretical % 2	M _w	M _N	Polydispersity	Number of 2	Actual % 2
95	38,430 ± 1081	37,887 ± 1021	1.0122	48.3 ± 2.3	87.7 ± 3.4
90	37,184 ± 1396	36,529 ± 1189	1.0084	46.0 ± 2.9	83.7 ± 5.2
75	36,388 ± 1750	35,775 ± 1752	1.0209	39.7 ± 3.7	72.3 ± 6.7
50	33,163 ± 1049	32,659 ± 979	1.0159	23.9 ± 2.2	43.5 ± 4.0
25	27,750 ± 1975	27,254 ± 1929	1.0192	13.7 ± 4.1	24.9 ± 4.8
10	25,920 ± 1120	25,516 ± 1080	1.0167	6.4 ± 2.3	11.7 ± 2.8
5	25,322 ± 1439	24,767 ± 1337	1.0267	1.7 ± 3.0	3.0 ± 4.8

Representative MALDI-TOF spectra are show in Figure 2.2 for both the partially and fully functionalized dendrimers of the 25% **5** and 75 % **2** functionalized G(4)-

PAMAM dendrimer, **6c**. Spectra of the other concentrations of **5** and **2** are shown in Appendix A.

A)



B)

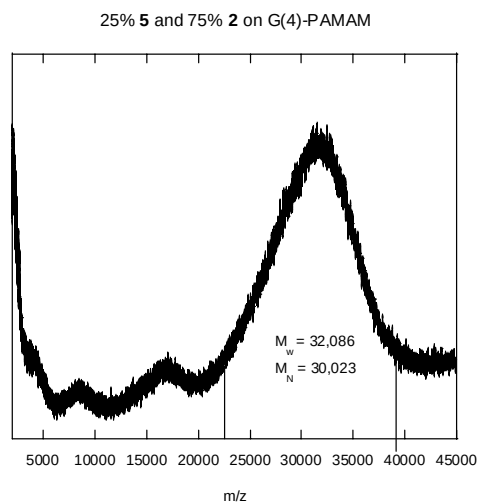


Figure 2.2 MALDI-TOF spectra of a) partially functionalized (**5** only) and b) fully functionalized (**5** and **2**) 25% **5** and 75% **2** G(4)-PAMAM dendrimer.

Characterization of Dendrimers where Mannose was Added First

Dendrimers **7a-g** were characterized by MALDI-TOF MS and analyzed by the methods discussed in the General MALDI-TOF MS Characterization of Heterogeneously Functionalized Dendrimers section of this chapter. Shown in Tables 2.4 and 2.5 are the data for partial and full loading of dendrimers **7a-g** respectively. The actual percent loadings are slightly different than the theoretical, but within reason given that the dendrimers themselves vary in the number of endgroups. All data was taken in triplicate and analyzed by the same methods. Shown in Tables 2.4 and 2.5 is the average of the three of more trials obtained. There are little or no discrepancies in the molecular weight data over three of more trials.

Table 2.4 MALDI-TOF MS data of partially functionalized dendrimers **7a-g**.

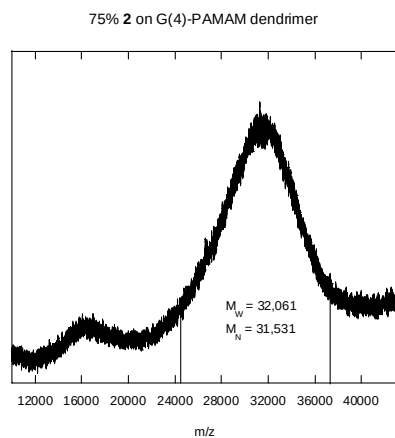
Theoretical % 2	M _W	M _N	Polydispersity	Number of 2	Actual % 2
95	36,645 ± 660	36,028 ± 660	1.0150	51.6 ± 1.4	93.8 ± 2.6
90	35,524 ± 644	34,806 ± 602	1.0155	48.5 ± 1.4	88.3 ± 5.1
75	32,285 ± 1937	31,721 ± 1885	1.0168	39.7 ± 4.1	72.2 ± 7.4
50	25,629 ± 306	25,214 ± 312	1.0169	27.2 ± 0.6	49.5 ± 3.2
25	20,398 ± 709	20,049 ± 700	1.0186	12.8 ± 1.5	23.2 ± 2.8
10	16,194 ± 109	16,553 ± 615	0.9753	6.2 ± 0.2	11.3 ± 0.3
5	15,701 ± 415	15,395 ± 465	1.0230	5.2 ± 0.9	9.4 ± 1.7

Table 2.5 MALDI-TOF MS data of fully functionalized dendrimers **7a-g**.

Theoretical % 5	M_w	M_n	Polydispersity	Number of 5	Actual % 5
5	$38,296 \pm 95$	$37,636 \pm 6$	1.0202	3.0 ± 0.4	5.5 ± 0.7
10	$37,756 \pm 188$	$36,872 \pm 68$	1.0170	5.9 ± 0.9	10.8 ± 1.6
25	$36,624 \pm 1682$	$35,942 \pm 1524$	1.0224	13.5 ± 3.5	24.6 ± 6.3
50	$33,557 \pm 1063$	$31,915 \pm 1055$	1.0181	30 ± 2.2	54.5 ± 4.1
75	$27,573 \pm 556$	$27,102 \pm 479$	1.0198	41.8 ± 1.2	76.0 ± 2.2
90	$25,574 \pm 1365$	$25,144 \pm 1287$	1.0174	48.8 ± 2.9	88.7 ± 5.3
95	$25,077 \pm 1396$	$24,586 \pm 1433$	1.0187	51.6 ± 2.9	93.9 ± 5.2

Representative samples of the MALDI-TOF spectra are show in Figure 2.3 for both the partially and fully functionalized dendrimers of the 25% **5** and 75 % **2** functionalized G(4)-PAMAM dendrimer (**7c**). Spectra of the other concentrations of **5** and **2** are show in Appendix A.

A)



B)

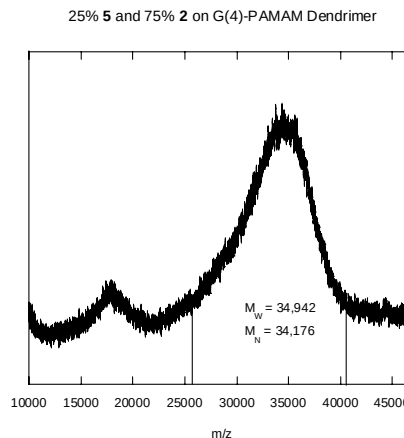


Figure 2.3 MALID-TOF spectra of a) partially (**2** only) and b) fully (**2** and **5**) functionalized 25% **5** and 75% **2** G(4)-PAMAM dendrimer (**7c**).

Characterization of Deacetylated Mannose/TEMPO Coated Dendrimers

Mannose/TEMPO coated dendrimers **8a-g** were characterized by MALDI-TOF MS similar to the way the Acetylated Mannose/TEMPO coated dendrimers **6a-g** and **7a-g** were characterized. The M_w calculated from the MALDI-TOF spectrum was compared to both the starting material (**6a-g** or **7a-g**) and the unfunctionalized dendrimer. The decrease in mass from **6a-g** or **7a-g** indicates that the acetyl groups were removed. If there was not a large enough decrease in mass, then the reaction was not complete and was allowed to react for longer times. Shown below in Figure 2.4 is the MALDI-TOF spectrum of a representative sample containing 75% mannose and 25% TEMPO on a G(4)-PAMAM dendrimer (**8c**).

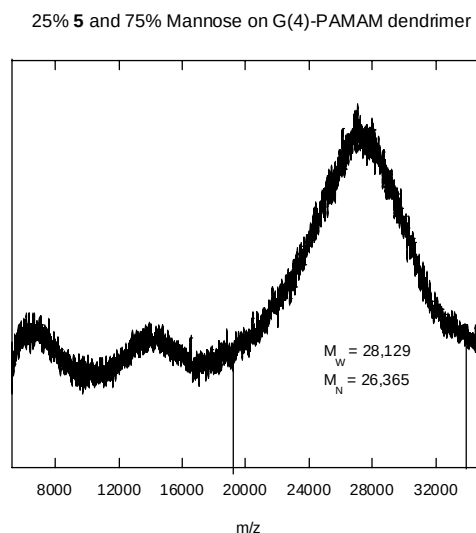


Figure 2.4 MALDI-TOF spectra of 75% mannose and 25 % **5** G(4)-PAMAM dendrimer (**8c**).

The MALDI-TOF MS was analyzed by comparing the mass of the acetylated mannose/TEMPO coated dendrimers with that of the products and by comparing the products to the mass of the unfunctionalized dendrimer. For example an acetyl deprotected dendrimer **8c** that contained 75% mannose and 25% TEMPO and had a mass of 28,129 g/mol was calculated to contain 39.1 mannose units (71.1%). The calculation was accomplished by subtracting the mass of the dendrimer from that of the starting dendrimer and the mass contribution from the TEMPO. Given that the starting dendrimer had a mass of 13,125 g/mol, the mannose has a molecular weight of 309 g/mol the TEMPO has a molecular weight of 213 g/mol and there are approximately 13.75 TEMPO residues on each dendrimer the calculation was $(28,129\text{g/mol} - 13,125\text{g/mol} - 13.75 \times 213\text{g/mol}) / 309\text{g/mol}$. This gave 39.1 (71.1%) mannose residues on the surface of the dendrimer. The other method of calculating the amount of mannoses deprotected was accomplished by comparing the MALDI-TOF spectrum of **6c** or **7c** with that of the **8c**. Knowing that the loss of all 4 acetyl groups results in a 168g/mol loss in mass per mannose, the calculation was done by subtracting the molecular weight of **8c** from that of **6c** or **7c**. For example the molecular weight of **8c** was 34,942 g/mol resulting in a loss of 6,813 g/mol in the molecular weight or 40.6 ($6,813\text{g/mol} \div 168\text{g/mol}$) mannoses were deprotected. This is equivalent with approximately 73.7% mannose on the dendrimer surface. Table 2.6 highlights the results of the deprotection for **8a-g**. Shown below are the average molecular weights obtained. The results were seen in triplicate prior to other studies on these compounds.

Table 2.6 MALDI-TOF MS data for **8a-g**.

Sample	% Mannose	M _w of 6 or 7	M _w of 8
a	95	38,363	29,252 ± 882
b	90	37,470	29,297 ± 724
c	75	36,506	28,706 ± 577
d	50	33,360	27,534 ± 648
e	25	27,662	25,085 ± 780
f	10	25,747	24,701 ± 1067
g	5	25,200	24,580 ± 596

EPR Analysis: Rationale and Previous Studies

Spin-spin interactions are seen in the EPR spectrum when two spins are in close proximity. In the case of stable free radicals such as 2,2,6,6-tetramethylpiperidine N-Oxide (TEMPO), line broadening effects can be seen in the spectra of a series of solutions of different concentrations. As the solution becomes more concentrated, the spin-spin interactions increase and the EPR spectrum broadens. The magnetic field of the EPR splits the spins into the α and β spin states. If the spins are interacting with each other, then there is further, smaller splitting in the energy levels, resulting in broadening of the spectrum. The more spins that interact, the more broadening occurs. An EPR spectrum of 12.5 mM **5** is seen in Figure 2.5. The two peaks above the base line are

labeled A and B. The broadening of the spectrum causes changes in the peak height ratio of A to B.

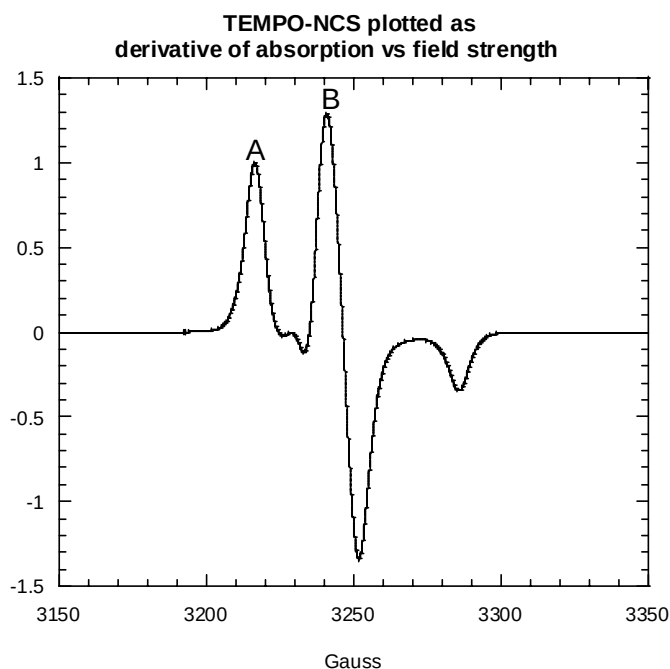


Figure 2.5 EPR spectrum of 12.5 mM TEMPO-NCS.

The amount of line broadening can be quantized by comparing the ratio of peak height A to peak height B. In looking at the integrals of the spectra there is little change in the slope where A absorbs over a wide range of TEMPO loading; however, there is a large change in the slope where B absorbs. This is illustrated in Figure 1.9 on page 10. The differences in slope in the integral spectrum correspond to differences in peak height ratio in the derivative spectrum. The changes in peak height ratios are equivalent to the changes in the slope. Figure 2.6 shows a change in the ratio between peaks A and B for different concentrations of TEMPO-NCS.

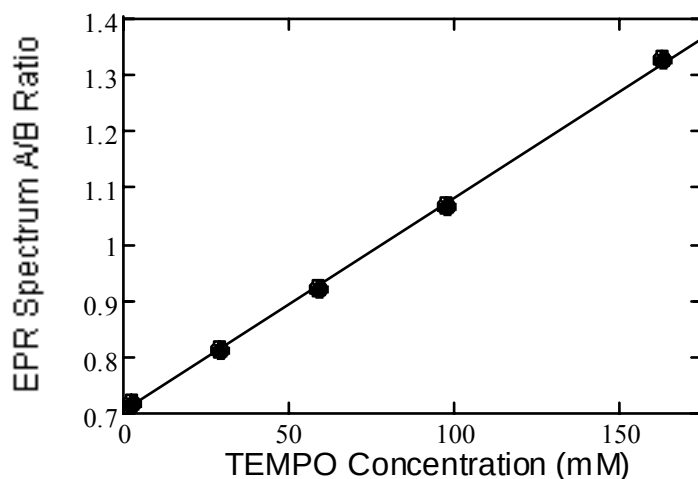


Figure 2.6 EPR concentration dependent trends of A/B ratios of 2,2,6,6-tetramethylpiperidine N-Oxide at 2, 30, 60, 100 and 160 mM in DMSO.

Several spin labels covalently attached to a single dendrimer (or other macromolecule) create a new scenario. The spin labels are no longer capable of orienting themselves so that they have the least number of interactions with other spin labels. Therefore at equivalent concentrations, more line broadening may be seen. This is seen on TEMPO functionalized dendrimers (**9a-g**). As more spin labels are attached to the dendrimer, more line broadening results.

PAMAM dendrimers are extremely flexible molecules. The surface groups can rearrange to be in different proximities to each other. There are three distinct possibilities for relative endgroup orientation that can occur; clustered, random and scattered. In the clustered scenario all the spin labels are as close together as possible, increasing line broadening and thus the A/B ratio. As more spin labels are attached to the dendrimer more broadening occurs and the A/B ratio increases more. The scattered case begins

with very little interaction between the spin labels; however as more spin labels are added they are forced to be closer and broadening occurs, increasing the A/B ratio. In the random case, the spin labels are randomly distributed on the surface. Again the spins interact more as more spins are added. The three theoretical scenarios are illustrated in Figure 2.7. The theoretical ratios were calculated by Eric Walter in David Singel's research group.

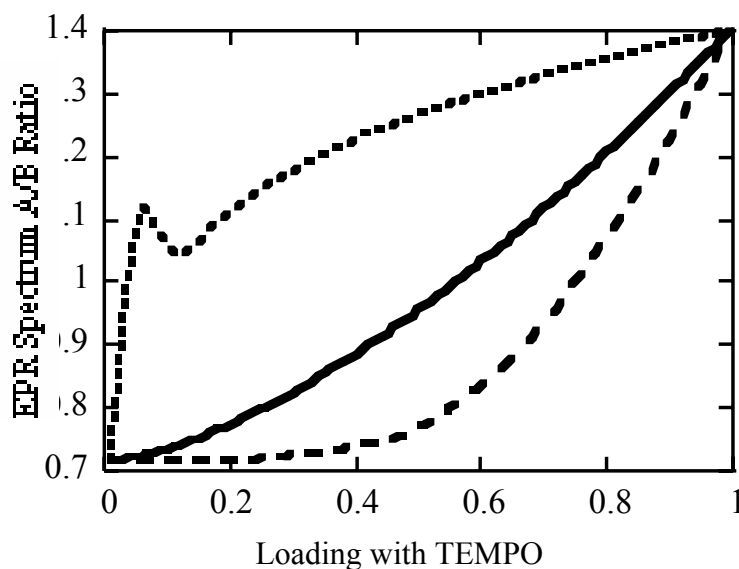
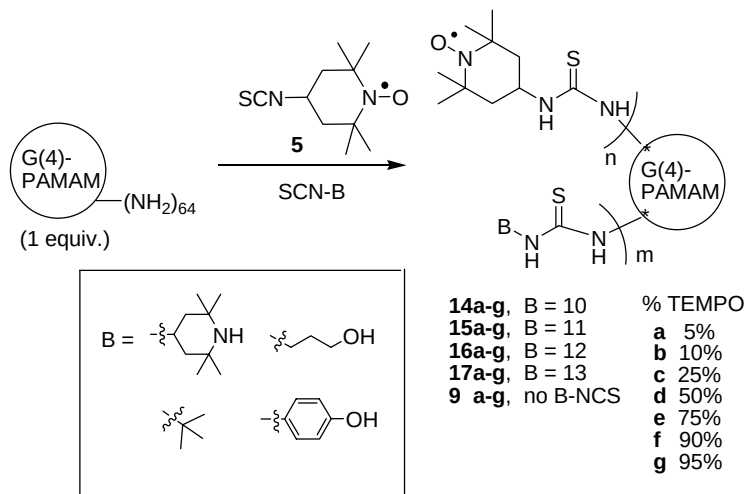


Figure 2.7 Amount of line broadening for different amounts of spin label on a G(4)-PAMAM dendrimer for clustered (dotted), random (solid) and scattered (dashed).

The theoretical possibilities of spin label arrangement and experimental results with TEMPO only on a G(4)-PAMAM dendrimer were used to determine the locations of other groups on the surface of heterogeneously functionalized dendrimers. Studies were completed by Eric Walter in the Singel research group at a range of percent loadings of

various groups and TEMPO on the surface of the dendrimer. The other functional groups included 2,2,6,6-tetramethylpiperidine (**10**), 1-propanol (**11**), tert-butyl (**12**) and para-phenol (**13**) and were synthesized by sequential or simultaneous addition. They are depicted in Scheme 2.7.



Scheme 2.7 Functionalization of G(4)-PAMAM dendrimer with **5** (**9**) and with **5** and another functional group (**14-17**).

A/B ratio comparisons indicated a random distribution for each of these molecules as seen in Figure 2.8. The data obtained for these functional groups indicates that the relative locations of the spin labels are random in all cases studied as determined by Karl Sebby and Eric Walter in the Singel research group. From this, it can be extrapolated that the relative locations of the other functional group is also random. The functional groups studied were a variety of different groups including primary, secondary, tertiary and aromatic with some containing a single hydroxyl unit.

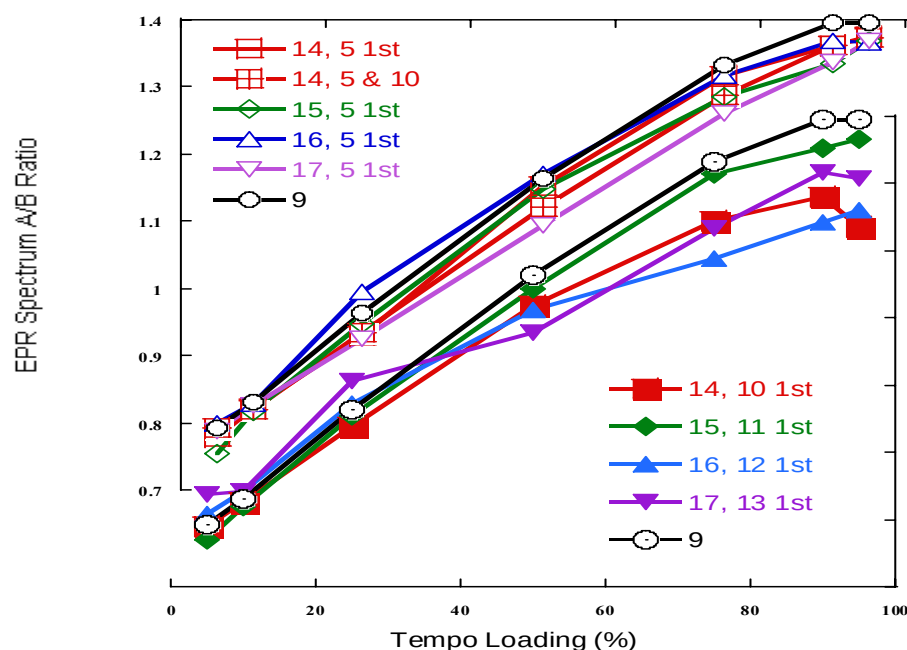


Figure 2.8 A/B peak height ratios for dendrimers **9** and **14-17**.

EPR Studies on the Mannose/TEMPO Coated Dendrimers

Although all the previous EPR studies concluded that the relative locations of the functional groups on the surface of a heterogeneously functionalized G(4)-PAMAM dendrimer were random, carbohydrates were not studied directly. The relative locations of carbohydrates on the surface of a heterogeneously functionalized dendrimer could be different. The primary reason for concern was the multiple hydroxyl groups that carbohydrates possess. Carbohydrates on the dendrimer's surface could rearrange due to hydrogen bonding as illustrated in Figure 2.9, whereas the other functional groups studied either could not or would not be as likely to rearrange.

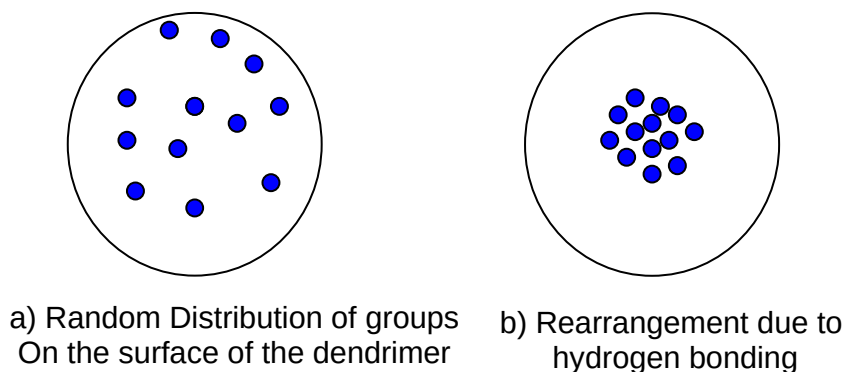


Figure 2.9 Schematic representation of possibilities for relative locations of the carbohydrates on the dendrimers surface of a) random and b) clustered orientations.

EPR studies on the spin-labeled dendrimers were conducted and analyzed in the same manner the previous studies had been. First, the studies were done on the O-acetyl protected mannose/TEMPO coated dendrimers **6a-g** and **7a-g**. The studies were conducted on **6a-g** and **7a-g** separately to compare to the MALDI-TOF MS data that indicated the order of loading was unimportant. Stackplots of the EPR spectra illustrating each percent of TEMPO with the loading of the other functional group changing in the same amount are made. Changes in the spectrum are seen as a higher loading of TEMPO is achieved. A qualitative comparison can be analyzed by looking at the normalized stackplots of **6a-g** and **7a-g**. As more spin labels are incorporated onto the surface of the dendrimer, more broadening is seen in the spectra and the peak height of B decreases in the normalized spectrum as seen in Figure 2.10 and 2.11.

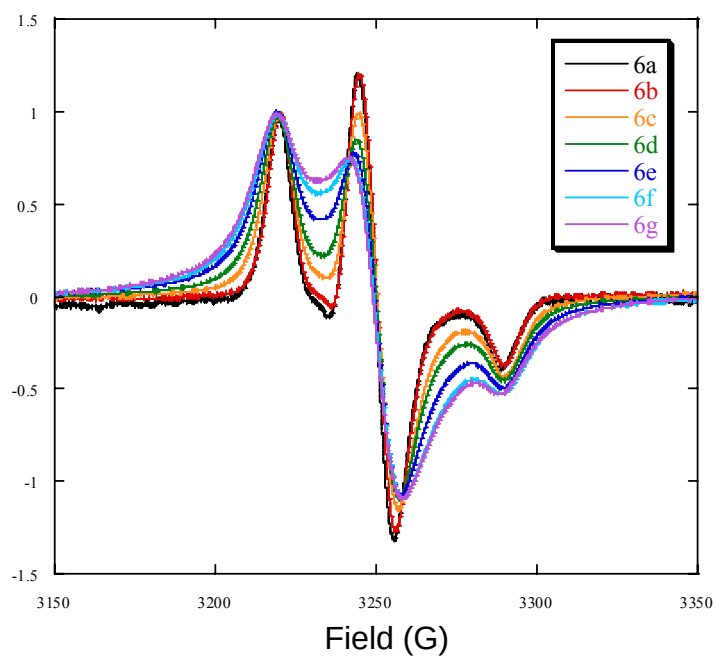


Figure 2.10 Normalized EPR stackplots of **6a-g**.

