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Synthesis and Characterization of Glyconanomaterials, and Their Applications in
Studying Carbohydrate-Lectin Interactions

by

Xin Wang

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Chemistry

Dissertation Committee:
Mingdi Yan, Chair
Andrea M. Goforth
Andres H. La Rosa
Shankar B. Rananavare
Jun Jiao

Portland State University
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Abstract

This dissertation focuses on the synthesis and characterization of glyconanomaterials, as well as their applications in studying carbohydrate-protein interactions. A new and versatile method for coupling underivatized carbohydrates to nanomaterials including gold and silica nanoparticles was developed *via* the photochemically induced coupling reaction of perfluorophenylazide (PFPA). A wide range of carbohydrates including mono-, oligo- and poly-saccharides were conjugated to the nanoparticles with high yields and efficiency. New analytical methods were developed to determine the binding affinities of glyconanoparticles (GNPs) with lectins; these include fluorescence-based competition assay, dynamic light scattering (DLS) and isothermal titration calorimetry (ITC). Results showed that the multivalent presentation of carbohydrate ligands significantly enhanced the binding affinity of GNPs by several orders of magnitude compared to the free ligands. Systematic studies were carried out to investigate the impact of ligand presentation, i.e., the type and length of spacer linkage, the ligand density and the nanoparticle size on the binding affinity of the resulting glyconanoparticles. We used gold GNPs to study interactions with anti-HIV lectin cyanovirin-N (CV-N), and dye-doped silica nanoparticles for labeling glycans and developing high-throughput screening technique.

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Table of Contents

Abstract.....	i
Acknowledgements	ii
List of Tables.....	v
List of Figures.....	vi
1. Introductions.....	1
1.1 Nanomaterials.....	1
1.2 Nanomaterials surface functionalization	6
1.3 Glyconanomaterials	11
1.4 Synthesis of glyconanomaterials	13
2. Development of a Photocoupling Method for the Synthesis of Glyconanoparticles	17
3. Quantitative Analysis of Binding Affinity of Glyconanoparticles with Lectins	44
3.1 Fluorescence-based competition assays	48
3.2 Dynamic light scattering.....	51
3.3 Isothermal titration calorimetry	65
4. Impact of Ligand Presentation on the Binding Affinity of Glyconanoparticles- Multivalency Effect	76
5. Applications of Glyconanomaterials in Bioanalysis	105
5.1 Signal enhancement study glycan-lectin interactions using anti-HIV lectins.	106

5.2 Labeling native glycans with dye-doped silica nanoparticles	119
5.3 High-throughput glycan screening on lectin microarray	136
6. Conclusion	155
References	157
Appendices	
Appendix A. List of Publications	170
Appendix B. Copyright Information.....	172

List of Tables

Table 1.1 Properties of typical nanomaterials and biomedical applications	3
Table 1.2 Typical complementary functional groups of covalent coupling chemistries	10
Table 2.1 Coupling efficiency and surface coverage of D-mannose immobilized on Au NPs using PFPA-disulfide 2 as the coupling agent	32
Table 3.1 LOD and K_D values of GNP-lectin interactions	60
Table 3.2 The thermodynamic parameters of GNP-lectin interactions at 25°C	74
Table 4.1 Coupling yield of mono-, oligo-, and poly-saccharides on Au NPs	88
Table 4.2 Binding affinity of different GNPs with Con A	94
Table 4.3 Binding affinity as a function of ligand density	96
Table 4.4 Binding affinity of GNPs with Con A	100
Table 4.5 Binding affinity of GNPs of varying sizes	102
Table 5.1 Affinities for Man2/3 (K_d) and GNP-M2/3 (K_D) binding to CVN ^{Q50C}	117
Table 5.2 Ligand density and coupling yield of glyco FSNPs	135
Table 5.3 Lectins and their corresponding carbohydrate binding ligands	145
Table 5.4 Association constants (K_a) of lectins and glycans	148
Table 5.5 Apparent K_D values of glyco-FSNPs with lectins obtained from super-microarrays	152

List of Figures

Figure 1.1. Modification of nanomaterials surface by non-covalent and covalent approaches	8
Figure 2.1 Simplified description of phenylazide photochemistry.	19
Figure 2.2 Synthesis of PFPA-Au NPs and subsequent coupling of α -1,4-mannobiose... ..	22
Figure 2.3 Synthesis of PFPA disulfides 1 and 2.	23
Figure 2.4 (a) UV-vis spectra (insert: Au nanoparticle solutions) b) TEM micrographs (scale bar: 200 nm) of Au NPs (A), PFPA-disulfide-functionalized Au NPs (B), Au NPs with surface-coupled α -1,4-mannobiose (C), and subsequent treatment with Con A (D). c) Schematic illustration of the interaction of mannobiose-coupled Au NPs with Con A, and the formation of Au NP aggregates.	30
Figure 2.5 UV-vis spectra of (a) monosaccharide-, and (b) disaccharide-functionalized Au NPs before and after binding with Con A.....	34
Figure 2.6 SPR peak shifts of carbohydrate-functionalized Au NPs after treating with various lectins	36
Figure 2.7 ^1H NMR spectra of PFPA-disulfide 1 and gold nanoparticles functionalized with PFPA-disulfide 1 (Au-PFPA) in CDCl_3 with TMS (0.0 ppm).	38
Figure 2.8 FT-IR spectra of PFPA-disulfide 1, gold nanoparticles functionalized with PFPA-disulfide 1 (Au-PFPA), and Au NPs subsequently coupled with α -1,4-mannobiose (Au-DiMan).....	39