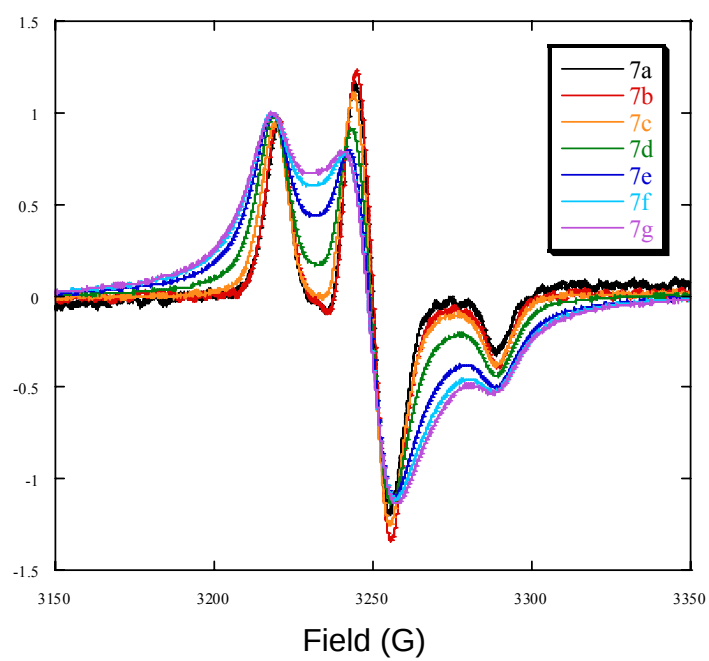


Figure 2.11 Normalized EPR stackplots of **7a-g**.

The quantitative method for determining whether the two series of compounds are the same is to compare the A/B ratio vs. percent loading for the two series. As can be seen in Figure 2.12 the A/B ratios are virtually the same for the two series of compounds. The MALDI-TOF MS data and the EPR data are consistent; the order of loading is not important.

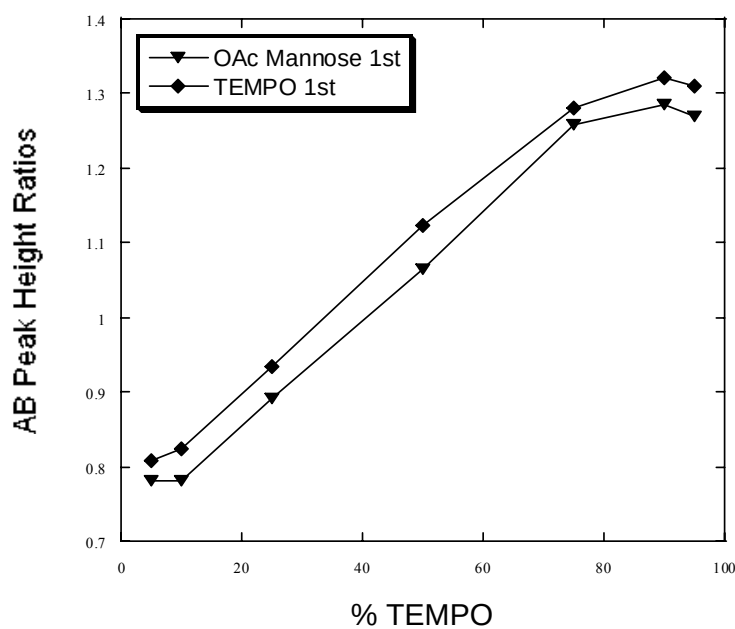


Figure 2.12 A/B Ratio vs % loading of spin label for heterogeneously functionalized dendrimers **6a-g** (squares) and **7a-g** (triangles).

Next, the series of heterogeneously functionalized dendrimers **8a-g** were studied via EPR. The normalized stackplot of the series was first compared to that of the acetylated dendrimer series. The stackplot of **8a-g** is seen in Figure 2.13.

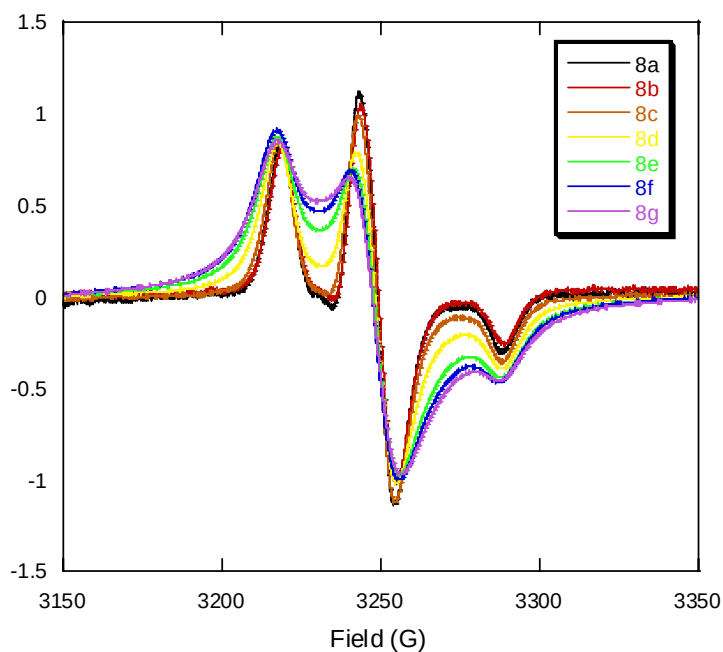


Figure 2.13 Normalized stackplot of EPR on compounds **8a-g**.

The quantitative analysis of the A/B ratio of **8a-g** was accomplished by the same methods as **6a-g** and **7a-g**. The linear regression indicates that the spin labels are randomly located on the surface of the dendrimer and thus the carbohydrates are also randomly located on the surface of the dendrimer. Since dendrimers **6a-g** and **7a-g** also have a random distribution, no rearrangement appears to occur when the hydroxyls are unmasked. As seen in Figure 2.14 there is little difference in the A/B ratio vs. percent loading from **6a-g** and **7a-g** (averaged) to **8a-g**. It can be concluded that the spin labels of all these dendrimers have relative locations that are random to one another and therefore the carbohydrate residues are randomly dispersed on the surface of the dendrimer. Also, it can be concluded that hydrogen bonding or any other interaction does not allow for the rearrangement of carbohydrates on the dendrimer's surface.

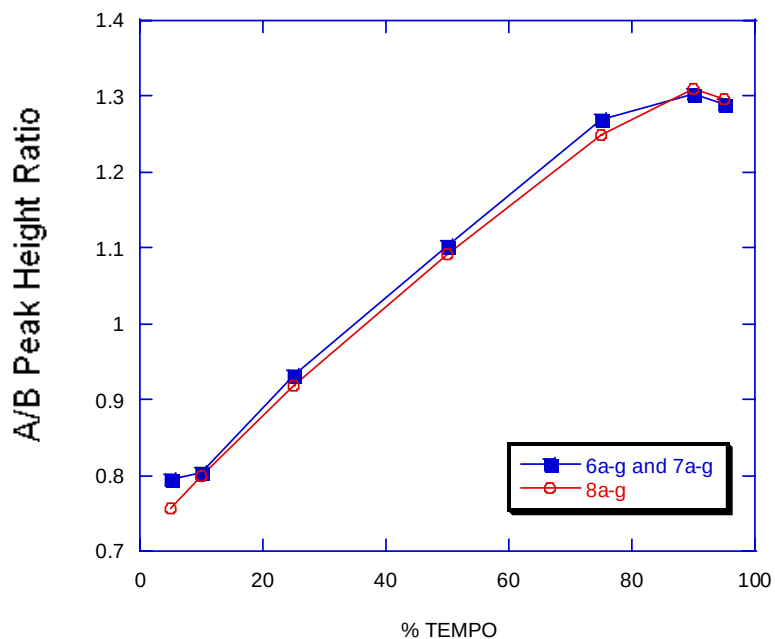


Figure 2.14 A/B ratio vs. % loading of spin label for average of all trials of **6a-g** and **7a-g** (squares) and an average of all trials of **8a-g** (circles).

The EPR data obtained clearly show that there is no significant difference in the relative locations of the spin labels for compounds **6a-g** and **7a-g** (Figure 2.12) and **8a-g**. That data complements the MALDI-TOF MS data, and it can be concluded that the order of isothiocyanate addition is not important. Comparing the average of the EPR data from **6a-g** and **7a-g** with that of **8a-g** indicates no change in the locations of the spin labels and therefore no change in the locations of the carbohydrates. As can be seen in Figure 2.14, the A/B peak height ratios vs. the percent loading of **5** is the same for both sets of molecules. It is evident that there is no rearrangement after the acetyl groups on the carbohydrate are removed.

Summary

Dendrimer functionalization to form **6a-g** and **7a-g** was successfully accomplished with the use of isothiocyanates **2** and **5**. In the course of analyzing the dendrimers, it was found that the percent loadings of the functional groups are independent of the addition order as shown both in the MALDI-TOF MS and EPR analysis. MALDI-TOF MS indicated addition order was not important because the number of each functional group was shown to be the same regardless of addition order. Also, percent loadings are very reproducible. EPR analysis indicated that the spin labels were randomly distributed on the surface of the dendrimer for both **6a-g** and **7a-g**. Therefore the carbohydrate residues must also be randomly distributed on the surface of the dendrimer. The EPR spectra indicate no change in line broadening between **6a-g** and **7a-g**.

The synthesis of dendrimers **8a-g** was accomplished in one synthetic step from **6a-g** and **7a-g**. MALDI-TOF MS was completed and analyzed to show that the hydroxyls on the carbohydrates were unmasked. The EPR analysis indicated that the relative locations of the spin-labels are random on dendrimers **8a-g**. From this conclusion it was decided that the relative locations of the carbohydrates are also random. Since the relative locations of the carbohydrates are random for both the dendrimers with the hydroxyls protected and those with the hydroxyls, there is no significant rearrangement of carbohydrates on the dendrimer surface after the hydroxyls are unmasked.

Experimental Procedures

General reagents were obtained from Aldrich, Sigma, and Acros chemical companies. Generation 4 PAMAM dendrimer was purchased from either Aldrich or Dendritech. Methylene Chloride was purified on basic alumina and $\text{BF}_3 \cdot \text{OEt}_2$ was distilled from CaH_2 (62 °C at 53 mm Hg) before use. The trans indol-acrylic acid was crystallized from warm absolute ethanol before use. All other solvents and reagents were used as supplied.

The General procedure for the synthesis of heterogeneously functionalized dendrimers (**6a-g** and **7a-g**) is as follows. The synthesis of **2** was completed by the experimental procedures previously published in our group.³³ The synthesis of the heterogeneously functionalized dendrimers (**6a-g** and **7a-g**) was carried out with a stock solution of G(4)-PAMAM in DMSO (500 μL , 25 mM in endgroups). Aliquots of a solution of NCS-TEMPO (**5**, 25 mM) and of **2** (25 mM) in DMSO are added to the dendrimer solution sequentially in a specific ratio (total added volume = 500 μL). The second reactant is added 48 h after the first (upon completion of reaction with the first isothiocyanate as shown by analysis of the MALDI-TOF spectrum). The mixture is allowed to react for an additional 48 h after the second reactant is added. Completion was again determined through the analysis of the MALDI-TOF spectrum. A 100 μL aliquot was taken for EPR spectra while the remaining solution was taken on to form dendrimers **8a-g**. The specific amounts are seen in Table 2.7.

Table 2.7 Experimental amounts used for **6** & **7(a-g)**.

6 & 7	Amt G(4)- PAMAM (μ L)	Amt. 2 (μ L)	% 2	Amt. 5 (μ L)	% 5
a	500	475	95	25	5
b	500	450	90	50	10
c	500	375	75	125	25
d	500	250	50	250	50
e	500	125	25	375	75
f	500	50	10	450	90
g	500	25	5	475	95

Table 2.8 MALDI-TOF MS and EPR data for **6a-g**.

6	M _w 5 only	Number of TEMPOS	M _w 5 and 2	Number of sugars	A/B Ratio
a	14,126	2.9	37,145	48.3	0.801
b	14,630	5.3	36,593	46.0	0.797
c	16,506	14.1	35,478	39.7	0.929
d	18,964	25.7	30,368	23.9	1.12
e	22,172	40.7	28,725	13.7	1.27
f	23,982	49.2	27,040	6.4	1.30
g	24,512	52.6	25,303	1.7	1.31

Table 2.9 MALDI-TOF MS and EPR data for **7a-g**.

7	M _w 2 only	Number of sugars	M _w 2 and 5	Number of TEMPOS	A/B Ratio
a	37,738	51.6	38,391	3.0	0.781
b	36,307	48.5	37,567	5.9	0.780
c	32,061	39.7	34,942	13.5	0.892
d	26,104	27.2	32,494	30	1.06

e	19,222	12.8	28,129	41.8	1.26
f	16,085	6.2	26,473	48.8	1.29
g	15,593	5.2	26,588	51.6	1.27

The general Procedure for deacetylation to form 1-O-(5-thiourea-3-oxapentyl)- α -D-mannopyranoside and 4-thiourea-2,2,6,6-tetramethylpiperidin N-Oxide heterogeneously functionalized G(4)-PAMAM dendrimers (**8a-g**) is as follows. The deprotection of the acetyl groups is completed by adding 0.3 M NaOMe in methanol (1.0 – 1.3 equiv./carbohydrate) to the DMSO solutions of **6a-g** or **7a-g**. After 48 h the completion is determined by MALDI-TOF MS. A 100 μ L aliquot is removed for EPR. The remaining solution is purified on either Sephadex G25 gel in DMSO or Amnicon centrifugation filters (MWCO = 5 kDa.). The DMSO is removed by lyophilization followed by repeated lyophilization with water until the product remains a constant mass. Specific amounts are given in Table 2.10.

Table 2.10 Experimental amounts used for **8a-g**.

8	μ L 6(a-g) or 7(a-g)	μ mol 2	μ L NaOMe (0.3M)
a	900	641	192
b	900	608	182
c	900	506	152
d	900	338	101
e	900	169	51
f	900	68	20
g	900	34	10

Table 2.11 MALDI-TOF MS and EPR data for **8a-g**.

8	M _w 6 or 7	M _w 8	A/B Ratio
----------	-------------------------------------	-------------------------	-----------

a	38,203	29,276	0.757
b	36,217	28,573	0.789
c	34,942	28,129	0.952
d	30,369	25,403	1.12
e	26,940	24,953	1.26
f	26,688	26,366	1.32
g	26,473	25,640	1.34

EPR spectra were taken by Karl Sebby or Eric Walter of the David Singel research group as previously described.³⁹ Primarily, the spectra were taken by Karl Sebby.

CHAPTER THREE

AFFINITY STUDIES ON MANNOSE/TEMPO FUNCTIONALIZED DENDRIMERS

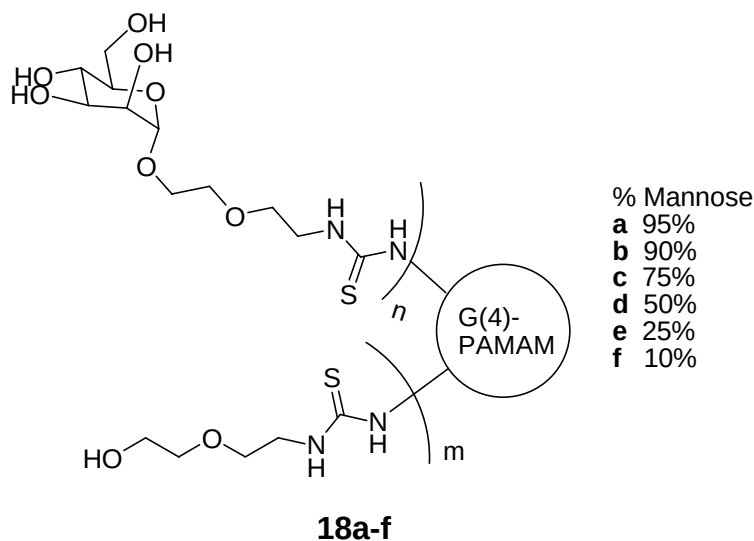
Background and Rational

Protein-carbohydrate interactions are an important part of several biological functions as discussed in CHAPTER ONE. Understanding these interactions is vital to increasing scientific knowledge. Multivalent binding plays a significant role in protein carbohydrate interactions. Previous results in the Cloninger research group show that the mannose functionalized G(4)-PAMAM dendrimers bind to Concanavalin A (Con A). These results are based primarily on a series of hemagglutination inhibition assays (HIA) in which a solution of the material is incubated with Con A, which was found to block the binding of Con A to red blood cells. The results of these assays are observed as a whole of the solution rather than as the individual parts of the solution. The relative binding measured may therefore be due to a number of interactions of differing binding strengths or a small amount of the material.

The EPR results from CHAPTER TWO made it possible to determine that the relative locations of the carbohydrates are randomly distributed on the surface of the dendrimer. Given that the functionalized dendrimers lack order, the HIA results seen previously in the Cloninger research group may be due to a single conformation or a combination of conformations. Also, the dendrimers are flexible and the mannose units may be able to orient themselves in the best conformation for binding. In this case it is conceivable that a small amount of the material was responsible for the bulk of the

activity that was observed in the HIA. To ensure that this was not the case, affinity chromatography was employed to determine whether the heterogeneously mannose coated dendrimer solution contains molecules of differing affinities.

Affinity chromatography separates oligosaccharides that do not have the same affinity for a given protein. The spin label on the molecule allows the study of apparent differences in the locations of the endgroups (if any) by EPR. As part of this study, EPR was used to observe the relative locations of the functional groups before and after the affinity column and to determine any differences between stronger and weaker binding fractions. Dendrimers **8a-g** were the ideal molecules to study because they contain both a spin label for EPR and the mannose residues that will interact with the Con A. Before the affinity chromatography began, dendrimers **8a-g** were studied by HIA to determine any affects the spin label may have on the results. The results of these assays were compared to previous results obtained in our group on G(4)-PAMAM dendrimers heterogeneously functionalized with **2** and **4** (**18a-f**) in the same % loadings as **8a-f**.



Hemagglutination Assays

The HIA is a simple assay first introduced by Osawa and Matsumoto in 1972.⁴⁰ The assay does not measure direct binding constants, but was an ideal first study for comparison of glycodendrimers with glycopolymers previously designed and studied. Erythrocytes contain carbohydrates on their surface that lectins bind. Extensive cross-linking of the lectins with the cells leads to agglutination, also called hemagglutination. Inhibiting hemagglutination by using a ligand, typically a sugar the lectin binds specifically, is the basis for the HIA. The concentration of ligand at which hemagglutination stops is the inhibiting dosage.

The protein used for HIA with the mannose coated dendrimers was Con A. Isolated from plant lectin of the jackbean, the function of Con A in the jackbean is unknown. Con A binds with carbohydrates in a non-covalent manner and selectively binds mannose and glucose but not galactose or fructose. Mannose binds more strongly to the protein than glucose does.⁴¹⁻⁴⁴ Con A contains 137 amino acids in the monomeric form and exists as a dimer in the pH range of 5.0 – 5.6 and a tetramer at pH higher than 7.0. Con A is a C-type protein which means the Ca^{2+} must be present in order for the protein to be active. Also, for Con A to be active, Mn^{2+} must be present.⁴⁵⁻⁵⁰ As a tetramer the carbohydrate binding sites are 65Å apart. The mannose functionalized G(4)-PAMAM dendrimer is large enough to bind 2 sites of the tetramer.

The HIA is done to determine whether a ligand will inhibit the interaction between Con A and red blood cells as shown in Figure 3.1. The HIA is done in a microliter plate with v-shaped wells. If only a solution of whole (rabbit) blood is added

to the well then the red blood cells settle out of solution and appear as a red “button” in the bottom of the well. A solution of Con A and whole blood leads to crosslinking of the Con A to glyoproteins on the erythrocyte’s surface. A cross-linking lattice is formed and the red blood cells do not settle out of solution. Inhibition of the Con A-red blood cell cross linking is reached when a mannose containing ligand (such as mannose functionalized dendrimers) is incubated with Con A prior to the addition of the blood solution. If the ligand concentration is high enough then the ligand will bind Con A and inhibit the interaction of Con A with the red blood cells. This results in hemagglutination being inhibited and the red blood cells settling out of solution.

A brief experimental description of the HIA follows; a detailed procedure can be found in Appendix B. Erythrocytes were added to a preincubated solution of Con A with varying concentrations of dendrimer. The lowest amount of dendrimer that caused inhibition of hemagglutination was determined. This was compared to methyl mannose. The reading of the wells to determine whether hemagglutination occurred or not can be a source of error. The wells were read consistently and a minimum of three trials were completed in order to combat this problem.

Hemagglutination Assay Results

HIA on dendrimers **8a-c** were performed using the procedure done by Dr. Eric Woller, formerly of the Cloninger research group. The assays are conducted in a pH 7.5 PBS buffer. Dendrimers **8d-g** are not soluble enough in water or PBS to obtain HIA results in the same manner. The results of HIA on dendrimers **8a-c** are highlighted below in Table 3.1.

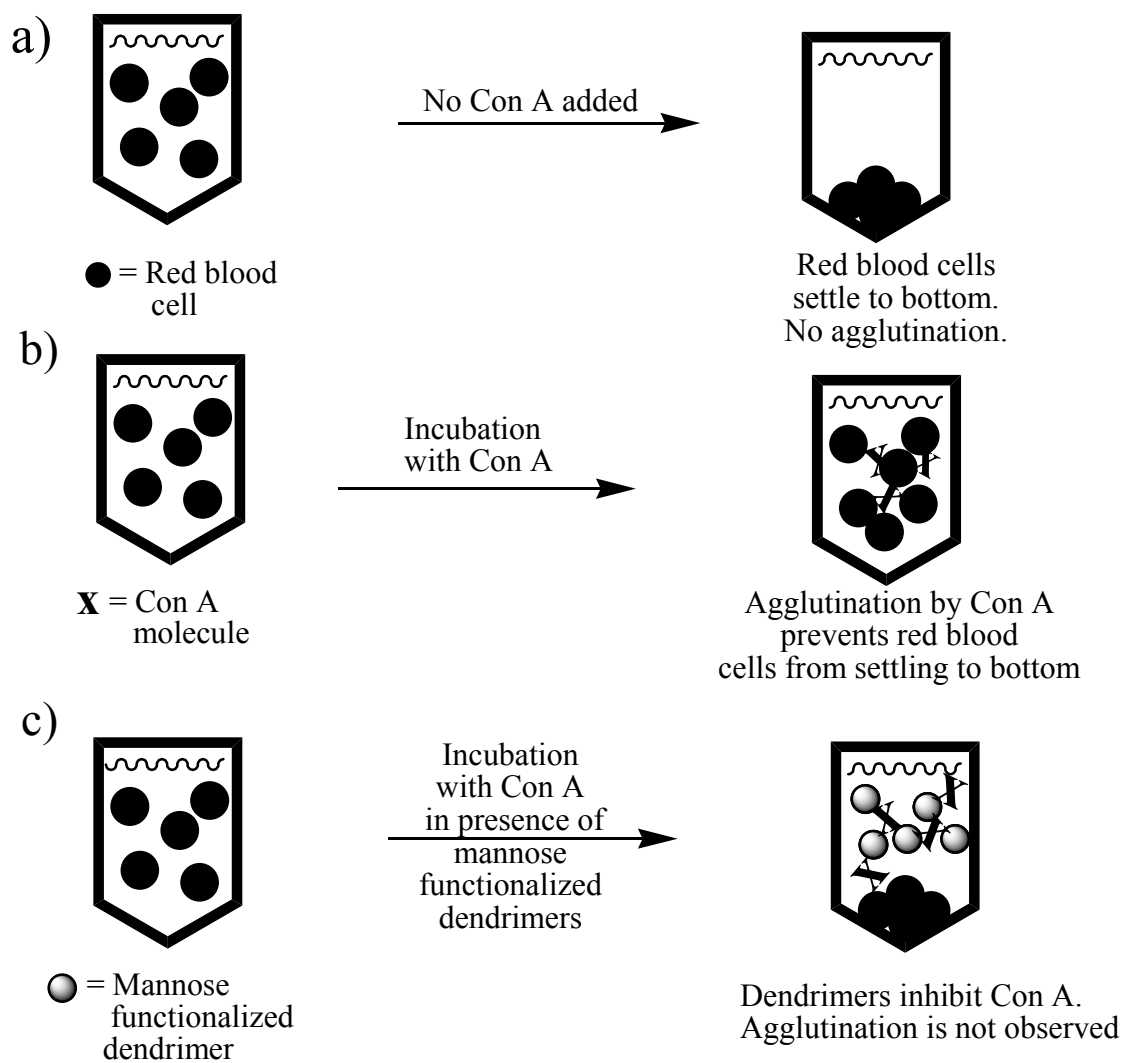


Figure 3.1 Schematic representation of HIA assay: a) red blood cells only b) red blood cells and Con A c) red blood cells inhibited by addition of a saccharide containing ligand.

Table 3.1 HIA results for dendrimers **8a-c**.

8	# Sugars	Relative activity/sugar	18	Relative activity/sugar
a	57	414 ± 309	a	204 ± 90
b	54	296 ± 121	b	184 ± 12
c	45	172 ± 75	c	203 ± 8

The relative activities/sugar of **8a-c** is on average slightly different from **18a-c**. This is due to combination of a two different operators performing the assays and fewer trials being done for **8a-c**. The three trials for **8a** contained three differing results of 173, 346 and 723 respectively. The three trials completed for **8b** were found to have an activity/sugar of 175, 351 and 363 respectively. The three trials completed for **8c** were found to have an activity/sugar of 101, 202 and 211 respectively. The first trials may have resulted in inaccurate reading of hemagglutination one well too far. The three trials were all in the same order of magnitude as each other and as the data obtained by Eric Woller. The trials were completed using different blood samples. Since HIA is an immunogenic assay, the concentrations of carbohydrates on the surface of the cells differs from rabbit to rabbit and even from the same rabbit at different times. Therefore it was concluded that the spin label had no consequential affect on the binding between Con A and **8a-c**.

The procedure was changed slightly to obtain hemagglutination assays on dendrimers **8d-g**. The assays were done in 1:1 DMSO:PBS instead of straight PBS to solublize the dendrimers. The Con A and red blood cell solutions were also changed to 1:1 DMSO:PBS. The other change that occurred is that the well plates were heated by

floatation in a 35 °C water bath during the incubation periods. The increase in temperature ensured complete mixing of the DMSO and PBS. Without heating two layers would form and the dendrimer would remain in the DMSO layer while the Con A was in the PBS layer and no inhibition was seen. Although the revised procedure worked for dendrimers **8d-e**, the higher loadings of mannose, it did not work well for dendrimers **8f-g**. These results are shown in Table 3.2 below.

Table 3.2 HIA results for dendrimers **8d-g** done in 1:1 DMSO:PBS.

8	# sugars	Relative activity/sugar	18	Relative activity/sugar
d	30	261 ± 115	d	256 ± 51
e	15	360 ± 41	e	124 ± 8
f	6	N.A.	f	31 ± 27
g	3	N.A.	g	N.A.

Results for dendrimers **8f-g** were unobtainable. This is contributed to the low solubility in any water solution regardless of cosolvent. The wells in the plate containing solutions of **8f-g** were cloudy by the end of the assay, indicating that the dendrimer was no longer in solution. The lack of solubility made it impossible to obtain these results. The results for dendrimers **8a-e** are comparable with previous results as shown in Tables 3.1 and 3.2. Note that the relative activities/sugar are different in some cases from those obtained for **18a-f**. The assay does not measure binding constants directly and allows for errors of difference such as two different people determining whether inhibition of hemagglutination occurred. It was concluded that the presence of a spin label does not

affect the binding between mannose and Con A. All experiments indicated that affinity chromatography on dendrimers **8a-g** would separate the components of the dendrimer solutions that partake in binding without any interaction from the spin label.

Affinity Chromatography Rational

Affinity chromatography was employed in order to separate the components of the **8a-g** and determine if the relative binding seen in the Hemagglutination assays was due to all molecules having the same affinity toward Con A or a combination of molecules with a combination of affinities toward Con A. There are several scenarios that could result in the HIA results seen. The most probable scenarios include: most or all of the material is binding with the same affinity to Con A; some of the material is binding weaker than other parts of the material; some of the material is binding strongly while other parts of the material are not binding at all; and lastly, some material is binding strongly, some weakly and some not at all. The parts of the solutions will elute off the column at different rates based on affinity. Parts of the solution with a higher affinity towards Con A will retain on the column longer than parts with a lower affinity. Parts with no affinity towards Con A will elute from the column immediately.

Affinity columns were completed on **8a-g** in triplicate. Since **8d-g** are not water soluble the columns were completed in two different solvents. Dendrimers **8a-c** were done in PBS buffer solutions in the same manner previously reported in the literature³⁶⁻³⁷ while **8d-g** were completed using DMSO as the solvent instead of PBS buffer.

Affinity Chromatography in Water

The affinity columns were done using a Con A attached to sepharose 4B gel with procedures the same as or similar to those previously reported.³⁶⁻³⁷ A detailed procedure is given in the experimental section; however, a brief procedure will be given here. Approximately 1 mL of Con A bound sepharose 4B was added to a 1 mL disposable syringe with a glass wool plug. The sepharose was eluted with 10 mL of PBS buffer prior to addition of the dendrimer solution. After the column was equilibrated, 100 μ L of a 30.0 mg/mL solution of one of **8a-c** was added to the top of the gel. The column was eluted with 20 1 mL fractions collected with PBS and 10 1 mL fractions collected with 0.1 M methyl mannose in PBS. The methyl mannose was used in relatively high concentration to elute any bound material from the column. After the fractions were collected they were desalted by a membrane purification technique against water and lyophilized to constant mass. The fractions that contained mass after this process were dissolved in DMSO to make a 12.5 mM solution in endgroups and analyzed by EPR. Analysis of the fractions by comparison of the EPR spectra to the starting material EPR spectra was completed. Also, the mass recovered from the column was compared with the mass added to the column.

Results of Affinity Chromatography in Water

The results of affinity chromatography on dendrimers **8a-c** were all the same. The majority of the mass was eluted between 21 and 22 mL for dendrimers **8a-c**. The

mass that was eluted at 21-22 mL was all or most of the total mass off the column as well as all or most of the mass expected off the column. Since 100 μ L of a 30.0 mg/mL sample was placed on the column, 3.0 mg was expected to be eluted from the column. In all the columns, approximately 3.0 mg was eluted, however obtaining an exact mass of 3.0 mg over 30 samples was challenging. Table 3.3 shows the mass eluted for dendrimers **8a-c**.

Table 3.3 Eluted masses of affinity columns on **8a-c** over 3 trials.

8	mL eluted trial 1	mg obtained trial 1 ^a	mL eluted trial 2	mg obtained trial 2 ^a	mL eluted trial 3	mg obtained trial 4 ^a
a	3&4	0.3	18	0.4	21	0.5
	7&8	0.8	22	1.9 ^b	22	1.6 ^b
	22	<u>2.0^b</u>	23	0.4	25	0.2
	Total mass	3.1	24	1.0	26	0.4
			29	0.2	27	<u>0.6</u>
			30	<u>0.3</u>	Total mass	3.3
			Total mass	4.2		
b	9&10	0.5	9&10	0.9	22	0.9 ^b
	16	0.2	15	0.2	28	0.2
	22	0.7 ^b	22	0.6 ^b	30	<u>0.2</u>
	23	0.6 ^b	28	0.3	Total mass	1.3
	28	<u>0.4</u>	29	<u>0.3</u>		
	Total mass	2.4	Total mass	2.3		
c	3&4	0.1	22	1.0 ^b	22	1.7 ^b
	5&6	0.3	23	0.1	23	0.1
	7&8	0.2	24	0.3	24	0.2
	9&10	0.5	25	0.4	26	0.4
	11	0.2	27	0.9	27	0.3
	12	0.1	28	<u>0.2</u>	28	0.2
	22	1.0 ^b	Total mass	2.9	30	<u>0.1</u>
	23	0.3			Total mass	3.0
	24	0.2				
	25	<u>0.1</u>				
	Total mass	3.0				

^aThe expected total mass off was 3.0mg.

^bOnly these fractions were EPR active.

A few minor mass fragments were obtained for other fractions that appeared to be random. These fragments were often not enough for an EPR sample to be taken, but the samples that were tested via EPR spectroscopy did not show activity. MALDI-TOF MS was attempted on a few of the small samples but no signal could be found. Conditions for the MALDI-TOF MS are particular and it is impossible to know whether no signal was seen because there was no large molecule present or because the conditions for ionization were not correct. It is possible that there was some dendrimer sample eluted at different affinity towards the Con A but that could not be confirmed. Other possibilities for this mass was that there was some salt or methyl mannose (between 20-30 mL) that was not removed during the purification process, that there was a contaminant in the vial or that the mass was off slightly due to variations in temperature or humidity. Even a particle of dust would be enough to observe a mass.

In addition to the mass eluted from the column, the EPR spectra before and after the column was studied. Since the majority of the mass came off at the same volume for samples **8a-c** over three trials, this material was the primary focus for study. If the EPR spectra after the column looked significantly different from the starting material then it is likely that there is another conformation in the solution that contributes to the EPR signal and probably the HIA. As shown in Figure 3.3 the EPR spectra before and after the column are close to identical.

The most likely reason for the post column spectra being slightly sharper is that a few radicals may have been quenched during the course of the separation. This is not surprising given the environment of the columns. Free radicals are sensitive to light and

protic solvents. In DMSO, a solvent cage is formed and the spin label is fairly stable to protonation. However, the solvent used for the columns was water, a protic solvent. The samples were kept frozen when not in use; when the experiment commenced, the samples were used as quickly as possible. Also, **8a-c** was stored and worked with in the dark. The A/B peak height ratios indicated little differences in the spectra before and after the affinity columns as shown in Table 3.4.

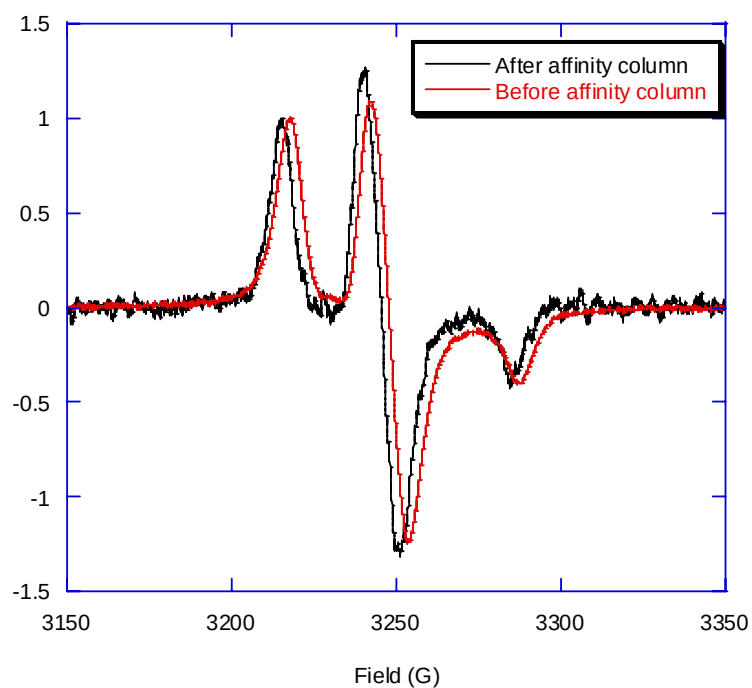


Figure 3.2 EPR spectra of **8c** before and after an affinity column.

Table 3.4 A/B peak height ratios for **8a-c** before and after affinity chromatography.

Sample (mL) ^a	A/B Peak Height Before column	A/B Peak Height After Column	A/B Peak Height After Column	A/B Peak Height After Column
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		Trial 1 mL A/B ratio		Trial 2 mL A/B ratio		Trial 3 mL A/B ratio	
8a	0.7675 (0.7966 for trial 1)	22	0.7700	22	0.7851	22	0.7315
8b	0.7855	22&23	0.7638	22	0.7661	22	0.6879
8c	0.9183	22	0.7940	22	0.8778	22	0.9130

^aOnly samples in which there was an EPR signal are shown in this table. For a complete list of where mass came off see Appendix B.

The results show that all the recovered material binds equally to the Con A. The data obtained indicates that there is not a mixture that contributes differently to the binding activity of Con A seen in the HIA. The results are promising; however, the data for the remaining part of the spectrum is necessary in order to make any general conclusions. Due to lack of solubility in water, affinity columns on dendrimers **8d-g** were conducted in DMSO.

Affinity Chromatography in DMSO

The procedure for affinity chromatography in DMSO was only slightly different. The DMSO was used without further purification of any added ions. The columns were washed with PBS buffer prior to equilibration with DMSO. The sepharose was found to be soluble in DMSO, so to retain the sepharose a piece of 50 kDa. molecular weight cut off (MWCO) dialysis tubing was attached to the bottom of the column. This allowed the dendrimer to pass through the dialysis tubing, but not the higher molecular weight Con A bound sepharose. More material than expected was obtained from the column. This was the first indication that there was something wrong with the column. Table 3.5 shows the average mass eluted at various volumes for **8d-f**.

Table 3.5 Eluted masses of affinity columns on **8d-g** over 3 trials.

8	mL eluted trial 1	mg obtained trial 1	mL eluted trial 2	mg obtained trial 2	mL eluted trial 3	mg obtained trial 3
d	1	1.0	1	1.7	1	2.0
	2	1.4	2	0.6	2	2.8
	3	2.0	5	2.9	3	0.9
	8	1.4	7	1.6	4	10.1
	19	0.1	10	1.4	5	4.1
	20	0.6	20	3.6	6	0.9
	21	0.1	21&22	1.0	7	0.2
	22	0.2	23&24	5.9	8	2.4
	23	0.1	27&28	<u>0.2</u>	9	0.4
	24	0.2	Total mass	18.9	21&22	3.1
	25	0.8			23&24	<u>0.1</u>
	26	0.8			Total mass	27.0
	27	<u>1.3</u>				
	Total mass	10.0				
e	1	1.2	1	1.8	1	1.3
	12	0.1	2	1.7	2	1.5
	14	1.2	3	0.8	8	0.7
	19	0.3	4	0.4	15	<u>0.5</u>
	21&22	<u>0.1</u>	5	0.1	Total mass	4.0
	Total mass	2.9	6	0.6		
			8	0.2		
			9	1.0		
			10	0.2		
			12	0.3		
			15	2.0		
			16	2.3		
			17	0.1		
			18	2.2		
			19	0.1		
			20	0.2		
			23&24	0.1		
			25&26	<u>0.1</u>		
			Total mass	14.2		
f	3	1.2	1	0.8	1	2.8
	4	2.7	2	1.4	2	1.0
	6	1.1	3	0.4	6	0.2
	7	1.8	4	0.1	15	0.2
	8	1.6	6	0.1	19	<u>0.1</u>
	9	<u>1.9</u>	7	0.7	Total mass	4.3
	Total mass	10.3	8	0.2		

			9	0.1		
			11	0.6		
			12	1.0		
			13	1.1		
			14	0.4		
			15	0.2		
			16	0.5		
			17	0.5		
			18	0.3		
			19	0.2		
			20	<u>4.2</u>		
			Total mass	12.8		
g	1	0.7				
	2	2.5				
	3	1.1				
	4	0.5				
	8	0.7				
	11	0.1				
	13	0.3				
	14	0.2				
	19	<u>0.3</u>				
	Total mass	6.4				

The results of affinity chromatography on **8d-g** were disastrous. First, the mass off was always too high compared to what was expected. The presence of white or yellow solid around the side of the vials indicated that there was material in the vials. Secondly, the dendrimers were eluted from the column in the first 10 mL. This was not expected given the results obtained for **8a-c** and for the HIA on dendrimers **18d-f**. Nonetheless, the EPR spectra were consistent with the pre-column spectra. The results of affinity chromatography are show in Table 3.6.

Table 3.6 Affinity Chromatograph results for **8d-g**.

Sample (mL) ^a	A/B Peak Height Before Column	A/B Peak Height After Column Trial 1 mL A/B ratio		A/B Peak Height After Column Trial 2		A/B Peak Height After Column Trial 3	
8d	1.1212	1-3	1.0585	1-2	1.0753	1-3 4-6	1.0695 0.9572
8e	1.2935	1 14	1.2608 1.207	1-3 4-6 8-10	1.2192 1.1717 1.1409	1-2	1.2160
8f	1.1866	3-4 6-7	1.2806 1.2692	1-2 6-7 11-12	1.3235 1.1437 1.1312	1 2	1.3288 1.2883
8g	1.2602	1-2 3-4 8	1.2567 1.6593 1.1074				

^aOnly samples in which there was an EPR signal are shown in this table. For a complete list of where mass came off see Table 3.5.

None of the affinity chromatography data in DMSO was useful while the data in water was useful. This indicated that the DMSO was the problem with obtaining useful data. In order to examine this hypothesis, two experiments were run. First, DMSO was run through an affinity column, followed by the normal PBS buffer used to equilibrate the column in the water method. It was found that most of the sepharose had dissolved and washed through the column. The sepharose bound Con A is too large to go through the 50 kDa dialysis tubing. The second experiment consisted of using DMSO to run an affinity column on **8b** and compare the results obtained with those in water. The results showed that the dendrimer was eluted from the column immediately along with the unexplainable extra mass.

These two experiments showed that affinity chromatography for this system does not work in DMSO. The most probable cause for failure in DMSO is that Con A is

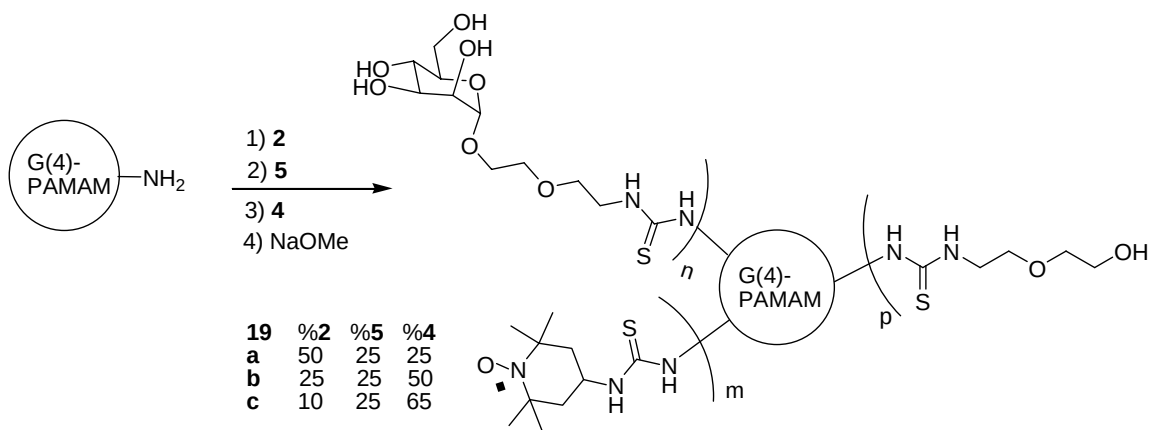
denaturing. If no Con A is present (because it was denatured) then the mannose on the dendrimers can not bind to the Con A and will pass through the column immediately. Also, the denaturing of Con A in DMSO would lead to small particles eluting from the column, through the 50 kDa plug. This would explain the extra mass obtained from the DMSO columns.

The data obtained from affinity chromatography on **8d-g** does not allow us to draw any meaningful conclusions. In order to make conclusions about the complete range of percent mannose loadings, the entire range needs to be studied. The major problem is the need to make the functionalized dendrimers water soluble. Since it is known that too much TEMPO creates a water insoluble molecule, less TEMPO would be needed on the surface of the dendrimer. In order to mimic the other studies all of the endgroups need to be functionalized, otherwise more flexibility could lead to different results. With this in mind, a series of tri-functionalized dendrimers were synthesized and studied via EPR, HIA and affinity chromatography.

Synthesis of tri-Functionalized Dendrimers

The synthesis of tri-functionalized dendrimers came about in search of greater water solubility. The dendrimers needed to be fully functionalized to most closely mimic the HIA studies done previously in the Cloninger research group; however, there could not be any more than 25 percent loading of TEMPO or the molecules would not be water soluble. The isothiocyanate **4** was chosen to be the third functional group on the dendrimer. It has been previously shown to increase water solubility in some

functionalized dendrimers and was readily available. Dendrimers were functionalized with 25 % **5** and 50, 25 or 10 % **2** while the remaining 25, 50 or 65 % of the endgroups were functionalized with **4** as shown in Scheme 3.1.



Scheme 3.1 Synthesis of tri-functionalized dendrimers **19a-c**.

The synthesis of **19a-c** was completed in the same manner as **8a-g**. Solutions of functional groups **2**, **5**, and **4** (25 mM, DMSO) were added sequentially to a stock solution of the G(4)-PAMAM dendrimer (1 mL, 25 mM in endgroups) in DMSO so that the total volume of the functional group solutions was equal to 1 mL. The amount of each functional group added was controlled to give the desired percent loading ratios. There was a 48 h reaction time between the addition of one functional group and the next. The reactions were monitored using MALDI-TOF MS as shown in Figure 3.5.

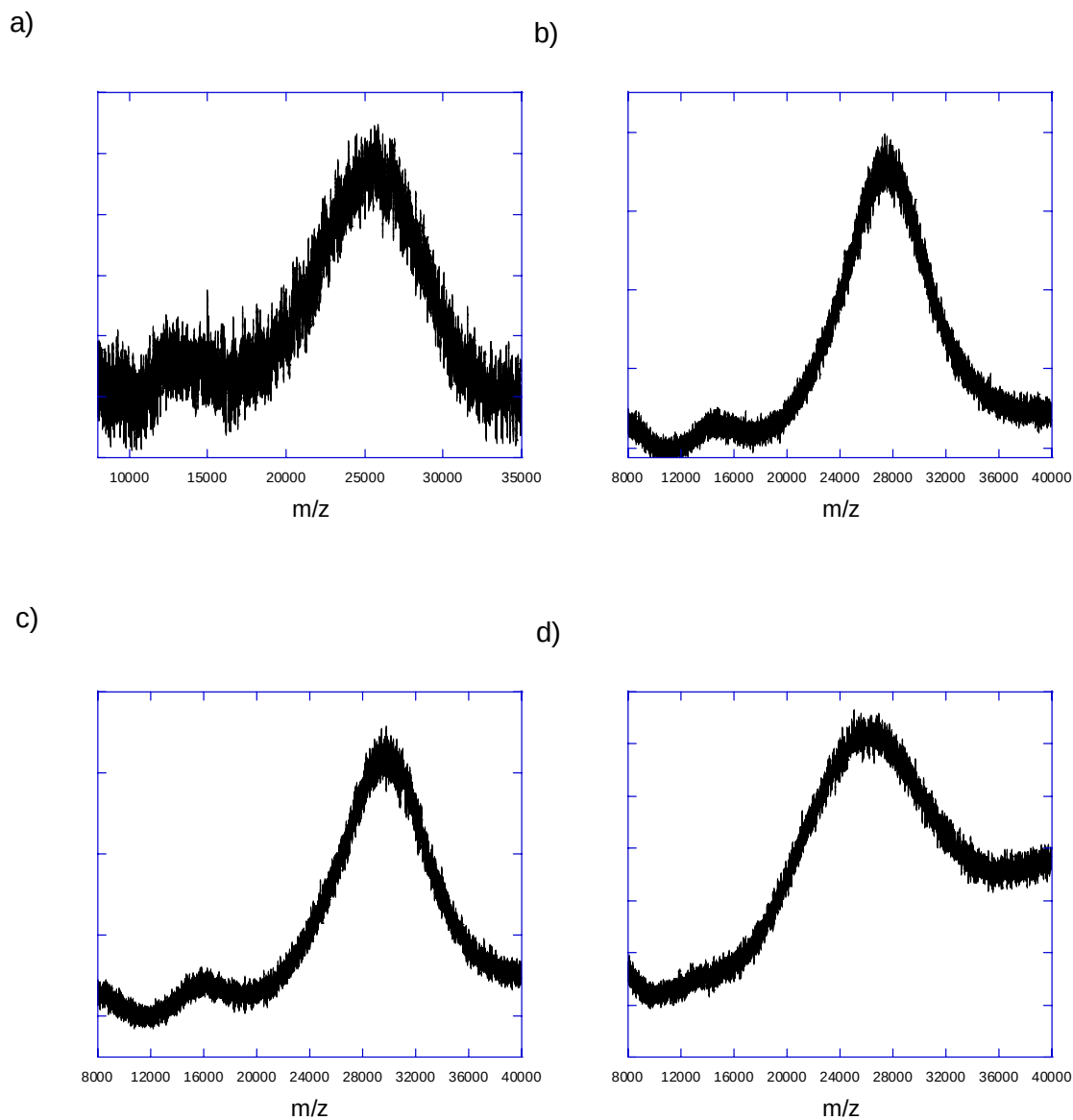


Figure 3.3 MALDI-TOF spectra for **19a** after addition of **2** (a), **5** (b), **4** (c) and NaOMe (d).

After functionalization was complete, 0.3M NaOMe in MeOH solution (1 equiv. per sugar) was added to remove the acetyl groups from the hydroxyls. A sample was removed for EPR and the remaining solution was purified with Amnicon filtration

centrifugation tubes (5 kDa MWCO) by slowly substituting water for DMSO. The products were lyophilized to dryness and found to be water soluble.

EPR analysis of the products indicated that the A/B peak height ratios were 0.9413, 0.9893 and 0.7824 for **19a**, **19b** and **19c** respectively. This is as expected for 25% loading of TEMPO. With water soluble variations of functionalized G(4)-PAMAM dendrimers with 50, 25 and 10 % mannose on the surface, affinity studies could be performed.