Figure 2.9 Calibration curve obtained by treating various concentrations of D-mannose with anthrone/sulfuric acid and measuring the absorption at 620 nm.	40
Figure 2.10 D-mannose molecules and Au NPs.....	41
Figure 2.11 UV-vis spectra of PFPA-functionalized Au NPs and after treating with 100 nM and 1000 nM Con A.....	43
Figure 3.1. Equilibria involved in the competition binding assay (left). Concentration dependent fluorescence intensity curve (right).....	49
Figure 3.2 (a) Synthesis of silica and Au GNPs: S-GNP and Au-GNP. (b) GNP aggregation upon addition of lectin	56
Figure 3.3 (a-d) DLS spectra and TEM images (inserts, scale bars: 50 nm) when 35-nm S-M-GNP was treated with varying concentrations of Con A. (e) The particle size vs. concentration of Con A.....	57
Figure 3.4 The change in particle diameter (ΔD) vs. lectin concentration: experimental data (circles) and the corresponding Hill fitting curves (lines) for a) 35 nm, b) 110 nm, and 470 nm silica NPs, respectively. The scale bars in the TEM images are a) 50 nm, b) 200 nm, and c) 500 nm respectively.	59
Figure 3.5 Percent increase in particle size (= increase in particle diameter (ΔD)/original particle diameter (D_0) x 100%) vs. concentration of Con A for various GNPs	61
Figure 3.6 (a) Diagram of ITC cells and (b) Typical ITC data. Top: raw ITC data. Bottom: binding isotherm curve.....	65

Figure 3.7 ITC graphs of GNP-Man with a) Con A and b) PNA. The experimental data (solid squares) were fit to theoretical titration curves (solid lines) using the software supplied by the ITC manufacturer	69
Figure 3.8 ITC titration graphs of Con A with (a) GNP-Man2I, (b) GNP-Man2II, and (c) GNP-Man2III. Solid squares: experimental data, lines: fitted titration curves..	71
Figure 3.9 ITC titration graphs of GNP-Man2 with CV-N Mut ^{DB}	73
Figure 4.1 Synthesis of PFPA-thiols a-d.	79
Figure 4.2 Functionalization of Au NPs with PFPA-thiol and subsequent coupling of carbohydrates.	87
Figure 4.3 a) UV-vis spectra of AuNP-a-Man upon addition of increasing concentration of Con A. (b) Absorbance of GNPs at 650 nm vs. Con A concentration.....	89
Figure 4.4 (a) The fluorescence-based competition binding assay; (b) two equilibriums established in the system; (c) Cheng-Prusoff equation, where IC_{50} = concentration of ligand displaying 50% of specific binding; $[M]$ = concentration of free ligand, K_{d1} = dissociation constant of the free ligand with Con A; and K_{d2} = dissociation constant of GNPs with Con A; (d) fluorescence spectra of the supernatant as a function of increasing concentration of AuNP-b-Man ; (e) concentration response curve, where the IC_{50} value was obtained.	91
Figure 4.5 Binding affinity vs. ligand density for Man (a), Man2 (b), and Man3 (c) with Con A.....	97

Figure 4.6 TEM images of gold nanoparticles in 4 different sizes: 7 nm (a), 14 nm (b), 22 nm (c) and 30 nm (d)	101
Figure 4.7 Schematic illustration of Con A and GNP	103
Figure 5.1 Synthesis of Man2- and Man3-conjugated AuNPs GNP-M2 and GNP-M3.....	111
Figure 5.2 UV-vis spectra of GNP-M3 before (solid line) and after (dotted line) treatment with a) CVN ^{Q50C} and b) CVN ^{MutDB} . TEM micrographs of GNP-M3 treated with c) CVN ^{Q50C} and d) CVN ^{MutDB}	113
Figure 5.3 Fluorescence competition assay. a) Schematic representation of binding scenario. b) Modified Cheng-Prusoff equation based on a competitive two site binding model, where [M] is the concentration of the free ligand, and K_{d1} and K_{d2} are the dissociation constants of the free ligand for the glycan-binding sites on Domains A and B of CVN ^{Q50C} , respectively. The data were fitted using the maximum bound fractions, f_{Bmax1} and f_{Bmax2} , corresponding to the two binding sites, and dissociation constants K_{D1} and K_{D2} as adjustable parameters. c) Concentration response curves of GNP-M2 and GNP-M3	114
Figure 5.4 Calorimetric titration of a) CVN ^{Q50C} (35 μ M) with Man2 (1.5 mM), and b) CVN ^{Q50C} (50 μ M) with Man3 (0.64 mM) at 30 °C. The raw data were obtained for 30 and 20 automatic injections, respectively. The integrated curves show experimental points (■) and the best fit (-). The buffer was 50 mM sodium phosphate, 200 mM NaCl, pH 7.5, containing 0.02% NaN ₃	116

Figure 5.5 (a) Synthesis of PFPA-functionalized FSNPs, and (b) high-throughput synthesis of glyco FSNPs	125
Figure 5.6 Fluorescence spectra of FITC-doped silica nanoparticles before and after UV irradiation for 10 min	127
Figure 5.7 TEM images after FSNP-Man was treated with <i>E. coli</i> strain ORN178 (a), or ORN208 (b). Insert: fluorescence image of the corresponding sample	129
Figure 5.8 (a) Preparation of lectin microarray, incubation with FSNP-Man, and fluorescence imaging. (b) Fluorescence image and (c) fluorescence intensities of lectin microarray after treating with FSNP-Man.	130
Figure 5.9 TEM image of FITC-doped SNPs	133
Figure 5.10 Size distribution of FITC-doped SNPs measured by DLS	133
Figure 5.11 Fluorescence spectra of FITC-doped silica nanoparticles before and after UV irradiation for 10 min	134
Figure 5.12 Fluorescence spectra of FITC before and after UV irradiation for 10 min.....	134
Figure 5.13 Schematics of lectin super-microarray fabrication, and the subsequent assays with glyco-FSNPs.....	137
Figure 5.14 Schematics of (a) high-throughput screening, and (b) competition assay on super microarrays.....	138
Figure 5.15 Interactions of Man2-FSNPs with super-microarray: (a) fluorescence intensity vs. the printing concentration of Con A (□); (b) fluorescence intensity vs.	