HIA on tri-Functionalized Dendrimers

Before affinity chromatography was to be employed, HIA analysis was obtained. This data was to ensure that the results were consistent with previous results. There was no reason to believe any inconsistency would exist, given that the spin label had already been shown to not affect the affinity towards Con A and the ethoxyethanol linker **4** was used in previous studies. The results of HIA on **19a-c** are shown in Table 3.7.

Table 3.7 Results of HIA on **19a-c**.

19	# sugars	Ave act/sugar	18	Ave act/sugar
a	30	206 ± 103	d	174 ± 44
b	15	111 ± 45	e	120 ± 44
c	6	N.A.	f	31 ± 27

As seen in Table 3.6, the HIA results for **19a** and **19b** are within reason to results found previously in the Cloninger research group. Results for **19c** could not be obtained due to lack of solubility. This dendrimer was soluble in water the first time it was

synthesized; unfortunately it was not soluble in the assay buffer. The material used for the HIA was synthesized separately from that used for affinity chromatography. As has been seen with other heterogeneously functionalized dendrimers in our group, solubility can vary for compounds synthesized separately. This could be due to a number of things such as differences in the number of endgroups on the dendrimer or lyophilizing too long. As can be seen in Table 3.6 the HIA results of **18f** show a lower activity/sugar than the dendrimers with more mannose residues. Dendrimer **19c** is likely to bind Con A with the equivalent binding activity of **18f**. The results on the HIA for dendrimers **19a** and **19b** were consistent enough with previous result to compare the HIA assays with the affinity chromatography results.

Affinity Chromatography on tri-Functionalized Dendrimers

The use of water soluble molecules ensured that the affinity chromatography data would be more reliable. With the molecules in hand, the studies were conducted the same as previous affinity chromatography columns in water. The results (shown in Table 3.7) indicate that for dendrimers **19a-b**, the affinities are consistent with the affinities of **8a-c**. The entire sample of EPR active material stayed on the column until eluted with methyl mannose, at which time all sample was removed. These results show that there is only one material contributing to the binding in the HIA studies. The complete data for the columns can be found in Table 3.7. Table 3.8 gives the A/B peak height ratios result for **19a-c** where EPR activity was found.

Table 3.8 Eluted masses of affinity columns on **19a-c** over 3 trials.

19	mL eluted trial 1	mg obtained trial 1 ^a	mL eluted trial 2	mg obtained trial 2 ^a	mL eluted trial 3	mg obtained trial 3 ^a
a	1	0.3	1&2	0.2	1&2	1.0
	13&14	1.2	3&4	0.1	5&6	0.7
	15&16	0.3	5&6	0.2	7&8	0.8
	19&20	0.2	7&8	0.2	13&14	1.2
	22	2.3 ^b	22	2.7 ^b	15&16	0.4
	23	0.4	23	0.1	22	2.9 ^b
	26	0.4	24	0.9	23	1.2
	30	<u>0.1</u>	25	0.8	24	0.6
	Total mass	5.2	27	0.2	25	1.4
			30	<u>0.1</u>	26	0.6
			Total mass	5.5	27	0.2
					28	0.1
					29	0.4
					30	<u>0.5</u>
					Total mass	12.0
b	21	0.7 ^b	7&8	0.2	5&6	0.1
	22	<u>1.0^b</u>	9&10	0.3	9&10	0.1
	Total mass	1.7	13&14	0.2	17&18	0.2
			15&16	0.2	21	0.3
			17&18	0.3	22	1.7 ^b
			21	0.6 ^b	24	0.1
			22	0.2 ^b	26	0.1
			23	0.1	27	0.1
			24	0.2	28	0.4
			25	0.5	30	<u>0.2</u>
			27	0.2	Total mass	3.3
			28	0.1		
			29	<u>0.2</u>		
			Total mass	3.3		
c	1	0.5 ^b	1	0.4 ^b	1	0.6 ^b
	3	0.6	2	1.2 ^b	2	0.8 ^b
	5	0.2	3	0.3	3	0.7
	7	0.2	7	0.1	4	0.5
	9	0.4	8	0.2	5	0.3
	11	0.1	9	0.2	6	0.3
	13	0.2	10	0.1	7	0.4
	15	0.1	11&12	0.2	11&12	0.4
	17	0.2	13&14	0.1	13&14	0.5
	21	0.2	15&16	0.1	15&16	0.5
	23	0.1	17&18	0.1	25&26	<u>0.5</u>

	27 Total mass	<u>0.1</u> 2.9	23&24 27&28 Total mass	0.2 <u>0.1</u> 3.3	Total mass	5.5
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^aThe expected total mass off was 3.0mg.

^bOnly these fractions were EPR active.

Table 3.9 Affinity chromatography results for dendrimers **19a-c**.

Sample (mL) ^a	A/B Peak Height Before column	A/B Peak Height After Column Trial 1	A/B Peak Height After Column Trial 2	A/B Peak Height After Column Trial 3	Ave A/B Ratio
19a (22)	0.9413	0.83324	0.75465	NA	0.79395
19b (22)	0.9893 (21-22 mL)	0.79073 ^b	0.98805 (21-22 mL)	1.0180	0.93226
19c (1&2)	0.7824 (1 mL only)	0.96724	0.73004	0.95402	0.8504

^aOnly samples in which there was an EPR signal are shown in this table. For a complete list of where mass came off see Table 3.3.

^bThe 21 mL sample had low EPR activity so an A/B ratio could not be determined.

The results for dendrimer **19c** are slightly different. Again since all the material is eluted at the same volume it appears that there is no mixture of higher and lower affinity molecules; however, the entire sample comes off the column in the first 1 -2 milliliters. This indicates that there is little or no binding with the Con A. Given that **19c** has only a 10% loading of mannose, which is 6 mannose residues per dendrimer, it is not surprising that less binding is seen. This is consistent with the HIA result seen for **18f**.

Summary

The results of the affinity studies indicate that the solutions are composed of one material that has a consistent affinity towards Con A. In the first part of this chapter, HIA results showed that incorporating a spin label into the heterogeneously functionalized

dendrimer resulted in no change in activity towards Con A. Next, affinity chromatography was employed to separate any mixtures that might have been contained in the solutions and their individual conformations were studied using EPR. It was found that **8a-c** consisted of material with a single affinity towards Con A. Unfortunately, **8d-g** were not able to provide any useful information about mixtures of components. This was due to lack of water solubility and the inability to perform affinity chromatography in non-aqueous media.

In the last half of the chapter, tri-functionalized dendrimers **19a-c** were synthesized and found to be water soluble. From HIA and affinity chromatography studies with **19a-c**, it was found that these dendrimers also consist entirely of material with the same affinity towards Con A. The combination of affinity chromatography from **8a-c** and **19a-c** indicated that there is only one material giving the HIA results seen previously in the Cloninger research group.

Experimental Procedures

General reagents were purchased from Sigma-Aldrich or Acros Organics. G(4)-PAMAM dendrimer was purchased from either Sigma-Aldrich or Dendritech Inc. The trans-3-indoleacrylic acid was purchased recrystallized from warm absolute ethanol. Concanavalin A was purchased from Calbiochem. Con A bound sepharose was purchased from Sigma-Aldrich. All other reagents were used as supplied.

Phosphate buffered saline (PBS), pH 7.2 (quantities for 4 L), consisted of 0.15 M NaCl (35 g), 0.007 M NaH₂PO₄ (3.86 g of dihydrate), 0.018 M Na₂HPO₄ (10.22 g of anhydrous or 19.3 g of heptahydrate).

The MALDI-TOF MS procedures are the same as described in the experimental section of CHAPTER TWO (pg 51).

Hemagglutination assay procedures are given in appendix B.

The general procedures using the affinity chromatography in water is as follows. A 1 mL disposable syringe with a glass wool plug was used as the column. A second 1 mL syringe was placed on top of the first and connected with Parafilm™ to hold the buffer. First the column was packed by running 1 – 2 mL of PBS buffered saline through the column and then slowly pipetting the Con A sepharose gel into the column so that the gel was on the 1 mL mark of the syringe. The column was wrapped in aluminum foil to avoid light reaching the sample (as the spin label is light sensitive). The column was washed with 10 mL of PBS buffer to equilibrate the column. The PBS was allowed to pass through and the column upon which 100 µl of sample (approximately 30 mg/mL in PBS) was added to the column via syringe. PBS buffer (20 mL) was run through the column while 1 or 2 mL fractions were collected. For the affinity columns performed on **8a-c**, 1 mL fractions were collected. For the affinity columns performed **19a-c**, 2 mL fractions were collected. After the 20 mL of PBS had run through the column, 10 mL of 0.1 M methyl mannose in PBS was run through the column while 1 mL fractions were collected. The fractions were desalted by either dialysis against water (MWCO = 3.5 kDa.) or via Amnicon centrifugation tubes with water (MWCO = 5 kDa.). After the

samples were desalted and the methyl mannose was removed the samples were transferred to vials that had been lyophilized to constant mass and lyophilized to constant mass. The fractions that contained enough mass to obtain an EPR spectrum were dissolved in DMSO to make a 12.5 mM solution of endgroups and scanned using EPR by Karl Sebby. Up to 4 continuous fractions were combined to make an EPR sample if needed.

The general procures using the affinity column in DMSO is as follows. A 1 mL disposable syringe with a glass wool plug was used as the column. A second 1 mL syringe was placed on top of the first and connected with Parafilm™ to hold the buffer. A small piece of 50 kDa MWCO dialysis tubing was attached to the bottom of the column in an attempt to keep the sepharose in the column. The column was packed by slowly pipetting the Con A sepharose gel into the column so that the gel was on the 1 mL mark of the syringe. The column was wrapped in aluminum foil to limit light exposure of the sample (as the spin label is light sensitive). The column was washed with 10 mL of DMSO to equilibrate the column. The DMSO was allowed to pass through the column upon which 100 µl of sample (approximately 30 mg/mL in PBS) was added via syringe to the top of the column. DMSO (20 mL) was run through the columns while 1 mL fractions were collected. After the 20 mL of DMSO had run through the column, 10 mL of 0.1 M methyl mannose in DMSO was ran through the column while 1 mL fractions were collected. The first 20 fractions were transferred to vials which had been lyophilized to a constant mass and lyophilized to a constant mass. In the last 10 fractions methyl mannose was removed via Amnicon centrifugation tubes with DMSO

(MWCO = 5 kDa.). After the methyl mannose was removed, the samples were transferred to vials that had been lyophilized to constant mass and lyophilized to constant mass. The fractions that contained enough mass to obtain an EPR spectrum were dissolved in DMSO to make a 12.5 mM solution of endgroups and given to Karl Sebby for EPR. Up to 4 continuous fractions were combined to make an EPR sample if needed.

The synthesis of the tri-functionalized dendrimers is as follows. The synthesis of the heterogeneously functionalized dendrimers (**19a-c**) is carried out with a stock solution of G(4)-PAMAM in DMSO (1 mL, 25 mM in endgroups). Aliquots of a solution of **5** (25 mM), **2** (25 mM) and **4** (25 mM) in DMSO are added to the dendrimer solution sequentially in a specific ratio (total added volume = 1 mL). The second reactant is added 48 h after the first and the third reacted 48 h after the second. The functionalization of each reactant is confirmed by analysis of the MALDI-TOF spectrum. The mixture is allowed to react for an additional 48 h after the third reactant is added. Completion is again determined through the analysis of the MALDI-TOF spectrum. After the three functional groups have fully added to the dendrimer solution, NaOMe is added so that there is 1.0 equivalent per mannose residue. The reaction is allowed to sit for 48 h and again checked with MALDI-TOF MS. Water is added to the mixture to quadruple the volume of the solution. The solutions were transferred to Amnicon centrifuge filters (MWCO = 5 kDa.) and purified with water. The samples are lyophilized to dryness and ready to use. The exact amounts are show in Table 3.10.

Table 3.10 Amount used in reactions to form tri-functionalized dendrimers.

19	mL G(4)	μ l 5	% 5	μ l 2	% 2	μ l 4	% 4

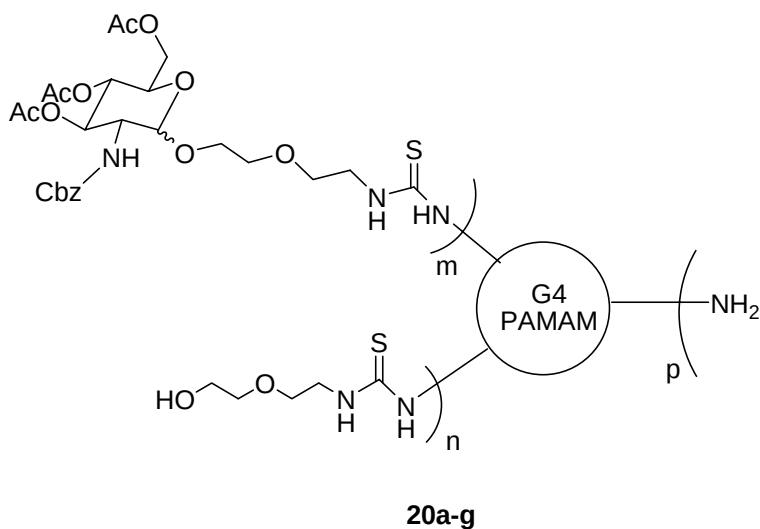
a	1 mL	244	25	500	50	250	25
b	1 mL	244	25	250	25	500	50
c	1 mL	244	25	100	10	650	65

CHAPTER FOUR

SYNTHESIS OF HETEROGENEOUSLY FUNCTIONALIZED GLUCOSE DENDRIMERS.

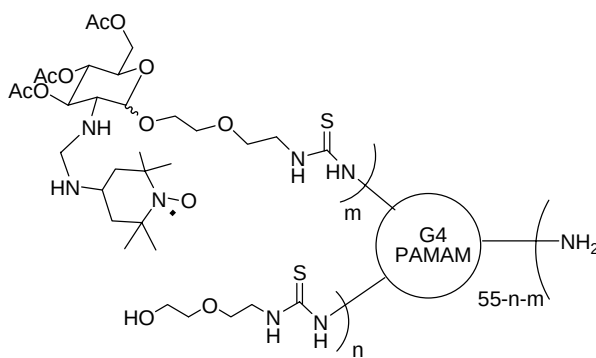
Background

In addition to the synthesis of heterogeneously functionalized dendrimers **6a-g**, **7a-g** and **8a-g** and **19a-c**, a series of heterogeneously functionalized generation 4.0 PAMAM-based thiourea-linked ethoxy ethanol and 1-O-(5-thiourea-3oxapentyl)-3,4,6-tri-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucoside dendrimers (**20a-g**) were synthesized.



Originally the intention was to remove the benzyloxycarbonyl group and attach TEMPO derivative **5** directly to the carbohydrate to make **26a-g**. In previous studies the relative

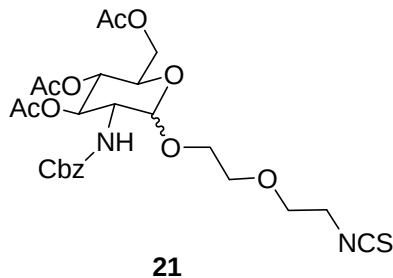
locations of the non-EPR active functional group was deduced from the relative locations of the spin-labels as discussed in CHAPTER TWO. Upon having a spin-label directly attached to the carbohydrate, EPR could be used to determine the relative locations of the carbohydrates directly. Unfortunately, the benzyloxycarbonyl group could not be removed after several attempts as will be discussed later in this chapter. This halted the synthesis of **20a-g**.



26a-g

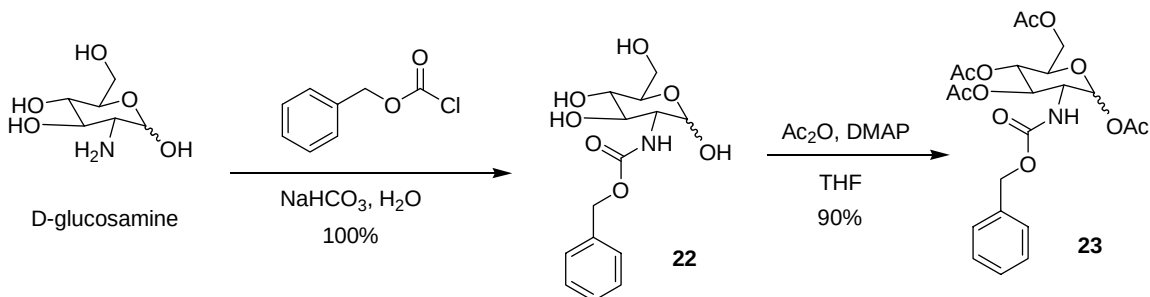
Synthesis of 1-O-(5-isothiocyaato-3-oxypentyl)-2-(amino-benzyloxycarbonyl)-2-deoxy-3,4,6-tri-O-Acetyl-D-glucoside (**21**)

Before dendrimer functionalization, the glucosamine derivative **21** was synthesized. The isothiocyanate **4** was then reacted with the surface of the dendrimer to form a thiourea linkage in the same manner as **6a-g** and **7a-g**. The synthesis of **21** described below was completed similar to the synthesis of **2**.



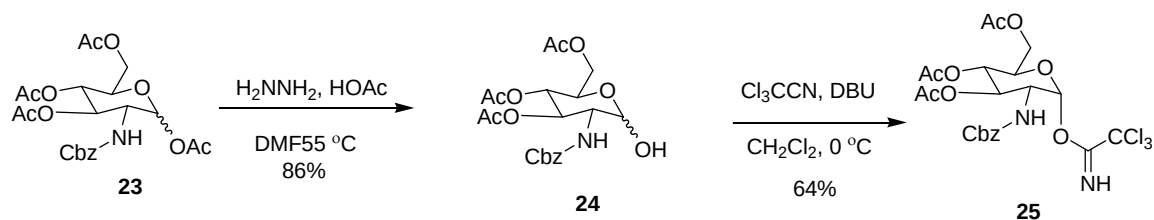
The synthesis of **21** began by reacting D-glucosamine hydrochloride with benzyl chloroformate and NaHCO_3 in water at $0\text{ }^\circ\text{C}$. The reaction was allowed to warm to r.t. and the white crystalline solid 2-(N-benzyloxycarbonylamino)-2-deoxy-D-glucosopyranoside (**22**) precipitated and was collected by filtration in close to quantitative yield. The product formed was difficult to characterize due to the presence of α and β anomers at the anomeric carbon as well as rotomers of the carbamate C-N bond. The ^1H NMR peaks are broad and difficult to integrate. In the ^{13}C NMR spectra there are more carbon peaks than carbons in the molecule. Both these affects are contributed to the rotomeric and anomeric mixtures. High Resolution Mass Spectrometry proved useful by giving the proper molecular weight and an analytical sample was recrystallized to determine the melting point, which agreed with the published data.⁵¹ Next the alcohols were acetylated with excess acetic anhydride and a catalytic amount of DMAP in THF. The crystalline solid from the previous step did not appear soluble in THF; however, as the reaction progressed, all the material became soluble. The substrate **22** is barely soluble in THF and the acetylated product **23** is extremely soluble in THF. Complete solubility is used as the indication that the reaction is complete, usually 4 h. A high yield of 90% is obtained for the acetylation reaction. This reaction was originally

attempted in pyridine as had been successful in the analogous mannose case, but low yields were obtained due to lack of solubility. The first two steps in this sequence are shown in Scheme 2.1.



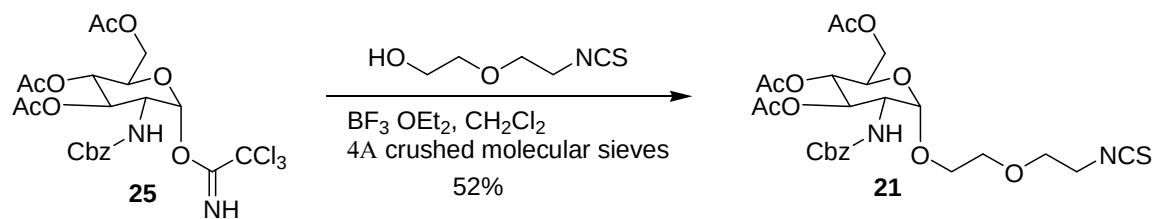
Scheme 4.1 Synthesis of 1,3,4,6-tetra-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranoside, **23** from D-glucosamine.

After all the hydroxyl groups were acetylated, the anomeric alcohol was selectively deprotected using hydrazine acetate in DMF at 55 °C for 30 min. An 86% overall yield of **24** was routinely accomplished in this reaction. The anomeric hydroxyl was converted into the trichloroacetimidate using trichloroacetonitrile in methylene chloride and in the presence of DBU at 0 °C. After 5 h, the solvent was removed in vacuo and the product was immediately purified by column chromatography to obtain a 64% yield of 1-O-(2-benzyloxyamido-3,4,5-tri-O-acetyl-2-deoxy- α -D-glucopyranosyl)trichloroacetimidate (**25**) as shown in Scheme 2.2.



Scheme 4.2 Synthesis of 1-O-(2-benzyloxyamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl)trichloroacetimidate, **25**, from **23**.

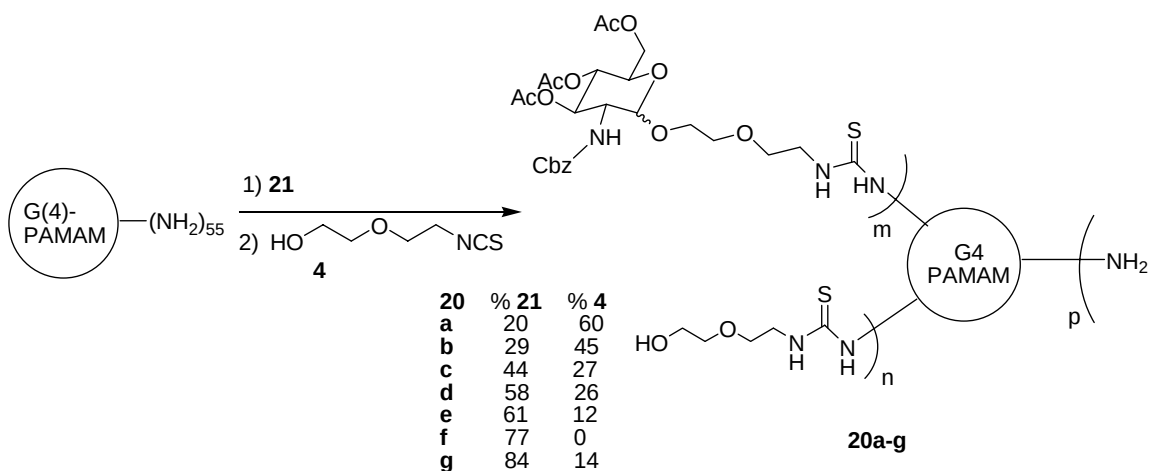
The final step in the synthesis was to couple the trichloroacetimidate **25** with isothiocyanate **4** as accomplished with the mannose derivative in CHAPTER TWO. The reaction was undertaken by the same conditions as in the final synthetic step of **2**; **23** and **4** dissolved in methylene chloride in the presence of $\text{BF}_3 \cdot \text{OEt}_2$ and crushed 4Å molecular sieves. Upon completion (8 h) the reaction was purified by silica gel chromatography to give a 52% yield of 1-O-(5-isothiocyanato-3-oxapentyl)- 3,4,6-tri-O-acetyl-2-benzyloxycarbonyl-2-deoxy-D-glucopyranoside (**21**). Although the yield was only moderate, it allowed for enough product formation to continue with dendrimer functionalization. The reaction is shown in Scheme 4.3. Upon completion of the synthesis of **21**, dendrimer functionalization was ready to commence.



Scheme 4.3 Synthesis of 1-O-(5-isothiocyanato-3-oxapentyl)- 3,4,6-tri-O-acetyl-2-benzyloxycarbonyl-2-deoxy-D-glucopyranoside, **21**, from **25**.

Dendrimer Functionalization

G(4)-PAMAM dendrimers were functionalized in the same manner as **7a-g** where the carbohydrate residue **21** was added first, followed by addition of **4** to complete dendrimer functionalization and form compounds **20a-g**. The benzyloxycarbonyl (Cbz) protecting group makes the carbohydrate more sterically hindered than the mannose of **2**. Because of the steric bulk, the reaction was slower and often incomplete. Compound **4** was added in excess; but, functionalization was usually not complete. As a result of this incomplete functionalization, there were remaining amines present on the dendrimer's surface. The synthesis of **20a-g** is illustrated in Scheme 4.4.



Scheme 4.4 Synthesis of heterogeneously functionalized generation 4.0 PAMAM-based thioura-linked ethoxy ethanol and 1-O-(5-thioureapentyl)-3,4,6-tri-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucoside dendrimers (**20a-g**).

The reactions illustrated in Scheme 4.4 had incomplete functionalization for each percent loading attempted. Desired amounts of **21** were reacted with the dendrimer first

and allowed to react until no more reaction occurred (15 – 20 days). The reactions were monitored by MALDI-TOF MS every 1-2 days. For the first 14 days the M_w of the dendrimer increased every 1-2 days, indicating that more functionalization was occurring. The usual increase was greater for the first 2-3 days and then equivalent to 1 or 2 of carbohydrate **21** adding to the dendrimer. After approximately 14 days no more increase was seen in the M_w over the course of 3-6 consecutive days. Heating the reaction to 35 °C resulted in no change in the outcome.