the concentration of Man2 -FSNPs (○); (c) fluorescence intensity vs. Man2-FSNPs (1.5 mg/mL) incubation time (inserts: fluorescence images of Con A spots (top panel) and SBA spots (bottom panel)). The concentration of Man2-FSNPs in (a) was 1.5 mg/mL, and the printing concentration of Con A was 1 mg/mL in (b)....	143
Figure 5.16 Fluorescence (a) and AFM images (b-d) of a printed Con A spot (1 mg/mL) after incubation with Man2-FSNP (1.5 mg/mL) for 2 h.....	143
Figure 5.17 Carbohydrate structures used in this study	146
Figure 5.18 Fluorescence image (a, c) and fluorescence intensity (b, d) of lectin super-microarrays interacting with glyco-FSNPs.....	149
Figure 5.19 (a) Fluorescence image of a lectin super-microarray after incubating with Man2-FSNP and varying concentrations of 2 α -Man2. (b-d) Fluorescence intensities vs. free 2 α -Man2 concentration for CVN-Q CVN-M (c) and Con A (d).....	151

Chapter 1. Introduction

1.1 Nanomaterials

Research on nanoscience and nanotechnology has increased exponentially over the past decade, with new investigations and discoveries appearing daily ¹. Unique properties of optical, electronic, magnetic, mechanical, and chemical reactivities have been discovered and are associated with nanomaterials solely because of their nanoscale sizes and shapes. These materials serve as model systems providing fundamental understanding of structure-property relationships at the nanoscale. The investigations in turn guide the creation of new structures, systems, and devices with novel properties, functions, and utilities. The interdisciplinary research on nanomaterials merges the fields of synthetic and materials chemistry, condense-matter physics, and fabrication engineering, solving problems in materials synthesis and characterization, and providing core frameworks for biomedical functions ². While challenges remain in improving the capabilities of characterization tools at the nanoscale, and the synthesis of nanomaterials of well-defined size, shape and composition, progress has already been made beyond fundamental research to the development of diverse and versatile nanomaterials-based biomedical devices adopting nanomaterials into current biomedical technologies ^{3,4}. For instance, chip-based microfluidic nanodevices enable high-throughput and exceptionally efficient analysis of gene sequences, greatly expanding the ability for the characterization of

genetic makeup and revolutionizing the specificity of diagnostics and therapeutics. Nanomaterials, having at least one dimension smaller than 100 nm, are comparable in size to many biological molecules. The nanosize dimension allows them to incorporate into cells for *in vitro* and *in vivo* imaging, drug-delivery, and targeting tumor cells ⁵. Nanosensors aid early detection and prevention of diseases, and nanodevices, used remotely and *in vivo*, show high promise for effective and low-cost home-based health-care, benefiting the well-being of the entire human society.

The field of nanomaterials is vastly diverse and is evolving rapidly. A great variety of nanomaterials are synthesized for biomedical applications (Table 1.1); a considerable number of which are polymeric nanomaterials formed either by applying the techniques of nanofabrication or via molecular self-assembly. These polymers, carrying multiple or multifunctional ligands by synthesis or physical encapsulation, have been used in biomedical imaging, as vehicles for drug delivery, and as scaffolds in tissue engineering ⁶. The functionalities on these polymeric nanomaterials are in general built into the material synthesis rather than by surface functionalization.

Table 1.1. Properties of typical nanomaterials and their biomedical applications

Nanomaterials		Intrinsic properties	Biomedical applications
Category	Examples		
Metallic	Au, Ag	SPR	Biosensing, drug delivery, bioimaging
Semiconductor	CdS, CdSe	Fluorescence, luminescence	Immunoassays, bioimaging, biosensing
Magnetic	Fe ₃ O ₄	Magnetism	MRI, drug delivery
Carbon-based	CNTs, Fullerene	Electronic and mechanical properties, conductivity	Drug and gene delivery, therapy, biosensing

Metal nanoparticles (NPs) such as Au and Ag NPs are excellent nanomaterials providing a powerful platform in biomedical applications of biomolecular recognition and sensing, drug delivery, and imaging ^{7,8}. Au NPs are among the mostly used and studied nanomaterials owing to their ease of preparation, stability, well-established surface functionalization chemistry, and their unique optoelectronic properties. The so-called surface plasmon resonance (SPR) absorption, produced by the collective oscillation of conducting electrons in the metal NP core upon interacting with the incident light, is dependent on the NP size and shape, the dielectric property of the media, and the distance between particles. This provides a unique and convenient platform for monitoring the molecular recognition event occurring at close to the

surface of the nanoparticles. Colorimetric bioassays have thus been achieved based on the SPR shift when molecular interactions take place at the surface of the nanoparticles, and have been employed to study fundamental biorecognition processes including cell-cell communication, enzymatic activity, protein-protein interaction, and DNA hybridization. When the ligand-receptor interaction causes additional aggregation of nanoparticles, very large SPR shifts occur producing intense color changes visible to the naked eyes ⁹. These optical properties, induced by single particles or interactions between particles, allow the highly sensitive detection of molecular binding events. In addition, the SPR absorptions are not subject to quenching/photobleaching that are frequently associated with organic fluorophores, or blinking that occurs in quantum dots. An early example was demonstrated by Mirkin and coworkers using oligonucleotide-capped Au NPs ¹⁰. Hybridization of the complimentary oligonucleotide strands induced the aggregation of Au NPs leading to a distinct solution color change easily visualized by naked eyes. Numerous examples can be found in the literature where bioconjugated Au NPs are used as colorimetric biosensors detecting proteins, viruses, and bacteria at an extremely sensitive level. An additional advantage of NPs is that the multiple ligands presented on the NP surface could drastically enhance affinities of specific monovalent interactions via the multivalent binding between NPs and the biological target. Lin et al. reported that the observed binding affinity of mannose-encapsulated Au NPs with Concanavalin A (*Con A*) was several orders of magnitude higher in comparison with that of mannose with *Con A* in solution ¹¹. In the study of Melander et al., SDC-1721, which is a

structural fragment of the HIV inhibitor TAK-77 and displays no inhibition activity in solution, became a potent inhibitor when coupled to 2-nm Au NPs ¹². The authors attributed the enhanced activity to the multivalency effect where multiple ligands presented on the nanoparticle surface greatly enhanced the overall binding affinity with the protein.

Besides metallic nanoparticles, quantum dots (QDs) are zero-dimension materials exhibiting quantum confinement in all three spatial dimensions. QDs have broad excitation spectra yet narrow and tunable emissions, and have thus been widely used as optical labels in a wide range of biomedical applications including immunoassays for proteins, nucleic acids, bacteria and toxin analysis ^{13,14}. Compared with the organic fluorescent dyes, QDs have additional advantages of high quantum yields and high photochemical stability, and offer improved detection sensitivity and application lifetime ¹⁵. Magnetic nanoparticles of iron oxides is another type of attractive nanomaterials that have a long history of investigation and have shown remarkable potentials in biomedical research, ¹⁶⁻²⁴ including magnetic resonance imaging (MRI) contrast enhancement ²⁵⁻²⁷, drug delivery ²⁸⁻³¹, hyperthermia ³²⁻³⁴, cell separation ³⁵⁻³⁸, and tissue repair ³⁹. Superparamagnetic iron oxide nanoparticles can furthermore improve the diagnostic value by enhancing the MRI contrast on surrounding healthy and pathological tissues, increasing the MRI resolution at the microscopic-level ^{40,41}.

An inherent feature of nanomaterials is their high surface areas, ie, high surface-to-volume ratio in comparison with their bulk material counterpart. For instance, for a CdSe QD of ~2 nm in diameter, ~90% of the atoms are located on the surface ⁴².

Nanomaterials thus have high surface energy resulting from increased surface curvature and a greater percentage of dangling bonds that lack nearest bonding neighbors. To minimize the surface energy, nanomaterials tend to adopt a spherical shape, and in addition, to agglomerate into large particles reducing the surface area and thus lowering the surface energy. Surface modification/passivation of nanomaterials is highly necessary where the surface layer serves to reduce the surface energy and at the same time acts as the protective coating preventing nanoparticles from agglomerating thus increasing their long-term stability ⁴³. The capping layer can be further derivatized with additional ligands or functional groups introducing diverse functions and properties to the nanomaterials ⁴⁴.

1.2 Nanomaterial Surface Functionalization

Bio-functions and bio-compatibility of nanomaterials are realized by introducing synthetic ligands or natural biomolecules onto nanomaterials, and combining ligand-receptor biological interactions with intrinsic nanomaterial properties. Common strategies of engineering nanomaterial surfaces involve physisorption or chemisorption of desired ligands. Surface modification of nanomaterials follows the general strategies of non-covalent and covalent approaches (Figure 1.1). The non-

covalent approach is a physisorption process where the ligand is adsorbed to the nanomaterials via the non-covalent forces, including electrostatic interactions, hydrogen bonding, and hydrophobic interactions. A popular method of non-covalent surface functionalization is the so-called steric stabilization that involves polymers or surfactants as the capping layer. The surface coating stabilizes individual nanoparticles, and at the same time, the steric repulsion inhibits agglomeration by keeping the nanoparticle dispersion intact. An added benefit of this process is that monodisperse nanoparticles can be synthesized. The polymer layer adsorbed on the surface of nanoparticles serves as a diffusion barrier to the growing species, resulting in a diffusion-limited growth in the subsequent growth of nuclei. Diffusion-limited growth would reduce the size distribution of the initial nuclei, leading to monodisperse nanoparticles. Furthermore, polymers as the coating materials provide high-density functional groups that can be subsequently derivatized with appropriate ligands for bioconjugation. In the example by Star et. al, a FET device was constructed using polymer-coated carbon nanotubes (CNTs) for the detection of protein binding⁴⁵. CNTs were coated with a mixture of poly(ethylene imine) (PEI) and poly(ethylene glycol) (PEG). PEI provided the functional groups, ie, -NH₂, for the covalent immobilization of the ligand, biotin, to CNTs. PEG, on the other hand, served as a non-fouling coating preventing the non-specific adsorption of proteins on the device, thus giving much increased sensitivity.

Figure 1.1. Modification of nanomaterial surface by non-covalent and covalent approaches.

The majority of surface functionalization methods are based on the covalent bond formation, which offers the advantage of robust linkage and the stability of the surface ligand. If the ligand possesses a functional group that is reactive towards the substrate materials, it can chemisorb to the nanomaterial surface and yield self-assembled structures. Typical examples of chemisorption include thiol/disulfide on metals (Au, Ag, Cu) and semiconductors (CdS, CdSe, ZnS), silanes on oxides (SiO₂, TiO₂), and phosphates on metal oxides (iron oxide, TiO₂). Depending on the nature of the substrate material, ligands possessing the corresponding functional groups are chosen and synthesized. Of the chemisorbed self-assembly systems, thiol/Au is the most

studied and used. The process is well-established and it produces well-behaved self-assembled monolayers that are stable, reproducible, and thoroughly characterized. The system is therefore widely used especially for proof-of-principle studies. The surface functionalization generally follows a simple solution process where the nanomaterial is immersed in a solution containing the ligand. The reaction occurs readily at room temperature. Excess ligands are then removed by rinsing with the solvent leaving behind nanomaterials that are surface-functionalized with the ligand. A highly effective surface engineering strategy is the so-called ligand exchange technique where the ligand of interest displaces the stabilizing capping layer on the nanomaterial. In this case the ligand should have at least equal or higher affinity than the capping molecule towards the nanomaterial in order to partially or fully displace them. In the synthesis of gold nanoparticles by the classic citrate reduction reaction of auric acid, the as-prepared Au NPs bear the citrate capping layer that can be subsequently replaced by a thiol or disulfide ligand ⁴⁶.

Covalent bond formation is also accomplished by reacting the complementary functional groups on the ligand and the surface of the nanomaterial. In this case, the nanomaterial is derivatized with a functional group, which then reacts with the ligand that either possesses the functional group in its native form or is derivatized by chemical synthesis. Table 1.2 shows typical complementary functional groups used for coupling ligands to nanomaterials ⁴⁷⁻⁵⁶.