Once it was evident that reaction with **21** was complete, multiple equivalents of **4** were added to each reaction in an attempt to functionalize all the remaining amines. Again the reaction was allowed to react until no more functional groups reacted with the dendrimer surface as shown in the MALDI-TOF spectra. Unfortunately, some terminal amines remained unreacted after several days of reacting. The best explanation for the incompleteness of the reaction is steric hindrance due to the bulky Cbz group on the carbohydrate blocking reaction of **4** with the amine. Figure 4.1 is an example MALDI-TOF spectrum of **20a**.

The final three synthetic steps of the sequence were to remove the benzyl carbamate (Cbz) via hydrogenation, add TEMPO-NCS **5** to the resulting amine and to remove the acetyl groups on the alcohols of the carbohydrate. This is highlighted in Scheme 4.5. The last two synthetic steps were almost identical to previous reactions completed in the synthesis of **8a-g**. It is expected that TEMPO-NCS would add to the amine on the carbohydrate instead of on the dendrimer because the carbohydrate would be more accessible than the dendrimer. This could have resulted in a slow reaction due to

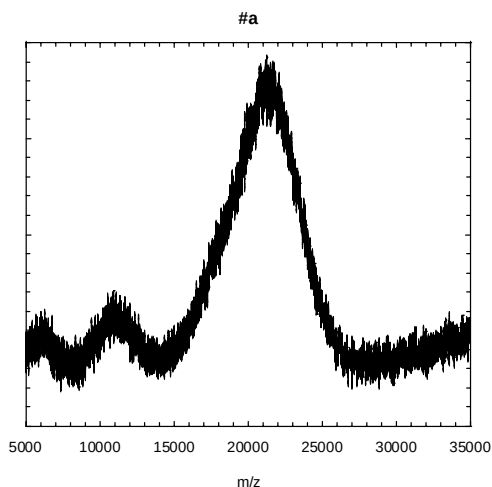
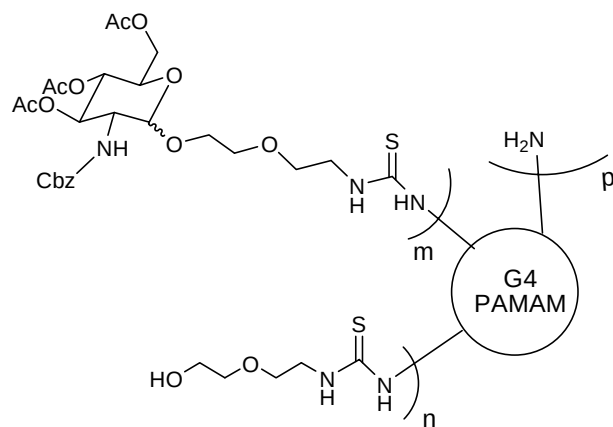
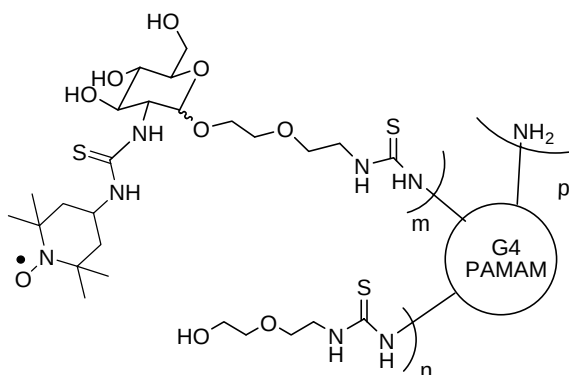
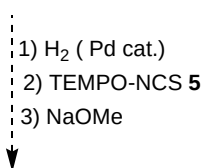


Figure 4.1 MADLI-TOF spectra of **20a**.

the fact that TEMPO-NCS is a secondary isothiocyanate and the carbohydrate amine was also secondary. Adding TEMPO-NCS to the primary amine of the dendrimer surface is slower than adding a primary isothiocyanate. It is still believed that the reaction would have worked, but possibly taken longer. Hydrolysis of the acetates with NaOMe would have been the last synthetic step. Although the carbohydrate was a glucose derivative as opposed to a mannose derivative there was expected to be no change in the outcome of the reaction.

Attempts to Remove the Cbz Protecting Group

The synthesis of **20a-g** was completed by the steps highlighted above. In the synthesis of **26a-g** the removal of the Cbz group was unsuccessful. The Cbz group is

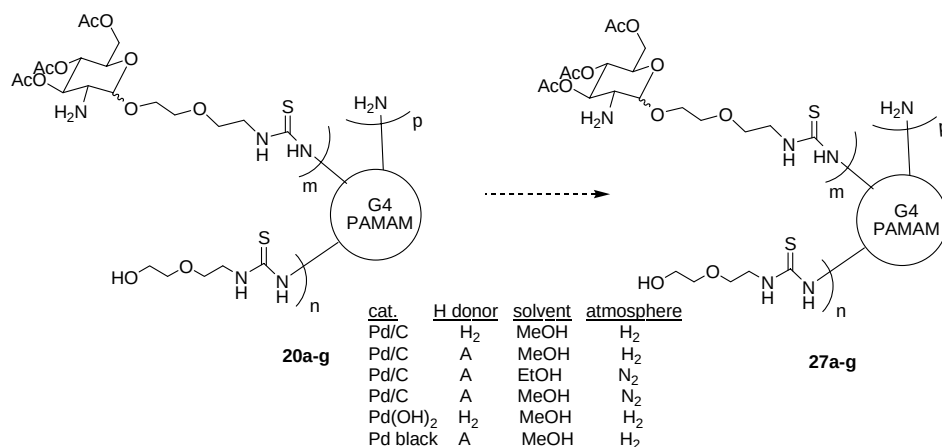
**20a-g****26a-g**Scheme 4.5 Proposed last three steps to form **26a-g**.

usually removed by hydrogenation. Unfortunately, the thiourea present in the reaction poisons the catalyst. The catalyst coordinates to the sulfur, rendering it inactive towards the hydrogenation. Since the dendrimers were heterogeneously functionalized with the carbohydrate **21** and the ethoxy ethanol **4** there was more than one sulfur per Cbz group.

Other typical conditions for removal of the Cbz group include the use of strong acids⁵² or bases⁵³; but they were not used because **20a-g** contain amide and thiourea bonds that degrade under strongly acidic and basic conditions and at elevated temperatures.

The first attempt at removing the Cbz group was through hydrogenation. The attempts at hydrogenation are highlighted in Scheme 4.6. Three different hydrogenation catalysts and a variety of conditions were attempted. The first catalyst was Pd/C, a common catalyst for hydrogenation. The reaction was stirred in methanol with solid Pd/C in the mixture under H₂ for several hours. Both MALDI-TOF MS and NMR indicated the presence of Cbz groups. The Cbz removal was then attempted by addition of 1,4-cyclohexadiene in ethanol with Pd/C. This reaction was done under both N₂ and H₂. The use of H₂ should not be necessary because 1,4-cyclohexadiene is a hydrogen donor, but was attempted nonetheless. The starting dendrimer **20a-g** was more soluble in methanol than ethanol so the reaction was also attempted with 1,4-cyclohexadiene in methanol under both H₂ and N₂. None of these conditions resulted in Cbz removal. Close to quantitative amounts of starting material were recovered in each reaction as characterized by MALDI-TOF MS and NMR.

Next, Pd(OH)₂/C was attempted as the catalyst for the reaction. After 18 h in methanol in the presence of Pd(OH)₂/C under H₂ no reaction had occurred. All of the above reactions with Pd/C and Pd(OH)₂ were attempted with huge excesses of catalyst, however no change in the outcome of the reaction occurred indicated by both MALDI-TOF MS and NMR.



Scheme 4.6 Attempts at removing the Cbz group on **20a-g** via hydrogenation.

The final catalyst attempted was Pd black. The literature indicated that it was possible to remove the Cbz group in the presence of sulfur with Pd black and 1,4-cyclohexadiene, but in low yield.⁵⁴ Pd black was used with 1,4-cyclohexadiene in methanol under H₂. In some cases MALDI-TOF MS indicated partial removal of the Cbz group and in other cases there was no indication of Cbz removal. An example MALDI-TOF spectrum is shown in Figure 4.2 where there was no indication of Cbz removal.

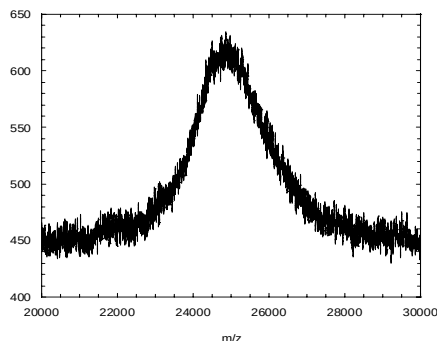


Figure 4.2 MALDI-TOF spectra of **20a** after attempted removal of the Cbz group with Pd black.

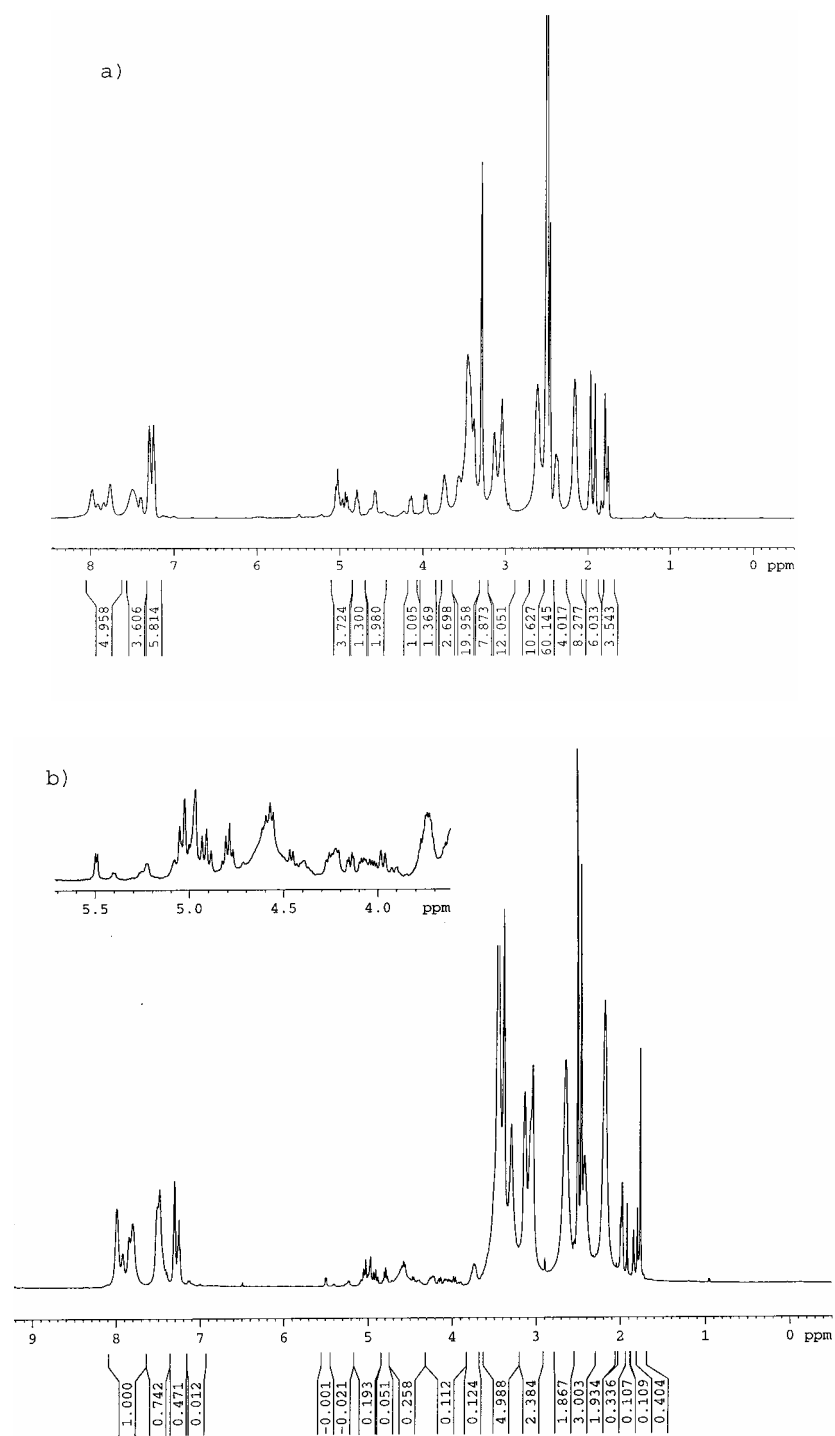
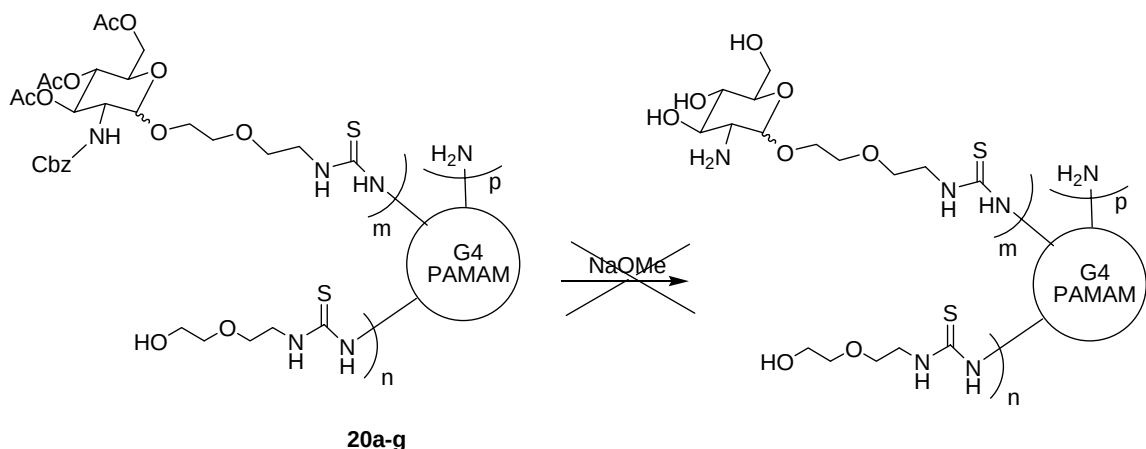


Figure 4.3 NMR spectra of **20a** a) before and b) after the attempted removal of the Cbz group with Pd black.

In a last resort three final attempts were made to remove the Cbz group. The first was through the addition of NaOMe as shown in Scheme 4.7.

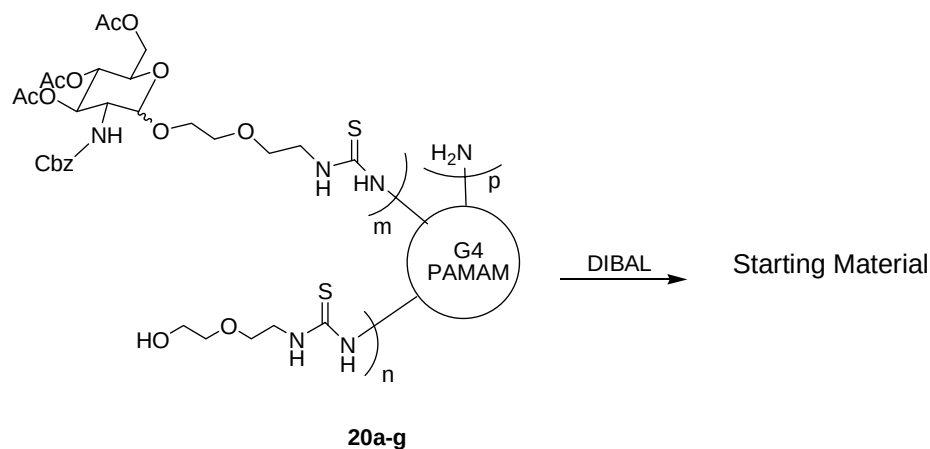


Scheme 4.7 Attempted removal of the Cbz group with NaOMe.

It was known that strong basic conditions would remove the Cbz group however no attempts at using NaOMe were found in the literature. The choice using NaOMe as the base was determined because it was known that at low enough concentrations of NaOMe, the dendrimer would not decompose. Although 2 equivalents of NaOMe were used per carbohydrate instead of the 0.9 equivalents for removal of the acetyl groups only destruction of the molecule occurred.

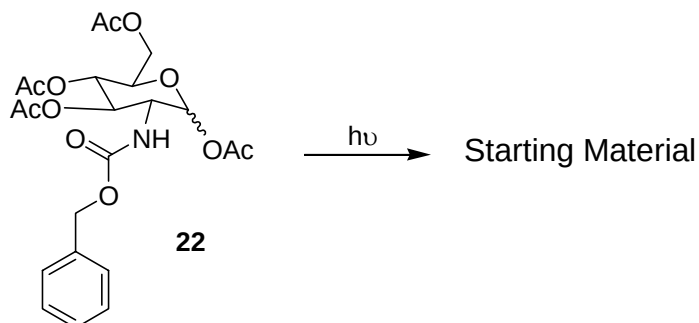
The second attempt at an approach other than hydrogenation was to reduce the carbamate to an amine using diisobutylaluminum hydride (DIBAL) as illustrated in Scheme 4.8. Two reactions were attempted with 0.9 and 4.5 equivalents of DIBAL. The reactions were performed in methylene chloride at -78 °C for 3 h, 0 °C for 2 h and r.t. for 17 h. Upon purification by dialysis, 60 and 48% of starting material was recovered for

0.9 and 4.5 equivalents of DIBAL used respectively. The NMR of the starting material and product are so similar that they can be perfectly overlaid.



Scheme 4.8 Attempted removal of the Cbz group using DIBAL.

The photolytic cleavage of the Cbz group was the last attempt to remove the protecting group. The reaction was attempted with the smaller derivative **22** as highlighted in Scheme 4.9 instead of the dendrimer. Given that the hydroxyls are protected the NMR is cleaner than when the deacetylated hydroxyls are present. The reaction was attempted in a 2:1 MeOH:H₂O solution in the presence of 254 nm light from a 450W medium pressure mercury lamp. Isolated product was 78% by mass of starting material with some decomposition product. There was no indication by NMR that any Cbz removal had occurred.



Scheme 4.9 Attempted removal of the Cbz group using $h\nu$.

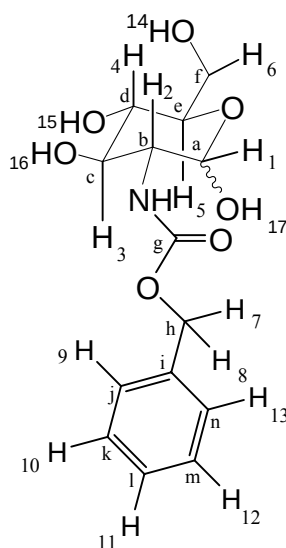
Summary

The synthesis of carbohydrate functionalized dendrimers with TEMPO residues attached to the carbohydrate was undertaken. Only functionalized dendrimers **20a-g** were successfully synthesized. In the final few steps the Cbz amine protecting group on the carbohydrate was unable to be removed. The synthesis of dendrimers **26a-g** would have allowed for further EPR studies on the locations of functional groups on the dendrimer's surface. Instead of having to infer the relative locations of the non-EPR active group, the relative locations would have been studied directly. The inability to remove the Cbz group made this study impossible. No further attempts to change the synthetic route were made as no other amine protecting groups looked especially promising.

Experimental Procedures

General reagents were obtained from Aldrich, Sigma, and Acros chemical companies. Generation 4 PAMAM dendrimer was purchased from either Aldrich or Dendritech. Methylene Chloride was purified on basic alumina and $\text{BF}_3 \cdot \text{OEt}_2$ was distilled from CaH_2 (62 °C at 53 mm Hg) before use. The trans indol-acrylic acid was crystallized from warm absolute ethanol before use. All other solvents and reagents were used as supplied.

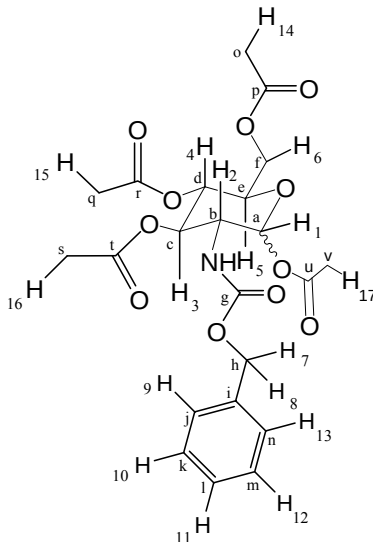
The procedures used to obtain MALDI-TOF spectra were the same as those given in the experimental section of CHAPTER TWO (pg. 52).



2-(N-benzyloxycarbonylamino)-2-deoxy-D-glucopyranoside (22)⁵¹

Glucosamine hydrochloride (26.1g, 121 mmol) and sodium bicarbonate (17.5 g, 209 mmol, 1.7 equiv.) were dissolved in water (250 mL) and cooled to 0 °C. Benzyl

chloroformate (45 mL, 314 mmol, 2.6 equiv.) was added dropwise over 5 min to this stirred solution. The reaction was stirred for 2.5 h at 0 °C. A white precipitate was filtered from the reaction and washed with 100 mL EtOAc. The filtrate was transferred back to the flask and stirred at room temperature overnight to ensure complete reaction. The resulting precipitate was filtered and washed with 100 mL EtOAc to afford a white crystalline solid. The combined product was dried under vacuum with P₂O₅ to leave 37.8 g (100%). The product was used without further purification. In the NMR spectrum both a mixture of anomers and rotomers were observed. m.p. 215 °C (recrystallized from 30% methanol in H₂O);⁵⁵ ¹H NMR (500 MHz, CD₃SOCD₃) δ 7.32 (d, J = 4 Hz, 3H), 7.27 (d, J = 4 Hz, 2H), 7.04 (d, J = 9 Hz, 0.4H, NH), 6.83 (d, J = 8 Hz, 0.6H, NH), 4.36 – 4.97 (m, 5H, including H_{1α} and OHs), 4.36 (d, J = 8 Hz, H_{1β}, integration included in previous entry), 3.52 – 3.65 (m, 2H), 3.36 – 3.46 (m, 2H), 3.26 (td, J = 3, 11 Hz, 1H, H₅), 3.18 (d, J = 8 Hz, 1H), 2.99 – 3.09 (m, 2H) ppm; ¹³C NMR (125 MHz, CD₃SOCD₃) 156.6, 156.5 (G), 137.8, 137.6 (I), 129.3, 129.1, 128.8, 127.1, 126.7 (J - N), 96.0, 91.1 (A), 77.2, 74.7, 72.5, 71.5, 71.4, 70.7, 65.7, 65.5, 61.7, 61.6, 59.2, 56.9 ppm; IR (thin film) 3440, 1713, 1654, 1542, 1250 cm⁻¹; HRMS C₁₄H₁₉NO₇ + Na⁺ calc. 336.2964, found 336.1049 amu.



1,3,4,6-tetra-O-acetyl-2-benzoyloxycarbonylmino-2deoxy-D-glucopyranoside

(23). Both THF (200 mL) and TEA (28 mL) were added to **(22)** (14.082 g, 45 mmol) to form a suspension. Acetic anhydride (28.4 mL, 301 mmol, 6.7 equiv.) was added dropwise over 5 min. to a stirred solution at 0 °C. A catalytic amount (about 10 mg) of DMAP was added. The reaction was warmed to r.t. as it stirred overnight. At this point all the initial solid was completely dissolved. The majority of the solvent was removed under vacuum. The remaining syrup was taken up in H₂O (100 mL) and extracted with ethyl acetate (2 x 100 mL). The organic layers were combined and washed with 5% HCl (2 x 100 mL), brine (2 x 100 mL), saturated NaHCO₃ solution (2 x 100 mL), and H₂O (2 x 100 mL). The organic layer was dried with MgSO₄. The solvent was removed under vacuum to afford 21.7 g (90%) of **23** as a white solid. This product was used without further purification. m.p. 148 °C (recrystallized from ethanol, lit 148 °C)⁵⁶; ¹H NMR^{55,57} (500 MHz, CDCl₃) δ 7.30 (m, 5H), 6.17 (d, J = 3.6 Hz, 1H, H₁), 5.05 – 5.20 (m, 3H, H₆ & H₄), 5.00 (d, J = 12 Hz, 1H, H₅), 4.87 (d, J = 10 Hz, 1H, NH), 4.21 – 4.24 (m, 2H, H₂,

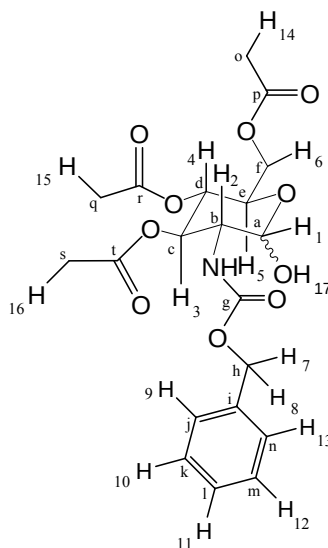
H_{7 or 8}), 3.95 – 4.03 (m, 2H, H₃, H_{7 or 8}), 2.13 (s, 3H, COCH₃), 2.05 (s, 3H, COCH₃), 2.00 (s, 3H, COCH₃), 1.90 (s, 3H, COCH₃) ppm; ¹³C (125 MHz, CDCl₃) δ 171.2, 170.7, 169.2, 168.7 (P, R, T, U), 155.6 (G), 135.9 (I), 128.6, 128.5, 128.4, 128.2, 128.1 (J-N), 90.8 (A), 70.6 (F), 69.7 (C), 67.6 (D), 67.3 (E), 61.5 (H), 52.8 (B), 20.9, 20.7, 20.6, 20.5 (O, Q, S, V) ppm; IR 3431, 3026, 2958, 1752, 1514, 1368, 1228 cm⁻¹; MS C₂₂H₂₇NO₁₁ + Na calc. 504.4452, found 504.1502.