Table 1.2. Typical complementary functional groups of covalent coupling chemistries.

(L: ligand)

Ligand	Substrate	Ligand decorated surface

1.3 Glyconanomaterials

Carbohydrates are the most abundant biomolecules in nature and essential elements in a wide range of processes in living systems. Besides their use as structural materials and energy sources, they are to large extents mediating recognition events through their interactions with proteins and other biological entities. Complex carbohydrate structures are thus involved in, for example, cell communication and trafficking, tumor genesis and progression, immune responses, fertilization, apoptosis, and infection.⁵⁷⁻⁶³ Many challenges are, however, associated with the study of these processes, and the development of glycoscience has been largely hampered by the complexity and low abundance of the glycan structures involved, and the weak affinities often associated with carbohydrate-protein interactions. The field has recently experienced a dramatic upsurge, much on account of the very strong developments in carbohydrate synthesis, glycan analysis methods, and nanotechnology.⁶⁴ New synthetic methods, such as automated strategies and enzyme-mediated protocols, have resulted in increased availability of complex carbohydrate structures promoting advances in the entire field.⁶⁵⁻⁶⁷

An important development in the field of glycoscience is the discovery that in biological systems, carbohydrates bind lectins, i.e., carbohydrate-binding proteins, in a highly cooperative manner to improve the weak affinity of individual carbohydrate ligands to the lectin.⁶⁸⁻⁷⁰ This cluster or multivalency effect involves multiple

carbohydrate ligands and lectins interacting with each other enhancing binding affinity by several orders of magnitude. For example, oligosaccharides exhibit higher binding affinity than monosaccharides towards the same lectin. In the biological system, lectins associate with cells by interacting with the multiple copies of carbohydrate ligands on the cell surface, exhibiting binding affinities significantly higher than those of the interactions between the lectins and the isolated carbohydrate ligands. Although the quantitative aspect of the multivalency effect is yet to be established, the fact that multivalency can significantly enhance binding affinity has helped fuel a renewed interest in fundamental glycoscience and glycomaterial development. Extensive work has been conducted for conjugating carbohydrates to the scaffolds of proteins, peptides, lipids, and synthetic polymers.⁷¹ Synthetic strategies are applied to control the number of ligands on the scaffold, the spatial display of the ligands, and the structure of the scaffold, which in turn impact the binding affinity of the resulting glycoconjugates with their binding partners. These synthetic multivalent glycoconjugates could bind to receptors competitively having the potential to serve as inhibitors displacing natural ligands in the applications of carbohydrate-based drug design and therapeutics.⁷² When the scaffold is a flat solid surface, efficient glycan microarrays can be generated facilitating the development of high-throughput analysis of ligand-protein interactions in applications of ligand screening and diagnostics.⁷³⁻⁸⁰ Nanomaterials as scaffolds for carbohydrate ligand display have recently emerged, and glyconanomaterials have thus been synthesized, demonstrating great potential in biomedical imaging, diagnostics, and therapeutics. Compared with molecular scaffolds,

nanomaterials as ligand carriers offer a number of attractive features. Nanomaterials, being small in size, have high specific surface areas and can therefore accommodate high-density ligands promoting multivalent interactions with their binding partners. The ligand density can be modulated by the size and shape of the nanomaterial, and multiple epitopes of the same ligand can be exposed and presented in a three-dimensional format. Nanomaterials possess unique optical, electronic, magnetic, and mechanical properties, as well as chemical reactivities. These properties, together with their nanosized dimensions allow them to be incorporated into cells for *in vitro* and *in vivo* imaging, drug-delivery, and targeting tumor cells. This opens up a wide range of possibilities, the potential of which is just emerging.⁸¹⁻⁸⁵

1.4 Synthesis of glyconanomaterials

Two general strategies for nanomaterial functionalization can be discerned, based on either non-covalent or covalent protocols. Both approaches are associated with advantages and drawbacks, although covalent protocols are generally preferred due to the considerably higher stabilities of the constructs.

1) Non-covalent attachment

A variety of glyconanomaterials based on physisorption of carbohydrate ligands to the material surface has been reported. The attachment relies on non-covalent interactions,

including, for example, hydrogen bonding, Coulombic interactions, and hydrophobic effects. A method for producing metallic glyconanoparticles through electrostatic adsorption was reported by Yang *et al.*, in which metal-chitosan nanocomposites were prepared on a range of different metals, including Au, Ag, Pt and Pd.⁸⁶ The nanoparticles were synthesized by reducing metal salts in the presence of chitosan, resulting in simultaneous ligand adsorption. Rosenzweig *et al.* synthesized dextran-coated quantum dots (QDs) where negatively-charged carboxymethyldextran was adsorbed onto QDs by mixing with positively-charged polylysine via electrostatic interactions.⁸⁷ As noticed from these examples, a notable advantage of the physisorption strategy is that the reaction conditions are relatively mild, and minimal chemical derivatization is required for the nanomaterials substrates and the carbohydrate ligands. Nevertheless, the physical adsorption is relatively random and disordered compared to covalent linkages. In addition, the strength of the association is not sufficiently strong, which may lead to potential bond breakage during interactions, as well as increased nonspecific or unexpected interactions with the target molecules. This can significantly affect the specificity and sensitivity in applications such as biological sensing and recognition. However, as demonstrated in the mentioned examples, oligomer/polymer-based ligands can to some extent circumvent the stability problems.

2) Covalent attachment

The most commonly used method for conjugating carbohydrate structures to nanomaterials is based on covalent attachment. Among the various nanomaterials, Au NPs are the most extensively used scaffold materials especially in fundamental studies due to their ease of preparation, exceptional stability, and high reproducibility.⁸⁸ Au NPs of different sizes, shapes, and controlled dispersity can now be synthesized using simple solution-based methods. The well-established thiol- and disulfide-Au chemistry, first applied to nanoparticles using a two-phase system by Brust *et al.*, allows the preparation of Au NPs with well-defined surfaces.⁸⁹ These surface ligands serve as a protective layer to provide high stability for the nanomaterials in media ranging from organic solvents to biological milieus. The chemistry has been widely adopted to prepare Au NPs modified with various functional groups, and biological molecules including DNA, proteins, peptides, and carbohydrates have all been successfully introduced into the system.^{10,90,91} Penadés *et al.* reported the first synthesis of carbohydrate-functionalized Au NPs (glyconanoparticles, GNPs).^{92,93} The trisaccharide determinant of the Lewis^x (Le^x) antigen was derivatized with an alkylthiol, and Le^x-coated Au NPs were prepared by reducing HAuCl₄ with NaBH₄ in presence of the thiol-derivatized Le^x. Based on this strategy, Au NPs functionalized with monosaccharides (glucose), disaccharides (maltose), and tetrasaccharides (Le^y) were prepared and applied to the studies of various biological interactions.^{94,95} Later, several other groups utilized a similar strategy to produce Au and Ag glyconanoparticles using thiolated carbohydrates.^{11,96-100} Furthermore, thiolated carbohydrate derivatives have been adopted in the preparation of glyco-quantum dots

(GQDs).¹⁰¹⁻¹⁰³ Additional coupling methods based on the reaction of complementary functional groups have also been developed to facilitate the conjugation of carbohydrates other than the thiolated derivatives. Examples include coupling *N*-hydroxysuccinimide (NHS)-functionalized dextran to amine-functionalized Ag NPs,¹⁰⁴ and amine-derivatized carbohydrates to aldehyde-functionalized Au NPs.¹⁰⁵

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Overall, the objective of my PhD project is as follows:

1. To synthesize glyconanomaterials using the photocoupling strategy to covalently attach a variety of underivatized carbohydrates onto nanomaterials;
2. To develop new analytical methods to study the binding affinity of glyconanomaterials with lectins;
3. To comprehensively investigate the effect of ligand presentation on binding affinity on glyconanomaterials;
3. To develop applications of glyconanomaterials in bioanalysis, such as lectin recognition, affinity enhancement, glycan labeling, and high-throughput screening.

These studies will provide a fundamental understanding of glyconanomaterials, and illustrate their importance in studying carbohydrate-involved biological interactions. The methodologies established should benefit the study of bio-functionalized nanomaterials and enable a wide range of biomedical applications.

Chapter 2. Development of a Photocoupling Method for the Synthesis of Glyconanoparticles

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Carbohydrate structures expressed on cell surfaces are vastly diverse. The matter is further complicated by the fact that half of all cellular proteins are post-translationally modified by the addition of structurally diverse and complex glycans with many types of chemical bonds and branch-chains. Therefore highly desirable for carbohydrate immobilization are effective coupling chemistries that are general and versatile, can accommodate ligand diversity and give stable interfaces, and yet are simple and reproducible. Current coupling methods involve chemical derivatization of carbohydrate structures to introduce functionality that then react with the functional groups on the nanoparticles, for example, by chemisorption of thiol-functionalized carbohydrates on gold nanoparticles.

In this chapter, a new technique has been developed and described for coupling underivatized carbohydrates on gold nanoparticles. The method is based on the photochemistry of perfluorophenylazides (PFPA), which upon light activation, undergoes C-H insertion reaction with neighboring molecules. Phenylazides and derivatives were the most popular photoaffinity labeling (PAL) agents, due to their high reaction efficiency, fast kinetics, excellent storage stability and ease of synthesis. Upon light irradiation, they decompose by releasing N_2 to give singlet phenylnitrene, a highly reactive intermediate which can undergo numerous nonselective reactions yielding a wide range of products. Three main processes of phenylnitrene reactions are shown in Figure 2.1: ring expansion, insertion/addition and intersystem crossing (ISC).¹⁰⁶

Figure 2.1 Simplified description of phenylazide photochemistry.

Because all carbohydrates and glycoconjugates possess C-H bonds, no chemical derivatization is required and carbohydrate structures can be directly coupled to solid substrates in their native forms. We have successfully attached monosaccharides and oligosaccharides on Au nanoparticles using this approach. The immobilized carbohydrates retained their recognition abilities with lectins, and the binding strength, measured by the SPR red-shift of Au nanoparticles, was consistent with the binding affinity of the free carbohydrate with the corresponding lectin. This general coupling chemistry together with the unique and sensitive optical property of the resulting nanoparticles serve as a label-free and rapid detection platform readily applicable to clinical diagnosis, sensing, and ligand screening.

Metal nanoparticles coupled with biological ligands have been widely used for monitoring activities of biomolecules and their interactions with ligands.¹⁰⁷ A unique characteristic of metal nanoparticles as the recognition probe is their remarkable and tunable optical property, the so-called surface plasmon resonance (SPR), determined by their size and shape, the dielectric property of the media, and the distance between particles. Colorimetric bioassays have thus been achieved based on the SPR shift when molecular interactions take place at the surface of the nanoparticles, and have been employed to study fundamental biorecognition processes including cell-cell communication, enzymatic activity, protein-protein interaction, and DNA hybridization.^{9,108,109} When the ligand-receptor interaction causes additional aggregation of nanoparticles, significant red-shift of the SPR absorption occurs producing intense color changes visible to the naked eye.¹¹⁰⁻¹¹³