Table 4.1 COSY NMR data for **23**

¹ H δ (ppm)	¹ H #	Coupled to ¹ H δ (ppm)	Coupled to ¹ H #
6.17	1	4.21 – 4.24	2
5.05 – 5.20	4	3.95 – 4.03	3
5.0	5	5.05 – 5.20	6
4.87	NH	4.21 – 4.24	2
4.21 – 4.24	2	3.95 – 4.03	3

Table 4.2 HMQC NMR data for **23**

¹³ C δ (ppm)	¹³ C letter	¹ H δ (ppm)	¹ H #
168.7, 169.2, 170.7, 171.2	P, R, T, V	1.9, 2.00, 2.05, 2.13 weakly coupled	14 - 17
128.1, 128.2, 128.4, 128.5, 128.6	J – N	7.30	9 - 13
155	G	No coupling	
135.9	I	No coupling	
90.8	A	6.17	1
70.6	F	5.05 – 5.20	6
69.7	C	3.95 – 4.03	3
67.6	D	5.05 – 5.20	4
67.3	E	5.00	5
61.5	H	4.21 – 4.24	7 or 8
61.5	H	3.95 – 4.03	7 or 8
52.8	B	4.21 – 4.24	2
20.5, 20.6, 20.7, 20.9	O, Q, S, V	1.9, 2.00, 2.05, 2.13	14 – 17



3,4,6-tri-O-acetyl-2-benzyloxycarbonylamino-2-deoxy-D-glucopyranoside

(24). A Hydrazine Acetate (1.65 g, 17.9 mmol, 1.5 equiv.) was added to a stirred solution of **(23)** (5.74 g, 11.9 mmol) in DMF (22 mL). The reaction was heated to 55 °C and maintained for 25 minutes. The majority of solvent was removed in vacuum. Water (50 mL) was added to the remaining syrup and extracted with EtOAc (2 x 50 mL). The organic layers were combined and washed with brine (2 x 50 mL), saturated NaHCO₃ solution (2 x 50 mL), and H₂O (2 x 50 mL). The organic layer was dried with MgSO₄. The solvent was removed in vacuum to leave 4.52 g (86%) of a fluffy white solid. This product was used without further purification. mp: 142-144 °C (recrystallized from 9:1 methanol: water); ¹H NMR (500 MHz, CDCl₃), 7.29 – 7.32 (m, 5H), 5.24 – 5.26 (m, 2H, H₁ & H₄), 4.99 – 5.16 (m, 4H, H₃, H₆, NH), 4.22 (d, J = 5 Hz, 1H, H_{7or8}), 4.07 – 4.19 (m, 2H, H₂ & H_{7or8}), 4.02 (td, J = 10, 3 Hz, 1H, H₅), 3.7 (bs, 1H, OH), 2.06 (s, 3H, COCH₃), 1.99 (s, 3H, COCH₃), 1.88 (s, 3H, COCH₃) ppm; ¹³C NMR (125 mHz, CDCl₃), 171.1, 170.9, 169.5, 155.9 (P, R, T, G), 136.2 (I), 128.5, 128.2, 128.1 (J - N), 91.9 (A), 70.9 (D),

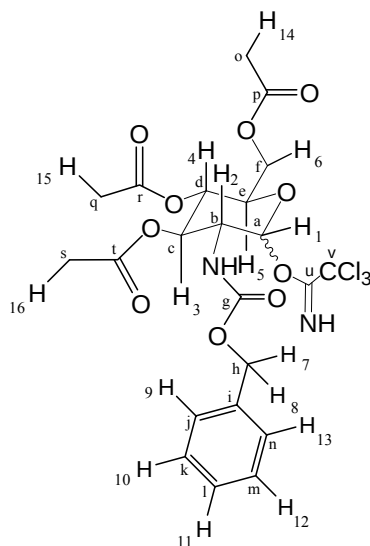
68.4 (C), 67.6 (B), 67.0 (F), 62.1 (H), 53.9 (E), 20.8, 20.63, 20.58 (O, Q, S) ppm; IR: 3434, 2959, 1746, 1516, 1368, 1233 cm^{-1} ; HRMS: $\text{C}_{20}\text{H}_{25}\text{NO}_{10} + \text{Na}$ calc: 462.4080, found: 462.1376.

Table 4.3 COSY NMR data for **24**

^1H δ (ppm)	^1H #	Couple to ^1H δ (ppm)	Coupled to ^1H #
5.2	1	3.7	OH
5.2	4	4.0	5
5.2	4	5.1	3
5.1	6	4.0	5
5.1	3 & NH	4.1 – 4.2	2
4.2	2	3.7	OH

Table 4.4 HMQC NMR data for **24**

^{13}C δ (ppm)	^{13}C Letter	^1H δ (ppm)	^1H #
128.1 – 128.5	I - N	7.23 – 7.32	9 - 13
91.9	A	5.24 – 5.26	1
70.9	D	5.24 – 5.26	4
67.0	F	4.99 – 5.16	6
68.4	C	4.99 – 5.16	3
67.6	B	4.07 – 4.19	2
62.1	H	4.22	7 or 8
62.1	H	4.07 – 4.19	7 or 8
53.9	E	4.02	5



1-O-(2-benzyloxyamido-3,4,5-tri-O-acetyl-2-deoxy- α -D-

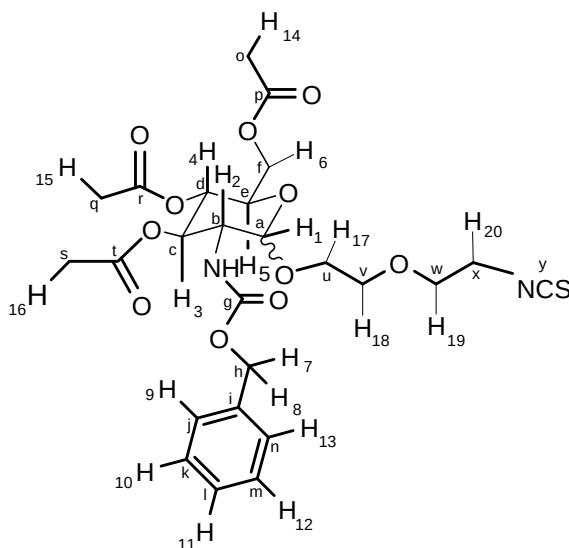
glucopyranosyl)trichloroacetimidate (25). A solution of **24** (5.564 g, 12.7mmol) and CH_2Cl_2 (52mL) was prepared. The mixture was cooled in an ice bath, under nitrogen. DBU (0.19 mL, 1.3 mmol, 0.1 eq) was added dropwise over 1 min. The reaction was stirred at 0 °C under nitrogen for 5 h. The solvent was removed under vacuum. The product was immediately chromatographed (6:4 hexanes: ethyl acetate on silica gel) to obtain 4.715 g (8.1 mmol, 64%) of **25**. m.p. 149 °C (recrystallized from H_2O); ^1H NMR (500 MHz, CDCl_3) δ 8.75 (s, 1H, HNCCl_3), 7.29-7.35 (m, 5H, 9 - 13), 6.37 (d, J = 3.5Hz, 2H, H_1), 5.27 (t, J -10 Hz, 1H, H_3), 5.20 (t, J = 9.8 Hz, 1H, H_4), 5.11 (d, J = 12.2 Hz, 1H, $\text{H}_{7\text{or}8}$), 5.0 (d, J = 12.2 Hz, 1H, $\text{H}_{7\text{or}8}$), 4.9 (d, J = 9.4Hz, 1H, NH), 4.2-4.3 (m, 2H, H_2 & H_5), 4.07-4.10 (m, 2H, H_6), 2.05 (s, 3H, COCH_3), 2.01 (s, 3H, COCH_3), 1.9 (s, 3H, COCH_3); ^{13}C (125 MHz, CDCl_3) δ 171.1, 170.6, 169.3 (P, R, T), 160.3 (G), 155.7 (U or V?), 136.0 (I), 128.5, 128.3, 128.1 (J - N), 94.5 (A), 70.6 (F), 70.2 (C), 67.5 (D), 67.2 (H), 61.5 (E), 53.5 (B), 20.7, 20.6, 20.5 (O, Q, S); IR: 3420, 3060, 1750, 1518, 1232, 1046 cm^{-1} .

Table 4.5 COSY NMR data for **25**

^1H δ (ppm)	^1H #	Coupled to ^1H δ (ppm)	Coupled to ^1H #
6.37	1	4.2 - 4.3	2
5.27	3	5.20	4
5.27	3	4.2 - 4.3	2
5.2	4	4.2 - 4.3	5
4.9	NH	4.2 - 4.3	2
4.2 - 4.3	5	4.1	6

Table 4.6 HMQC data for **25**

^{13}C δ (ppm)	^{13}C Letter	^1H δ (ppm)	^1H #
128.5, 128.3, 128.1	J - N	7.29 - 7.35	9 - 13
94.9	A	6.37	1
70.2	C	5.27	3
67.5	D	5.20	4
67.2	H	5.11	7
53.5	B	4.2 - 4.3	2
70.6	F	4.1	6
61.5	E	4.2 - 4.3	5
20.7, 20.6, 20.5	O, Q, S	2.05, 2.01, 1.9	14 - 16



1-O-(5-isothiocyanato-3-oxapentyl)- 3,4,6-tri-O-acetyl-2-benzyloxycarbonyl-2-deoxy-D-glucopyranoside (21). The trichloroacetimidate **25** (2.38 g, 4.1 mmol) was dissolved in CH₂Cl₂ (16mL). Crushed 4Å molecular sieves and isothocyanate **4** (741 mg, 5.0 mmol, 1.2 equiv.) were added to the solution. The reaction was purged with N₂. Freshly distilled BF₃•OEt₂ (0.52 mL, 0.41 mmol) was added dropwise. The reaction was stirred at r.t. for 8 h under N₂. It was then filtered through a plug of silica, solvent was removed under reduced pressure and the resulting syrup was purified by column chromatography (1:1 hexanes: EtOAc on silica gel) to give 1.21 g. of **21** (52% yield). ¹H NMR (500 MHz, CDCl₃) 7.29 – 7.34 (m, 5H), 5.12 – 5.24 (m, 6H), 4.68 (bs, 1H), 4.25 (dd, J = 4.7, 12 Hz, 1H), 4.12 (dd, J = 1.9, 12 Hz, 1H), 3.94 (dt, J = 3.4, 12 Hz, 1H, H₅), 3.45 – 3.76 (m, 8H), 2.07 (s, 3H), 2.00 (s, 3H), 1.94 (s, 3H) ppm; ¹³C NMR (125 MHz, CDCl₃) 170.73, 170.66, 169.5, 155.9 (P, R, T, G), 136.4, 128.5, 128.2, 128.1 (I - N), 101.3, 91.9 (A), 72.3, 71.8, 70.6, 69.3, 69.2, 68.6, 66.9, 62.1, 56.1, 45.2 (B – F, H, U - X), 20.8, 20.61, 20.56 (O, Q, S) ppm; IR 3018, 2399, 1748, 1518, 1368, 1215 cm⁻¹; HRMS for C₂₅H₃₂N₂O₁₁S + Na Calc = 591.55, found 476.12 (591.55 – 116.16 (C₄H₆OS)).

Table 4.7 COSY NMR data for **21**

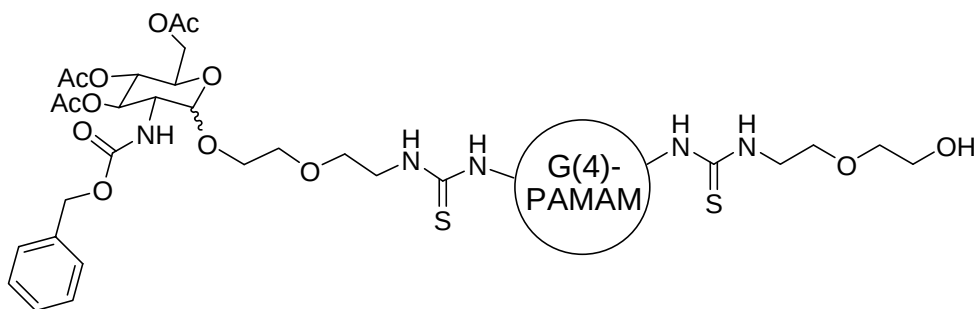
¹ H δ (ppm)	Coupled to ¹ H δ (ppm)
4.25	5.12 – 5.23
4.12	4.25
3.94 (H ₅)	5.12 – 5.23
3.94 (H ₅)	3.45 – 3.76
3.45 – 3.76	5.12 – 5.23
3.45 – 3.76	4.25

Table 4.8 HMQC data for **21**

^{13}C δ (ppm)	^1H δ (ppm)
128.1 – 128.5 (I-N)	7.29 – 7.34 (H9-13)
101.4	4.68
91.9	5.23
71.8	3.45 – 3.76
70.5	3.45 – 3.76
69.3 (E)	3.94 (1H5)
69.2	3.45 – 3.76
68.7	5.01 (5.12 – 5.24)?
66.9	5.06 (5.12 – 5.24)?
62.1	4.25
62.1	4.12
56.1	3.45 – 3.76
45.2	3.51 (3.45 – 3.76)
20.56 – 20.76 (O, Q, S)	1.85 – 2.13 (14-16)

The general procedure for heterogeneously functionalized generation 4.0

PAMAM-based thiourea-linked ethoxy ethanol and 1-O-(5-thiourea-3oxapentyl)-3,4,6-tri-O-acetyl-2-benzoyloxycarbonylamino-2-deoxy-D-glucoside dendrimer (**20a-g**) follows.



An approximately 25% w/w solution in water of G(4)-PAMAM Dendrimer was

lyophilized to dryness and dissolved in 2 mL of DMSO. Varying amounts of sugar **21**

(210 mM solution in DMSO) were added to the stirred dendrimer solution as shown in

Table 4.9 below. The reaction was allowed to stir for 15 days, until the M_w was constant in the MALDI-TOF MS. A 3.0 M solution of **4** in DMSO was added to each reaction to give approximately 2 equivalents per remaining surface amine. MALDI-TOF MS was obtained. After 3 days 100 μ l of **4** was added to each reaction. MALDI-TOF MS was obtained and were consistent with the previous spectra (indicating that there were no more available amines). The reactions were stirred for 2 days longer, purified by dialysis (M_w cutoff = 3.5 kDa against DMSO) and lyophilized to dryness.

Table 4.9 Dendrimer Functionalization: amounts used, % loadings and % yields.

20	mg G4	μ mol dendrimers (μ mol surface NH_2 groups)	μ mol surface NH_2 groups ^a	mL 21 (μ mol)	% 4 (# 4)	% 21 (# 21)	mg Product (%) ^b
a	167	12 (660)	660	0.698 mL (148)	60 (36)	20 (12)	404.3 mg (140)
b	138	10 (550)	550	1.01 mL (215)	45 (27)	29 (17.6)	453 mg (175)
c	145	11 (605)	605	1.52 mL (322)	27 (16.1)	44 (26.6)	209 mg (64) ^c
d	135	10 (550)	550	2.12 mL (450)	26 (15.6)	58 (34.7)	635 mg (187)
e	130	9.6 (528)	528	1.20 mL (480)	12 (7)	61 (36.8)	289 mg (81)
f	126	9.3 (511)	511	2.38 mL (504)	0 (0)	77 (46)	289 mg (81)
g	42.0	3.1 (187)	187	1.75 mL (373)	14 (8.4)	84 (50.2)	166 mg (124)

^a μ mol surface NH_2 groups (μ mol dendrimer x 60)

^bSome DMSO remained, lyophilization to dryness resulted in insoluble material

^cspilled flask

Due to severe peak overlap and rotamer and anomer formation, peak normalization to whole #'s was not always possible.

20a: ^1H NMR (500 MHz, DMSO): 7.80 – 7.99 (m, 10.3 H), 7.40 – 7.49 (m, 7.6H), 7.4 – 7.31 (m, 5H), 5.49 (d, $J = 5.7$ Hz, 1 - 2H), 5.23 (m, 1 - 2H), 4.89 – 5.05 (m, 1.9H), 4.79 – 4.81 (m, 0.5H), 4.45 – 4.58 (m, 2.7H), 3.96 – 4.22 (m, 0.74H), 3.74 (bs, 1.2H), 3.04 – 3.46 (m, 49.6H), 2.65 (bs, 19.2H), 2.36 – 2.54 (m, 30.8H), 2.18 (bs, 10.4H), 1.76 – 2.05 (m, 6.5H) ppm; MALDI-TOF MS: Sugars **21** only $M_w = 19,142$ g/mol (equivalent with 11 sugars on average), Sugars **21** and **4** $M_w = 24,385$ g/mol (11 sugars **21** & 36 of **4** on average).

20b: ^1H NMR (500 MHz, DMSO): 7.80 – 7.98 (m, 6.5H), 7.40 – 7.49 (m, 4.9H), 7.24 – 7.31 (m, 5.2H), 5.49 (s, 0.1H), 4.91 – 5.05 (m, 2.5H), 4.79 (t, $J = 9.5$ Hz, 0.85H), 4.45 – 4.58 (m, 1.8H), 3.96 – 4.21 (m, 2H), 3.74 (bs, 1.8H), 3.03 – 3.46 (m, 52.8H), 2.64 (bs, 12.1H), 2.43 – 2.50 (m, 19.6H), 2.18 (bs, 12.0H), 1.76 – 2.00 (m, 9.9H) ppm; MALDI-TOF MS: sugar only $M_w = 23,157$ g/mol (equivalent to 17.6 sugars **21** on average), sugars and **4** $M_w = 27,131$ g/mol (equivalent to 17.6 sugars **21** and 27 of **4** on average).

20c: ^1H NMR (500 MHz, DMSO): 7.78 – 7.98 (m, 4.5H), 7.40 – 7.48 (m, 4.1H), 7.24 – 7.30 (m, 5.6H), 4.88 – 5.50 (m, 2.9), 4.79 (t, $J = 9.6$ Hz, 0.9H), 4.57 (d, $J = 8.2$) & 4.46 (d, $J = 8.4$ Hz) (1.5H, 2 signals), 4.09 – 4.21 (m, 1.2H), 3.97 (d, $J = 11.4$ Hz, 0.9H), 3.75 (m, 2.1H), 3.04 – 3.57 (m, 48.9H), 2.64 (bs, 8.7H), 2.36 – 2.58 (28.7H), 1.76 – 2.05 (m, 16.8H) ppm; MALDI-TOF MS: sugars **21** only $M_w = 28,273$ g/mol (equivalent to

26.6 sugars **21** on average); sugars **21** and **4** MW = 30,641 g/mol (equivalent to 26.6 sugars **21** and 16.1 of **4** on average).

20d: ^1H NMR (500 MHz, DMSO): 7.87(bs, 2.7H), 7.39 – 7.48 (m, 3H), 7.25 – 7.30 (m, 5H), 5.03 – 5.05 (m, 2H), 4.92 (d, $J = 12.5$ Hz, 1H), 4.79 (t, $J = 9$ Hz, 0.8H), 4.57 (d, $J = 9$ Hz, 0.9H), 4.14 – 4.15 (m, 1H), 3.97 (d, $J = 11.2$ Hz, 1H), 3.75 (m, 2H), 3.04 – 3.57 (m, 24.3H), 2.63 (bs, 5.2H), 2.36 – 2.56 (m, 13.4H), 2.17 (bs, 5.2H), 1.97 (s, 3H), 1.91 (s, 3H), 1.79 (s, 3H) ppm; MALDI-TOF MS: Sugars **21** only $M_w = 32,860$ g/mol (equivalent to 34.7 sugars **21** on average), Sugar **21** and **4** MW = 35,152 g/mol (equivalent to 34.7 sugars **21** and 15.6 of **4** on average).

20e: ^1H NMR (500 MHz, DMSO): 7.96 (bs, 1.1H), 7.76 (bs, 1.1H), 7.39 – 7.48 (m, 3.8H), 7.23 – 7.30 (m, 5H), 5.01 – 5.05 (m, 1.9H), 4.92 (d, $J = 12.8$ Hz, 0.9H), 4.79 (t, $J = 9.6$ Hz, 0.8H), 4.57 (d, $J = 8.2$ Hz, 1H), 4.14 – 4.16 (m, 0.9H), 3.97 (d, $J = 11.4$ Hz, 1H), 3.75 – 3.77 (m, 2.7H), 3.21 -3.58 (m, 26.4H), 2.96 – 3.14 (m, 7.2H), 2.32 – 2.64 (m, 35.6H), 2.17 (bs, 6.2H), 1.97 (s, 3.2H), 1.91 (s, 3.2H), 1.79 (s, 2.6H) ppm; MALDI-TOF MS: Sugars only $M_w = 34,406$ g/mol (equivalent to 36.8 sugars **21** on average), Sugars **21** and **4** $M_w = 35,431$ g/mol (equivalent to 36.8 sugars **21** and 7 of **4** on average).

20f: ^1H NMR (500 MHz, DMSO): 7.96 (bs, 1H), 7.75 (bs, 1H), 7.39 – 7.47 (m, 3H), 7.23 – 7.30 (m, 5H), 5.00 – 5.05 (m, 2H), 4.91 (d, $J = 12.8$ Hz, 1H), 4.79 (t, $J = 9.6$ Hz, 1H), 4.56 (d, $J = 8.6$ Hz 1H), 4.14 (d, $J = 8.0$ Hz, 1H), 3.97 (d, $J = 11.3$ Hz, 1H), 3.75 – 3.77 (m, 2H), 3.05 – 3.56 (m, 19 H), 2.36 – 2.64 (m, 24H), 2.16 (bs, 4H), 1.96 (s, 3H), 1.91 (s, 3H), 1.79 (s, 3H) ppm; MALDI-TOF MS: 39,269 g/mol (equivalent with 46 sugars **21** and 0 of **4** on average).

20g: ^1H NMR data not available. MALDI-TOF MS: Sugars only $M_{\text{W}} = 41,991$ (equivalent to 50.2 sugars **21** on average), Sugars **21** and **4** $M_{\text{W}} = 43,231$ g/mol (equivalent to 50.2 sugars **21** and 8.4 of **4** of average).

Attempts at Cbz removal to form 1-O-(5-thiourea-3-oxapentyl)-3,4,6-tri-O-acetyl-2-deoxy-D-glucoside/ethoxy ethanol G(4)-PAMAM dendrimers are described below.

In the first attempt the catalyst Pd/C (13.6 mg) was added to a solution of **20f** (13.5 mg, 16 μmol s Cbz) in 1,4-cyclohexadiene (292 μl , 43 μmol s) and MeOH (600 μl). The system was stirred at r.t. under H_2 for 7h. The reaction was filtered to remove Pd/C and the solvent was removed by vacuum. The reaction was purified by dialysis (MWCO = 3.5 kDa.) against DMSO and lyophilized to near dryness by repeated lyophilization with nanopure water. Lyophilization to dryness would have resulted in insoluble material. The reaction resulted in 13.9 mg of material. The M_{W} was found to be comparable to the starting material.

The catalyst Pd/C (~10 mg) was added to a solution of **20f** (36 mg, 42 μmol s Cbz) in MeOH (1.5 mL). The system was stirred at r.t. under H_2 for 48 h. The reaction was filtered to remove Pd/C and the solvent was removed by vacuum. The reaction was purified by dialysis (MWCO = 3.5 kDa.) against DMSO and lyophilized to near dryness by repeated lyophilization with nanopure water. Lyophilization to dryness would have resulted in insoluble material. The reaction resulted in 25.4 mg of material. The M_{W} was found to be comparable to the starting material.

The catalyst Pd/C (12.6mg) was added to a solution of **20g** (37.8mg, 44 μmol s Cbz) in 1,4-cyclohexadiene (500 μl , 74 μmol s) and EtOH (1.0 mL). The system was

stirred at r.t. under N₂ for 16h. The reaction was filtered to remove Pd/C and the solvent was removed by vacuum. The reaction was purified by dialysis (MWCO = 3.5 kDa.) against DMSO and lyophilized to near dryness by repeated lyophilization with nanopure water. Lyophilization to dryness would have resulted in insoluble material. The reaction resulted in 28.9 mg of material. The M_w was found to be slightly lower than the starting material (MW = 42,134 g/mol) indicating removal of 8.1 Cbz groups on average. The NMR data indicates no decrease in aromatic ¹H integration as compared to the acetyl methyl ¹H signals. Attempts at re-reacting the 28.9 mg of material recovered resulted in equivalent MALDI-TOF MS and NMR data.