Naturally occurring carbohydrates, glycoproteins, and glycolipids are present at the surface of nearly every cell in living systems, and play crucial roles in biological events as recognition sites between cells and different binding partners. They for example mediate various phenomena including cell growth, inflammatory responses or viral infections, and changes in glycosylation are often involved in disease states, including cancer. Efficient analysis and control of such events are of high importance. Carbohydrate-based detection platforms have recently emerged as highly useful analytical and diagnostic tools, and have demonstrated tremendous potential to superior sensitivity, selectivity, and stability.^{68,114,115} A key technology requirement in

receptor/ligand-based sensing and detection is the surface conjugation chemistry that can effectively couple ligands to solid substrates. Carbohydrate structures expressed on cell surfaces are vastly diverse and complex. Despite the development of new synthetic and enzymatic protocols, obtaining large quantities of glycans with the required functionality and precise glycosylation pattern is still a major challenge. Highly desirable are effective coupling chemistries that are general and versatile, can accommodate carbohydrate diversity and give stable interfaces, and yet are simple and reproducible. Ligands attached to solid surfaces through a covalent bond are more stable than those that are physisorbed by weaker forces. Of the reported covalent coupling chemistry, the most popular involves chemisorption of thiolated carbohydrates on Au nanoparticles.^{11,105,116-120} Other conjugation methods include coupling *N*-hydroxysuccinimide (NHS)-functionalized dextran to amine-functionalized Ag NPs,¹⁰⁴ and amine-functionalized carbohydrates to aldehyde-functionalized Au NPs.¹⁰⁵ Coupling chemistry that does not require chemical derivatization of the carbohydrates is appealing. A few examples have been reported to conjugate underivatized carbohydrates on flat substrates for microarray construction, although the protocols have not been adapted for Au NPs. One approach used hydrazide-modified gold substrates where the hydrazide reacted with the terminal aldehyde group of the carbohydrates.^{56,121} A similar approach employed amine-functionalized surfaces and the coupling with carbohydrates took place by reductive amination to yield an amine conjugate.¹²² In both cases, reducing carbohydrates are

necessary, and for monosaccharides, the coupled products often became acyclic and lost their binding affinity.

Figure 2.2 Synthesis of PFPA-Au NPs and subsequent coupling of α -1,4-mannobiose.

We have successfully employed PFPAs in surface modification, targeting polymeric materials that lack reactive functional groups for surface coupling.¹²³⁻¹²⁶

Carbohydrates are another class of compounds that are well-suited for the PFPA photocoupling chemistry. The design of our approach is to prepare PFPA-functionalized Au NPs which can be subsequently be used to covalently couple, in principle, any carbohydrate structures by way of the insertion reactions of photochemically activated nitrene species. (Figure 2.2) This coupling chemistry does not require chemical derivatization of the carbohydrate, which can be complex when multiple protection and glycosylation reactions are involved. PFPA has been successfully utilized to immobilize hyaluronan on polystyrene (PS) beads.¹²⁷ PS beads with surface amino groups were treated with NHS-functionalized PFPA, and

hyaluronan was then attached to the bead surface by UV irradiation. In this chapter, we report that PFPAs can be employed to conjugate monosaccharides and oligosaccharides on Au NPs. The surface-bound carbohydrates retained their binding affinity with the corresponding lectins, and the ranking of binding affinity was consistent with that observed for free ligands in solution. A colorimetry method was developed to determine the density of the carbohydrates attached to Au NPs. Results showed that the coupling chemistry is efficient and high yielding.

Experimental details

Figure 2.3. Synthesis of PFPA disulfides **1** and **2**.

Synthesis.

PFPA-disulfide **1** was prepared following a previously reported procedure.¹²⁸

11,11'-Disulfanediyldi(undecane-11,1-diyl)bis(4-azido-2,3,5,6-tetrafluorobenzoate)

(PFPA-disulfide **2**). 11-Mercapto-1-undecanol (90 mg, 0.42 mmol) in absolute ethanol (15 mL) was titrated with a saturated solution of iodine in ethanol until the brown color of iodine persisted. The solution was concentrated to 2 mL and then water (15 mL) was added. The solution was extracted using diethyl ether (3 x 15 mL), and the combined ethereal extracts were washed with brine, dried over NaSO₄, and the solvent was removed under reduced pressure to afford the disulfide **4** as a brown oil. A solution of **3**¹²⁹ (100 mg, 0.42 mmol) in CH₂Cl₂ (15 mL) was cooled to 0 °C, and *N,N'*-dimethylaminopyridine (DMAP) (5.2 mg, 0.042 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (88.6 mg, 0.046 mmol) were added. The disulfide **4** was then added and the solution was stirred for 1 h, after which the solution was allowed to warm to room temperature, and stirred for 12 h. The product was recovered by extraction with CH₂Cl₂. The organic layer was washed with water, brine, and dried over Na₂SO₄. Purification of the crude product was carried out by flash column chromatography with 10/1 v/v hexane/ethyl acetate to afford PFPA-disulfide **2** as clear oil (74.1 mg, 42%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 4.36 (t, *J*=7.4 Hz, 2H), 2.68 (t, *J*=7.2 Hz, 2H), 1.76-1.72 (m, 4H), 1.41-1.28 (m, 14H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 159.4, 146.0 (d, *J*_{C-F}=258 Hz), 140.4 (d, *J*_{C-F}=255

Hz), 124.7, 107.1, 63.8, 38.7, 34.4, 31.5, 29.8, 28.8, 28.1, 27.7, 25.1. Anal. Calcd for $C_{36}H_{44}F_8N_6O_4S_2$: C, 51.42; H, 5.27; N, 9.99. Found: C, 51.50; H, 5.31; N, 10.01.

Sample preparation

The gold nanoparticles were prepared following a modified procedure of the two-phase system. A 0.25 mM aqueous solution (100 mL) of $HAuCl_4$ (Aldrich) was heated to boiling and 1 wt% sodium citrate solution (1.8 mL) was added quickly under vigorous stirring. The solution was allowed to boil for an additional 5 min until the color of the solution became dark purple and finally light red. A 1.7 mM solution of PFPA-disulfide **1**¹²⁸ or **2** in acetone (5 mL) was added slowly to the Au NP solution, and the solution was stirred for 10 hours when it turned to burgundy color. Toluene (15 mL) was subsequently added, and the mixture was vigorously stirred for 1 hour leaving behind a light pink aqueous phase. The toluene layer was then separated, concentrated to 5 mL using a rotary evaporator at 45 °C, and then diluted with acetone (20 mL). The diluted solution was kept in refrigerator overnight, and centrifuged at 14,000 rpm for 30 min. Precipitates were collected and re-dissolved in acetone by sonication for 1 min, and was further centrifuged. The re-dissolution and centrifugation processes were repeated 3 times to remove the excess PFPA-disulfide. The functionalized Au NPs were kept in acetone for storage. To determine the concentration of functionalized Au NPs, an aliquot of the solution was centrifuged, and the precipitate collected, dried, and weighed.