The catalyst Pd/C (12.7mg) was added to a solution of **20g** (11.5 mg, 13.4 μmols Cbz) in 1,4-cyclohexadiene (295 μl, 31 μmols) and MeOH (600 μl). The system was stirred at r.t. under N₂ for 8.5 h. The reaction was filtered to remove Pd/C and the solvent was removed by vacuum. The reaction was purified by dialysis (MWCO = 3.5 kDa.) against DMSO and lyophilized to near dryness by repeated lyophilization with nanopure water. Lyophilization to dryness would have resulted in insoluble material. The reaction resulted in 16.9 mg of material. The MALDI-TOF spectrum gave an extremely broad peak ranging from 25-53 kDa. From the MALDI-TOF spectrum no data was conclusive; however, the NMR data indicates no decrease in aromatic ¹H integration as compared to the acetyl methyl ¹H signals.

The catalyst Pd(OH)₂/C (~10 mg) was added to a solution of **20g** (6.2 mg, 7.2 μmols Cbz) in MeOH (400 μl). The system was stirred at r.t. under H₂ for 18.5h. The reaction was filtered to remove Pd/C and the solvent was removed by vacuum. The

reaction was purified by dialysis (MWCO = 3.5 kDa.) against DMSO and lyophilized to near dryness by repeated lyophilization with nanopure water. Lyophilization to dryness would have resulted in insoluble material. The reaction resulted in 11.5 mg of material. The M_w was found to be comparable to the starting material.

The catalyst was freshly prepared by a literature method.⁵⁸ To prepare the catalyst PdCl_2 was dissolved in 500 μL of 2N HCl (heating required). This solution was transferred to 5 mL of boiling water. The mixture was brought to a boil, 20 μL of formic acid was added followed by the addition of 1.6 mL 10% KOH to adjust the pH to 7.5. The pH was then readjusted to 6.0 with the addition of formic acid. The solution was boiled for 5 min until the orange solution turned clear and a black solid precipitated. The catalyst was filtered, washed with water (6 x 2 mL) and MeOH (6 x 2 mL). Care was taken to ensure the catalyst never completely dried. The catalyst was immediately transferred to the reaction followed by rinsing the sides of the reaction flask with methanol to ensure all the catalyst was under MeOH.

The catalyst Pd black (~34mg) was added to a solution of **20d** (66.8mg, 66 μmol s Cbz) in 1,4-cyclohexadiene (3.1 mL, 46mmols) and MeOH (1 mL plus amount needed to add Pd black). The system was stirred at r.t. under H_2 for 23h. The reaction was filtered to remove Pd/C and the solvent was removed by vacuum. The reaction was purified by dialysis (MWCO = 3.5 kDa.) against DMSO and lyophilized to near dryness by repeated lyophilization with nanopure water. Lyophilization to dryness would have resulted in insoluble material. The reaction resulted in 32 mg of material. The M_w was found to be

comparable to the starting material. NMR data indicates no decrease in aromatic ^1H integration as compared to the acetyl methyl ^1H signals.

In the second attempts 0.3M NaOMe in methanol (228 μL , 68 μmol) was added to a solution of **20f** (31 mg, 34 μmol Cbz) and 1:1 MeOH:H₂O (3 mL). The reaction was stirred 22.5 h, neutralized by addition of amberlite acid resin and filtered. The methanol was removed in vacuo. The remaining solution was transferred to dialysis membrane (MWCO = 3.5 kDa.) and dialyzed against H₂O. The water was removed by lyophilization to give 11.9 mg of solid material. No MALDI-TOF MS data could be obtained and the NMR data indicates destruction of material. This would also account for the low mass of recovery.

Finally, the starting material **20d** (42.2 mg, 41 μmol Cbz) was dissolved in clean dry CH₂Cl₂ (1 mL). The mixture was cooled to -78 °C in a dry ice/acetone bath. The reaction was purged with N₂. A 1.0M solution of DIBAL (184 μL , 184 μmol) was added. The reaction was stirred at -78 °C for 3 h, warmed to 0 °C and stirred for 2 h, warmed to r.t. and stirred for 17 h. A saturated potassium sodium tartrate solution (355 μL) and ethyl ether (355 μL) were added to the reaction. The reaction was stirred overnight then transferred to dialysis tubing (MWCO = 3.5 kDa.). The reaction was purified by dialysis against H₂O and then lyophilized to remove the solvent to give 20.3 mg of material. The NMR spectrum of the recovered material is identical to the starting material (**20d**).

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

EPR Studies

TEMPO/mannose functionalized dendrimers were synthesized and characterized by MALDI-TOF MS. EPR was employed for the determination of endgroup locations. The methodology for determining the relative locations of functional groups on dendrimers was developed with our collaborators; however, the series of functional groups that was previously studied did not include carbohydrates. Expanding the series to include mannose/TEMPO dendrimers has given valuable information about the behavior of carbohydrate functionalized dendrimers in solution. With the knowledge that the locations of the carbohydrates are random, the interpretation of previous and future results in the study of protein-carbohydrate interactions in the Cloninger research group benefit.

The observation that the locations of the carbohydrates are random and not clustered shows that the carbohydrates on the dendrimer's surface can reach two binding sites on Con A, provided the functionalized dendrimer is large enough. Since previous results show a large increase in binding relative to methyl mannose for mannose-functionalized PAMAM Generations (4-6), the interpretation that this is multivalent binding holds.

Affinity Chromatography

Affinity chromatography was used to evaluate binding of randomly-functionalized mannose/TEMPO dendrimers to Con A. First we compared binding of mannose/TEMPO dendrimers with Con A to that of previously reported mannose/hydroxyl dendrimers using HIA. Results with and without spin label were comparable. Then affinity chromatography was used to separate the binding components of the solutions. Since components are separated based on binding strength and only one EPR-active component was isolated we were able to determine that there was only one main binding component in the solution. Since the bulk of the material came off in one fraction and the EPR spectra were equivalent before and after affinity chromatography it was concluded that there is only one binding component in the solution. Although the structure of the dendrimer is heterogeneous due to omissions in the structure and the synthetic methodology used, this does not result in a change in the binding affinity.

Summary

The results of both the EPR studies and the affinity studies probe the solution phase behavior of carbohydrate functionalized dendrimers. The dynamic nature of the dendrimers overcomes any minor differences in the structures from dendrimer deformations and differences in synthesis and allows them to behave similarly. Previous and future affinity studies on carbohydrate functionalized dendrimers can be analyzed as containing a single affinity toward the protein.

APPENDICES

APPENDIX A

MALDI-TOF SPECTRA FOR
DENDRIMERS **6(a-b)**, **7(a-g)**, **8(a-g)** AND **19(a-c)**

MALDI-TOF Spectra for Dendrimers **6a-g**

The MALDI-TOF Spectra for **6(a-g)** both partially and fully functionalized G(4)-PAMAM dendrimers are shown below. The details for the synthesis of these dendrimers are given in CHAPTER TWO.

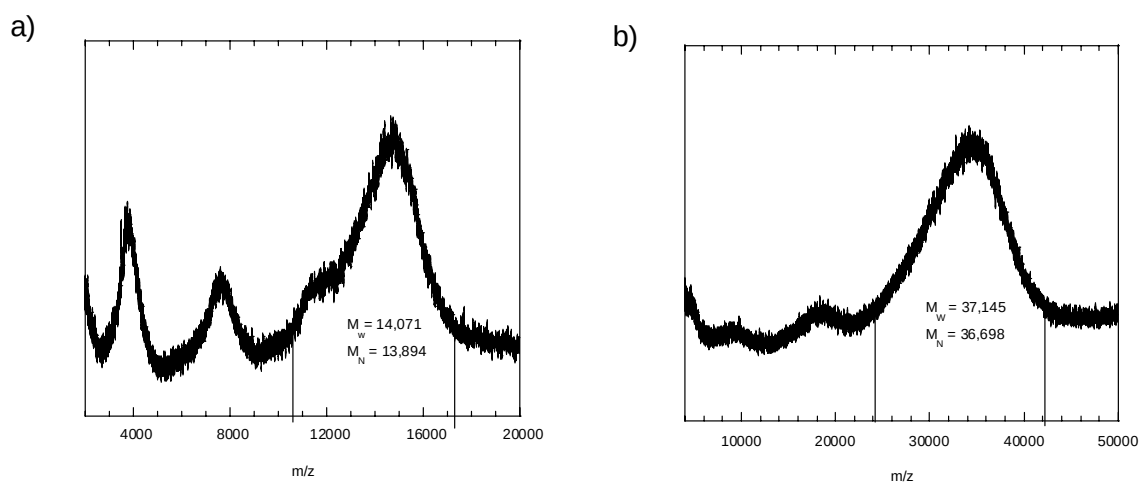


Figure A.1 MALDI-TOF spectra of a) partially (**5** only) and b) fully (**5** & **2**) functionalized 5% **5** and 95% **2** G(4)-PAMAM dendrimer (**6a**).

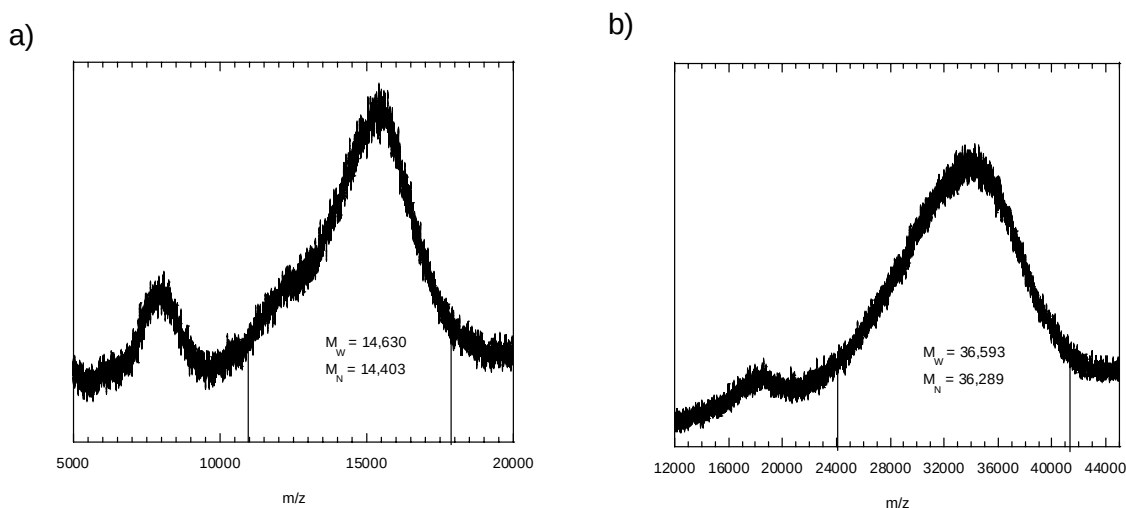


Figure A.2 MALDI-TOF spectra of a) partially (**5** only) and b) fully (**5** & **2**) functionalized 10% **5** and 90% **2** G(4)-PAMAM dendrimer (**6b**).

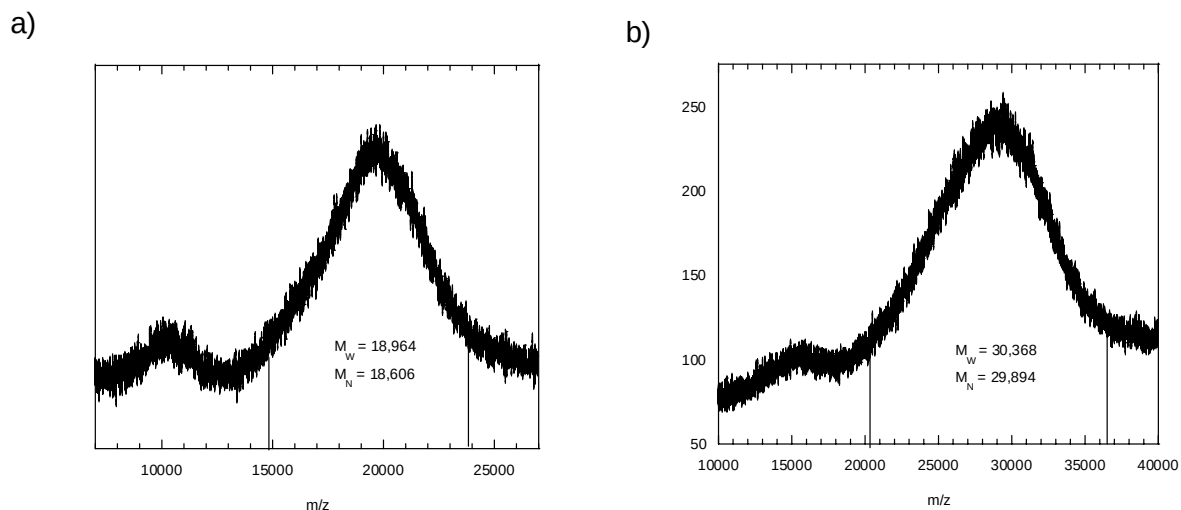


Figure A.3 MALDI-TOF spectra of a) partially (**5** only) and b) fully (**5** & **2**) functionalized 50% **5** and 50% **2** G(4)-PAMAM dendrimer (**6d**).

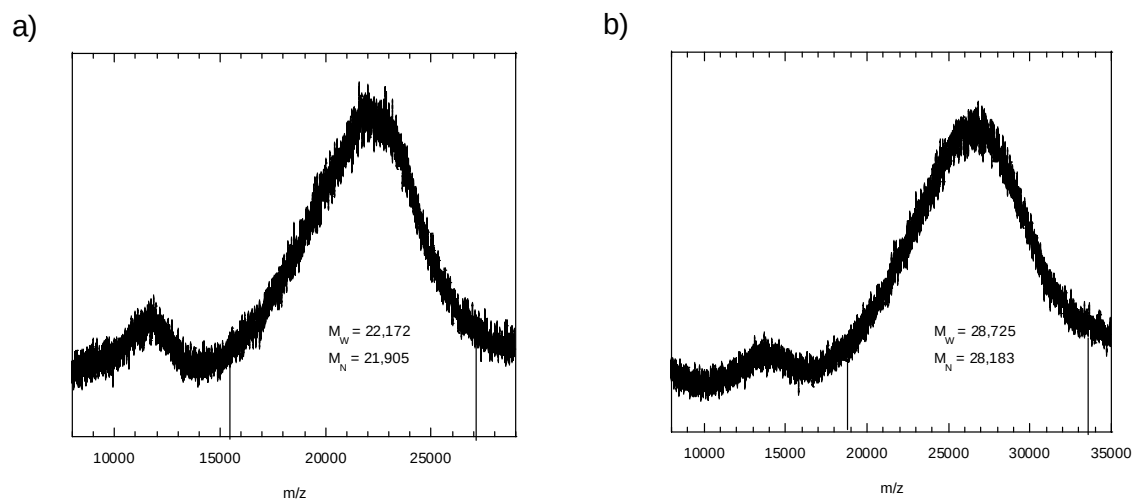
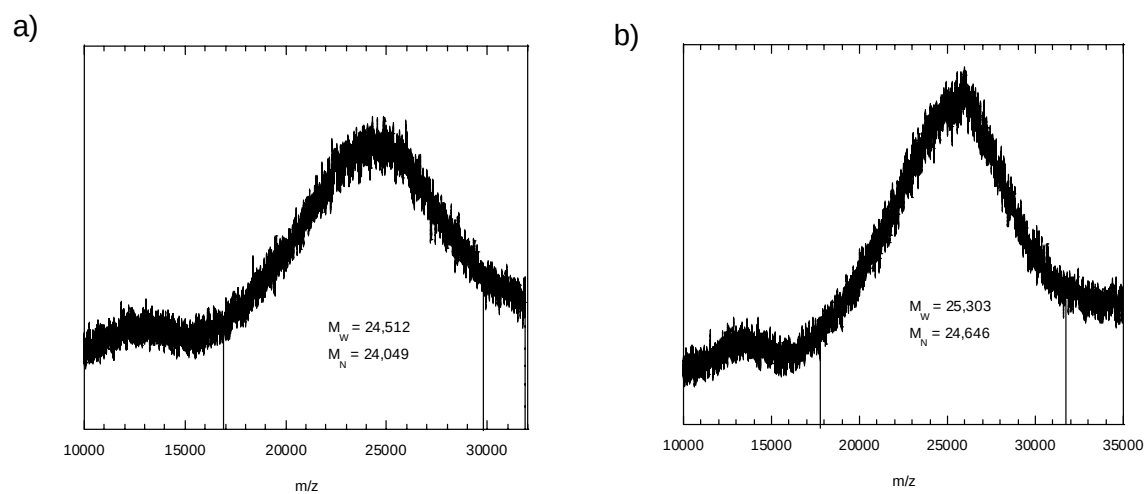
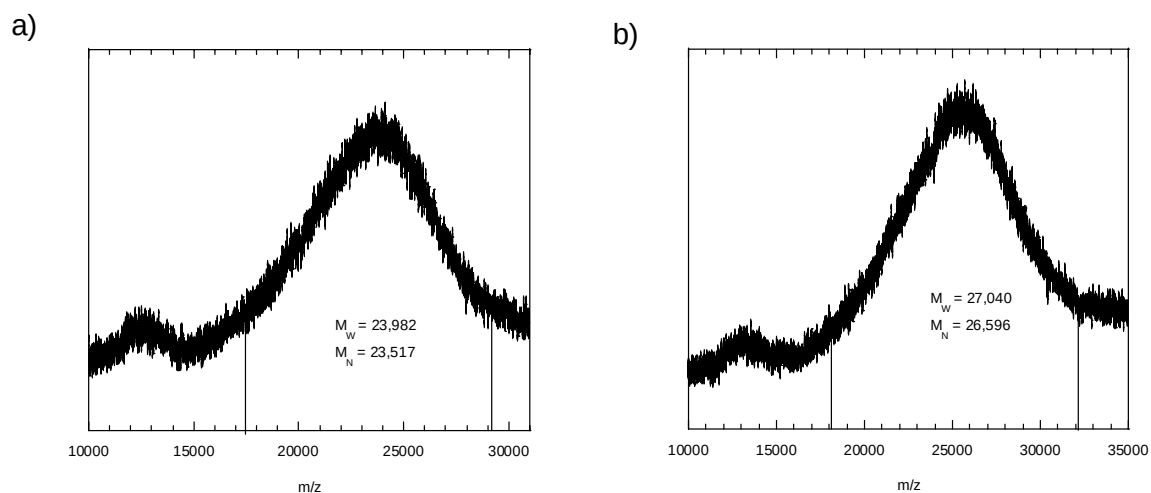


Figure A.4 MALDI-TOF spectra of a) partially (**5** only) and b) fully (**5** & **2**) functionalized 75% **5** and 25% **2** G(4)-PAMAM dendrimer (**6e**).



MALDI-TOF Spectra for Dendrimers **7a-g**

The MALDI-TOF Spectra for **7a-g** both partially and fully functionalized G(4)-PAMAM dendrimers are shown below. The details for the synthesis of these dendrimers are given in CHAPTER TWO.

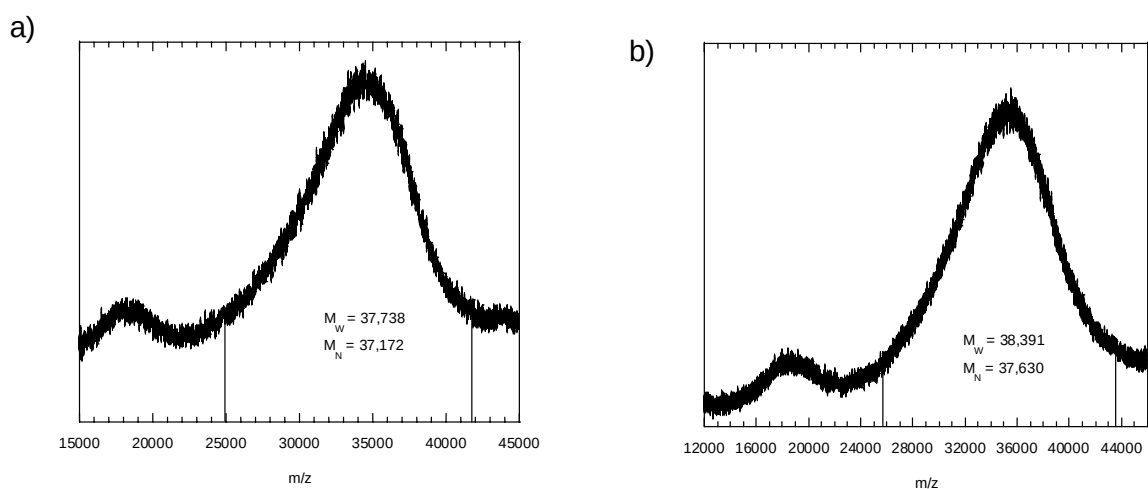


Figure A.7 MALDI-TOF spectra of a) partially (**2** only) and b) fully (**2** & **5**) functionalized 5% **5** and 95% **2** G(4)-PAMAM dendrimer (**7a**).

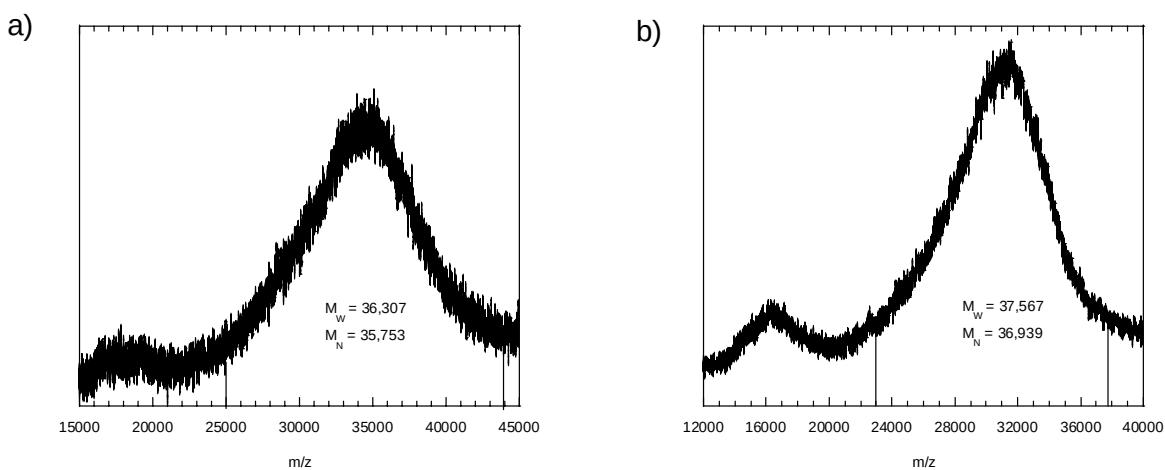


Figure A.8 MALDI-TOF spectra of a) partially (**2** only) and b) fully (**2** & **5**) functionalized 10% **5** and 90% **2** G(4)-PAMAM dendrimer (**7b**).

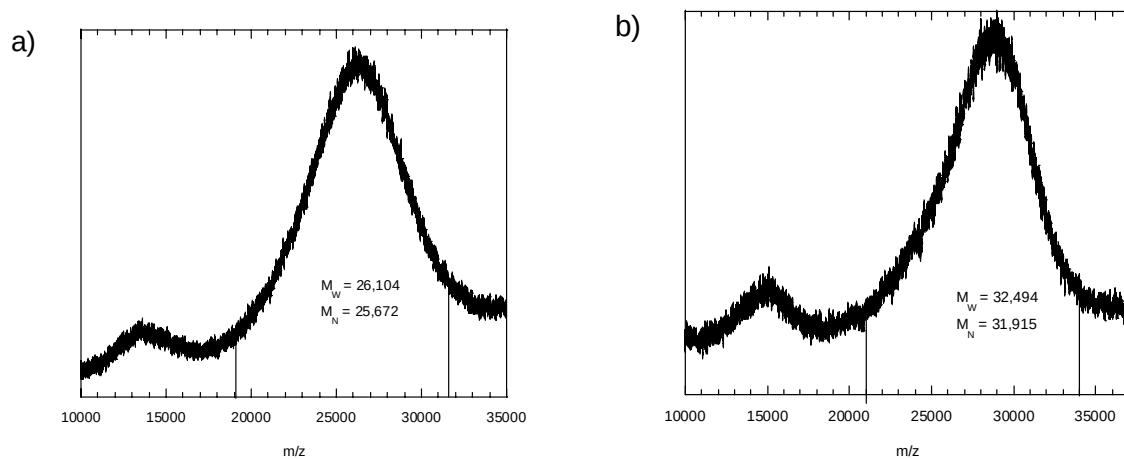


Figure A.9 MALDI-TOF spectra of a) partially (**2** only) and b) fully (**2** & **5**) functionalized 50% **5** and 50% **2** G(4)-PAMAM dendrimer (**7d**).

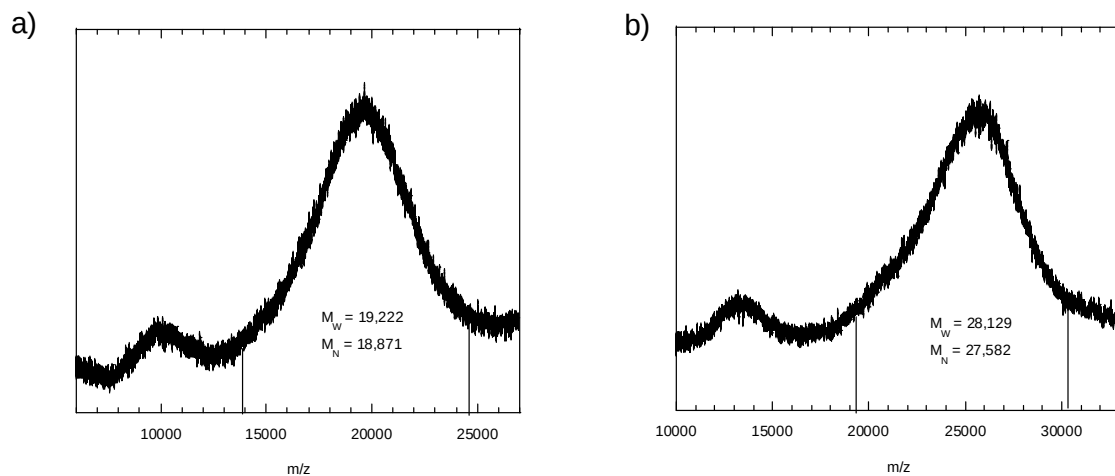


Figure A.10 MALDI-TOF spectra of a) partially (**2** only) and b) fully (**2** & **5**) functionalized 75% **5** and 25% **2** G(4)-PAMAM dendrimer (**7e**).

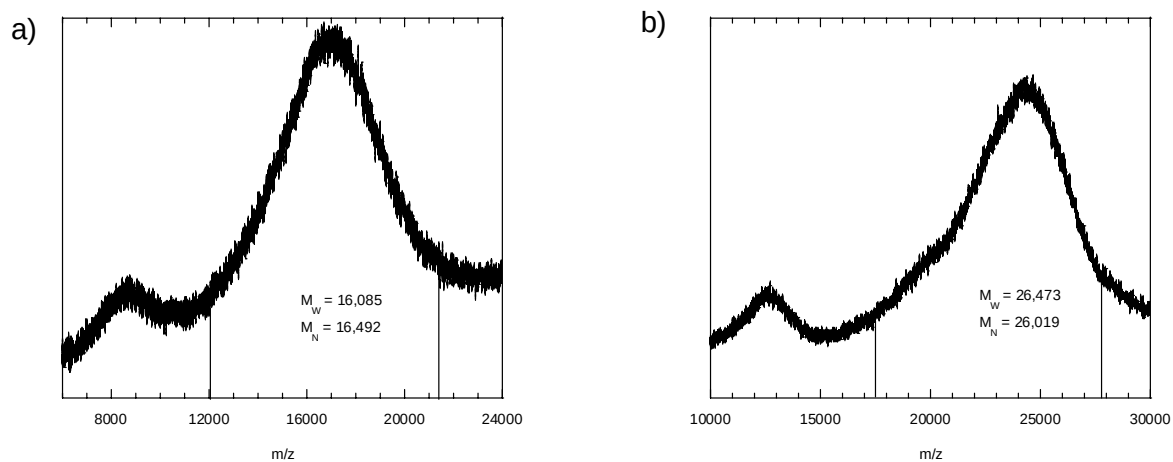


Figure A.11 MALDI-TOF spectra of a) partially (**2** only) and b) fully (**2** & **5**) functionalized 90% **5** and 10% **2** G(4)-PAMAM dendrimer (**7f**).

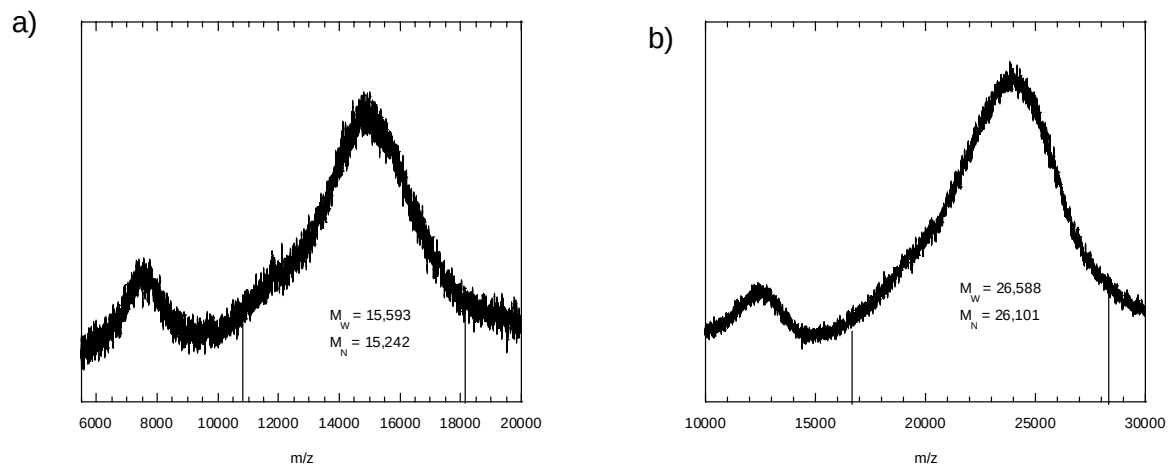


Figure A.12 MALDI-TOF spectra of a) partially (**2** only) and b) fully (**2** & **5**) functionalized 95% **5** and 5% **2** G(4)-PAMAM dendrimer (**7g**).

MALDI-TOF Spectra for Dendrimers **8a-g**

The MALDI-TOF Spectra for **8a-g** heterogeneously functionalized G(4)-PAMAM dendrimers are shown below. The details for the synthesis of these dendrimers are given in CHAPTER TWO.

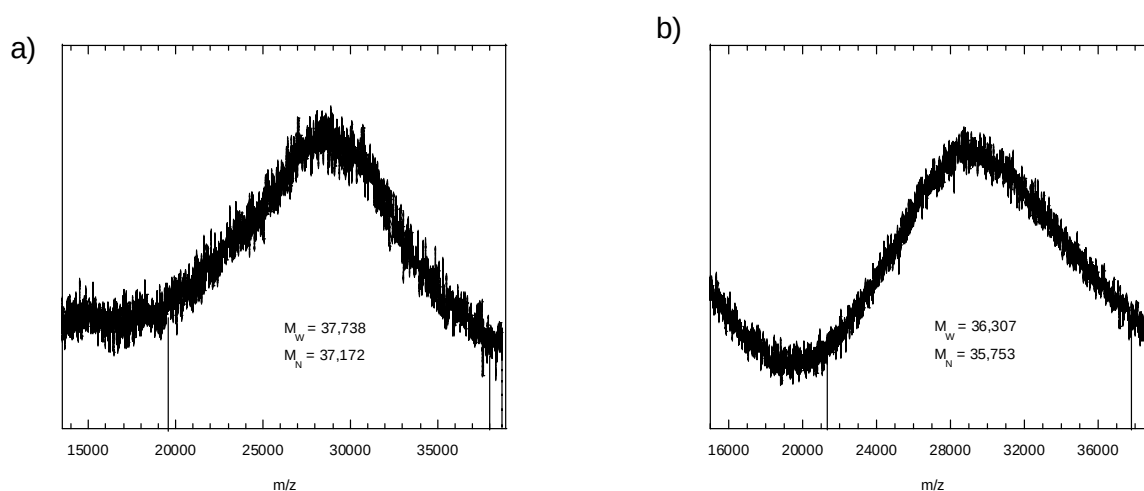


Figure A.13 MALDI-TOF spectra of a) 5% **5** and 95% mannose and b) 10% **5** and 90% mannose heterogeneously functionalized dendrimers.

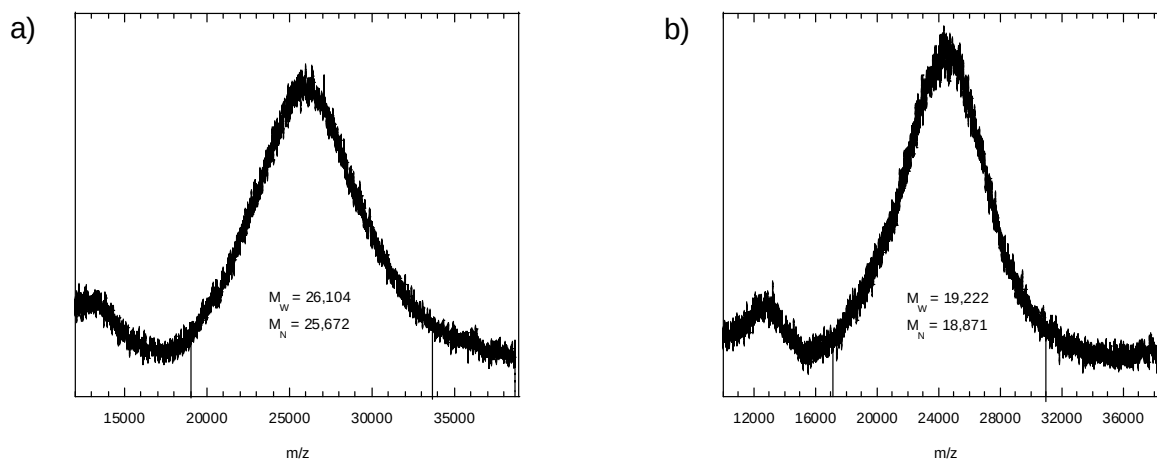


Figure A.14 MALDI-TOF spectra of a) 50% **5** and 50% mannose and b) 75% **5** and 25% mannose heterogeneously functionalized dendrimers.

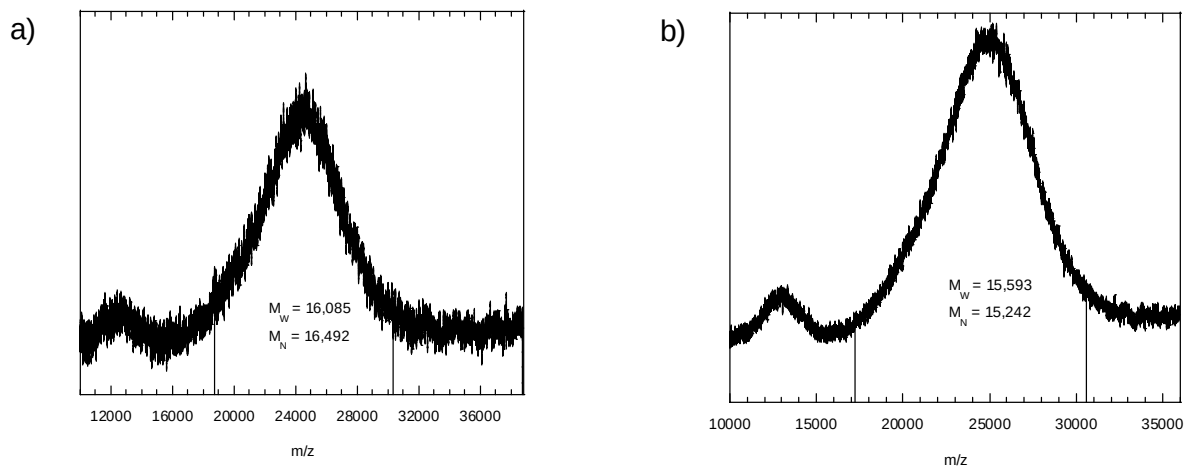


Figure A.15 MALDI-TOF spectra of a) 90% **5** and 10% mannose and b) 95% **5** and 5% mannose heterogeneously functionalized dendrimers.

MALDI-TOF Spectra for Dendrimers **19b-c**

The MALDI-TOF Spectra for **19b-c** heterogeneously functionalized G(4)-PAMAM dendrimers are shown below. MALDI-TOF Spectra for **19a** is in CHAPTER THREE. The details for the synthesis of these dendrimers are given in CHAPTER THREE.

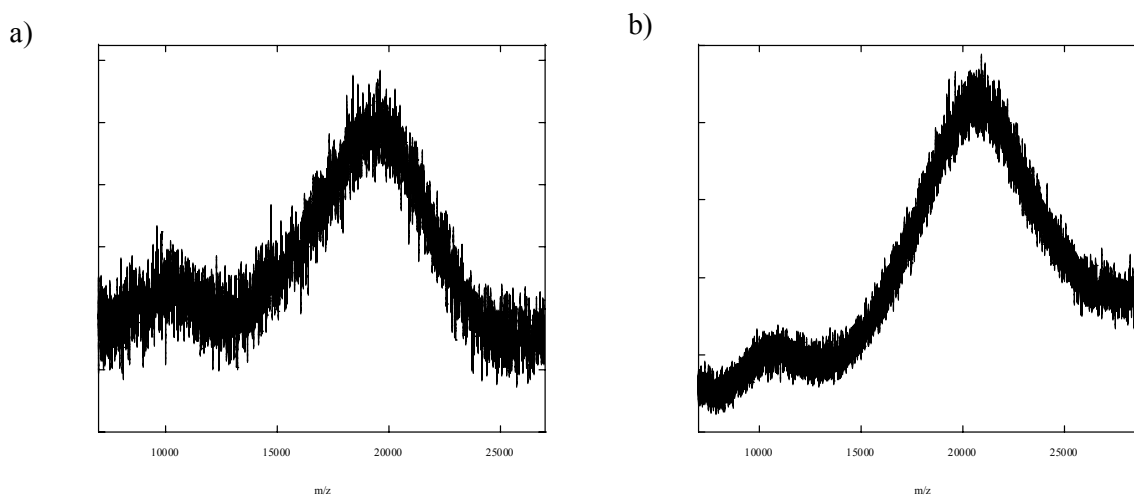


Figure A.16 MALDI-TOF spectra of a) 25% **2** and b) 25% **2** and 25% **5** heterogeneously functionalized dendrimers (**19b** partially functionalized).

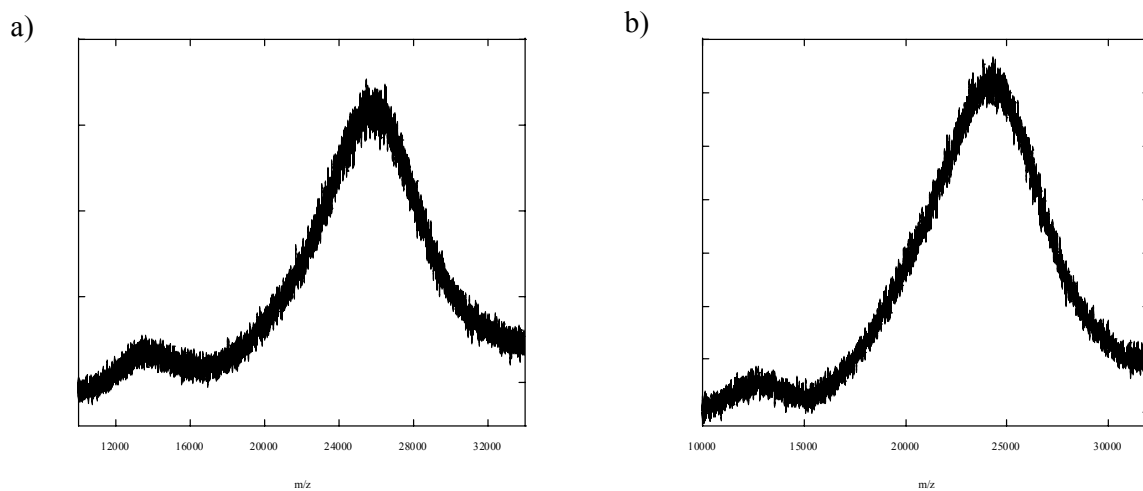


Figure A.17 MALDI-TOF spectra of a) 25% **2**, 25% **5** and 50% **4** and b) 25% mannose, 25% **5** and 50% **4** heterogeneously functionalized dendrimers (**19b**).

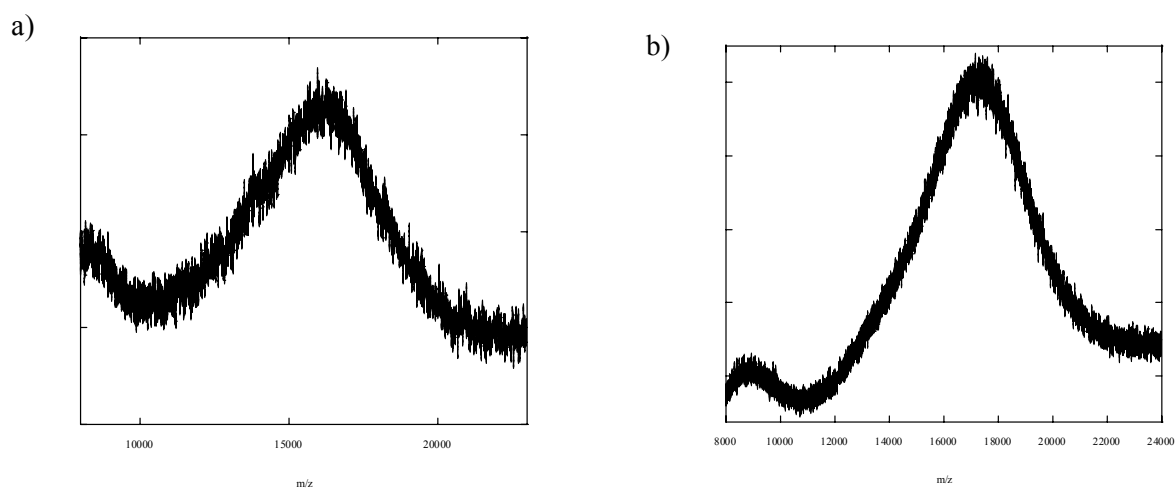


Figure A.18 MALDI-TOF spectra of a) 10% **2** and b) 10% **2** and 25% **5** heterogeneously functionalized dendrimers (**19c** partially functionalized).

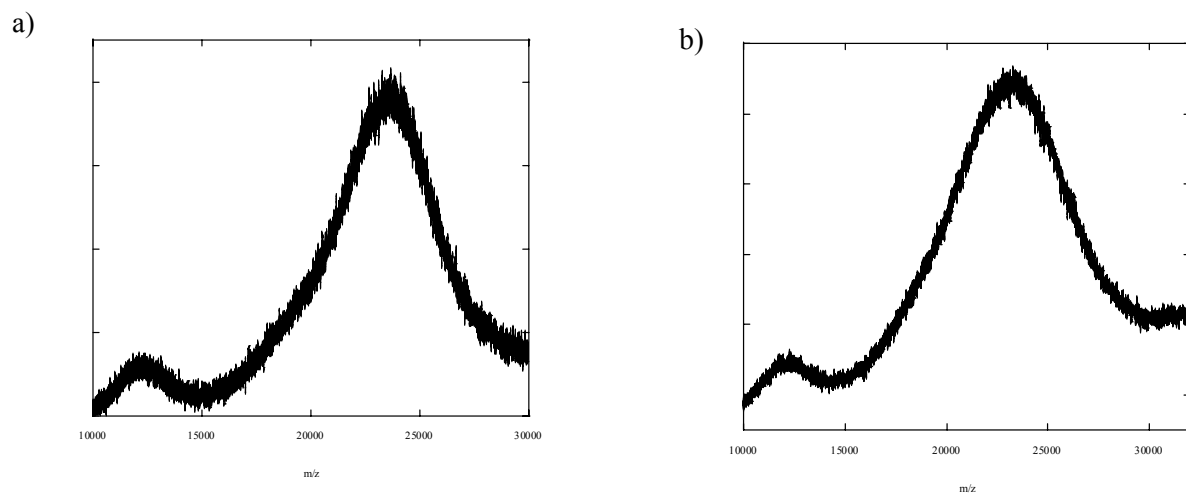


Figure A.19 MALDI-TOF spectra of a) 10% **2**, 25% **5** and 65% **4** and b) 10% mannose, 25% **5** and 65% **4** heterogeneously functionalized dendrimers (**19c**).

APPENDIX B

HEMAGGLUTINATION ASSAY PROCEDURE

The procedure for Hemagglutination was taken verbatim from Eric Wollers dissertation (p. 120 – 123).

To prepare the Concanalin A, in a 10 mL centrifuge tube, approximately 5 mg of Concanavalin A (Con A) was dissolved without agitation in 10 mL of HEPES buffer with 100 μ M CaCl_2 (pH = 8.5). The tube was stored at 4 °C for 8 hours to allow the Con A to dissolve. Afterwards, the solution was placed in a dialysis tube and dialyzed against 1 L of tris-buffered saline (TBS) for 4 hours. This was repeated with fresh TBS solution, followed by dialysis against 1 L of phosphate-buffered saline (PBS) for 8 hours. The dialysis was done to remove any excess Ca^{2+} from the lectin solution. The Con A solution was removed from the tube and stored at 4 °C until needed (the Con A solution can be stored at temperatures below 0 °C for longer periods of time).

To prepare the red blood cells, fresh whole rabbit blood was obtained from the MSU animal care center. It was drawn into a tube containing EDTA. Alsever's solution was added to the blood to make a 60% v/v solution of blood in Alsever's solution. The blood solution was separated into 2 mL aliquots in 15 mL centrifuge tubes. Care was taken to avoid hemolysis of the red blood cells. The red blood cells were diluted to 12 mL with Alsever's solution. The cells were pelleted by centrifugation (1100 rpm x 10 min.). The layer of white blood cells and plasma proteins was removed by pipet. The process was repeated 2 more times using PBS instead of Alsever's solution. Finally, the volume of the red blood cells was estimated (usually about 0.3 mL) and a 2% v/v suspension was made using the assay buffer (0.5% w/v BSA in PBS).

To titrate the Con A, decreasing amounts of Con A were incubated with red blood cells to determine the lectin concentration required to agglutinate the cells. Serial two-fold dilutions were made in the wells of a 96-well V-bottomed microliter plate. The two-fold dilutions were made by adding 50 μ l of Con A solution in the first well. In all of the other wells (typically, a total of 24 wells, is used), 50 μ l of assay buffer was added. In the second well, 50 μ l of the Con A solution was mixed with the assay buffer. After mixing, 50 μ l of the solution in the third well was added to the fourth well, etc... This was repeated until the Con A was diluted through all of the wells of interest. In the end, there should be 50 μ l of solution in each well. To each of the wells, 50 μ l of the red blood cell suspension was added (the suspension was frequently agitated gently to ensure consistent mixing of the red blood cells). Once the blood has been added, the wells were incubated for 1.5 hours at 22 °C (ideally 1h @ 25 °C). After this time, the wells were examined. The minimum concentration required to fully agglutinate the 2% cell suspension was determined. This was considered 1 unit.

The wells in which hemagglutination occurred were differentiated from those in which hemagglutination did not occur. When hemagglutination did NOT occur, the red blood cells settled to the bottom to form a red "button." This was best demonstrated with a control that does not contain Con A. When hemagglutination occurred, the solution in the well stayed as a cloudy suspension. Often, there was an intermediate well that was borderline.

For the assay with inhibitor, 8 times the minimum concentration (8 Units) was used. To determine this concentration, the corresponding well was found, and based on

the serial two-fold dilution, the necessary dilution of the stock Con A was calculated. About 15 mL of the 8 Unit concentration Con A was made. The concentration of this Con A solution was determined spectrophotometrically at 280 nm using $A^{1\%, 1\text{cm}} = 13.7$ @ pH = 7.2 and expressed in terms of the monomer (26,500). Example: for $A = 0.616$, then $0.616 \times 10 \text{ mg/mL} \div 13.7 = 0.45 \text{ mg/mL}$. Since, the FW = 26,500, then $0.45 \text{ mg/mL} \div 26,500 \text{ mg/mmol} = 0.000017 \text{ M}$ or $17 \text{ }\mu\text{M}$.

To determine the inhibiting dose, starting with a concentration of about 10mg/mL, serial two-fold dilutions of various inhibitors were made in the same fashion as the two-fold dilutions of the Con A described above. The inhibitor solutions (50 μL) were incubated with the 8 Unit Con A solution (50 μL for 3 hours at 22 °C (ideally 2 h @ 25 °C). After which, 50 μL of the red blood cell suspension was added. The wells were mixed and incubated for 1h at 22 °C. The minimum concentration causing inhibition of the Con A was determined from the inspection of the wells. This is the inhibiting dose.

Buffers were made using the amount shown below.

Alsever's solution, pH 6.0 (quantities for 1L), contained NaCl (4.2g), sodium citrate (8.0 g, 2.72 mmol), and glucose (20.5g).

Phosphate buffered saline (PBS), pH 7.2 (quantities for 4L), consisted of 0.15 M NaCl (35g), 0.007 M NaH_2PO_4 (3.86 g of dihydrate), 0.018 M Na_2HPO_4 (10.22 g of anhydrous or 19.3 g of heptahydrate).

HEPES buffer, pH 8.5 (quantities for 1L), consisted of 100 μM CaCl_2 (14.7 mg) and 10 mM HEPES (2.38 g HEPES, N-[2-hydroxyethyl]piperazine-N-2-ethanesulfonic acid)]. The pH was adjusted to 8.5 using NaOH.

Tris buffered saline (TBS), pH 7.4 (quantities for 4L), consisted of 0.15 M NaCl (35 g), 0.042 M Trizma HCL (26.44 g), 0.008 M Trizma base (3.88 g). The pH was adjusted to 7.4 using HCl if necessary.

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