Carbohydrates were coupled to Au NPs according to the following general procedure using α -1,4-mannobiose as the example. A solution of PFPA-functionalized gold nanoparticles (1.5 mL) was mixed with 2.9 mM of α -1,4-mannobiose aqueous solution (0.1 mL, V-Labs) in a short flat beaker. The mixture was covered with a 280-nm long-path optical filter (WG-280, Schott Glass) and was irradiated with a 450-W medium pressure Hg lamp (Hanovia) for 5 min under vigorous stirring. Centrifugation of the solution at 14,000 rpm for 15 min separated the mannobiose-attached gold nanoparticles as precipitates. Excess mannobiose was removed by rinsing the nanoparticles with water 3 times and centrifugation.

Carbohydrate density determination

A freshly-prepared anthrone solution in concentrated H_2SO_4 (0.5 wt%, 1 mL) was added into various concentrations of D-mannose in water (0.5 mL) in ice bath under stirring. The solution was then heated to 100°C and stirred for 10 min. After cooled to room temperature, the UV-vis spectra of the resulting solutions were recorded on a Perkin-Elmer Lambda 45 UV-vis spectrometer. The absorbance of the solution at 620 nm was measured and the data were plotted against the concentration of D-mannose. The result was used as the calibration curve for the calculation of the ligand density on Au NPs. Ligand density experiments for Au NPs were carried out by dissolving freshly-prepared D-mannose-conjugated Au NPs (0.3-0.5 mg, measured by drying the Au NPs solution under the reduced pressure) in 0.5 mL Milli-Q water, and the solutions were treated with anthrone/ H_2SO_4 following the same protocol described

above. The absorbance of Au NPs were deducted from the total signals measured as the background, and the density of D-mannose immobilized was then determined using the calibration curve.

Lectin binding assay

The binding affinity of carbohydrates conjugated on Au NPs was evaluated using Con A according to the following procedure. The binding studies with other lectins (lectin from *Griffonia simplicifolia* (GS II), lectin from *Arachis hypogaea* (peanut) (PNA), lectin from *Glycine max* (soybean), Sigma) were carried out in the similar manner. In the experiment, the nanoparticles were incubated in a 10 mM pH 7.4 PBS buffer solution containing 0.1% Tween 20 and 3% bovine serum albumin (BSA) for 30 min, centrifuged, and incubated in a pH 7.4 PBS solution without BSA for another 20 min. The nanoparticles were subsequently treated with 10 µg/mL Con A (from *Canavalia ensiformis* (Jack bean), Sigma) solution in pH 7.4 PBS buffer (0.05 mL) containing 10 mM MnCl₂ and CaCl₂ for 1 hour while shaking. In cases where aggregation was induced after binding with Con A, the suspension was transferred to a centrifuge tube and centrifuged at 14,000 rpm for 15 min.

Results and discussion

PFPA-disulfides **1** and **2** were synthesized by coupling PFPA-COOH **3** with the corresponding diol using EDAC (Figure 2.3). Diol **4** was prepared by oxidizing the hydroxythiol with I₂.¹³⁰ The two disulfides were chosen differing in the length of the

spacer linkage. A one-pot procedure was developed to simultaneously synthesize and functionalize Au NPs with PFPA. Colloidal Au NPs, ~20 nm in diameter, were prepared using the citrate reduction reaction of HAuCl₄. The resulting citrate-stabilized Au NPs were light red in color exhibiting the surface plasmon absorption peak at ~520 nm in the UV-vis spectrum. The Au NPs were subsequently functionalized with PFPA-disulfide (Figure 2.3) via a modified phase-transfer ligand-exchange reaction, after which the PFPA-functionalized Au NPs migrated to the organic phase, indicating that the hydrophilic surface of citrate-stabilized Au nanoparticles became hydrophobic. The successful functionalization of Au NPs with PFPA was confirmed by ¹H NMR and FTIR (see Figures 2.7 and 2.8 in Supporting Information). The subsequent coupling of carbohydrates to PFPA-functionalized nanoparticles was carried out by mixing an aqueous solution of the carbohydrate with the nanoparticles in acetone, and irradiating the mixture with >280 nm light. FTIR spectra of the resulting nanoparticles show that the characteristic -N₃ absorption at 2125 cm⁻¹ disappeared (see Figure 2.8), indicating that the azido groups were activated. The mannobiose coated NPs could be redispersed in water without significant change in optical density due to coating of high-density carbohydrate ligands, and excessive unattached carbohydrate was completely removed through water rinses. Accompanied by each step of the surface functionalization is the color change of the nanoparticle solution, shown in Figure 2.4a where α-1,4-mannobiose was coupled on Au NPs. The UV-vis spectra of PFPA-Au and mannobiose-Au both underwent red-shifts after

surface functionalization (8 nm and 4 nm, respectively), likely due to the slight size growth and the change of environment around the nanoparticles.

The amount of carbohydrate ligands coupled to Au NPs was determined by a colorimetry method using anthrone/sulfuric acid.^{131,132} This is a well-established assay for the quantitative analysis of carbohydrates, and has been adopted in glyconanoparticle analysis.¹³³ We investigated the ligand density on Au NPs using D-mannose. A calibration curve was obtained by treating various concentrations of D-mannose with anthrone/sulfuric acid, and the absorption at 620 nm was measured (Figure 2.9 in Supporting Information). Au NPs with D-mannose immobilized were subjected to the same assay and the absorptions at 620 nm were recorded. The amount of D-mannose attached to Au NPs was subsequently derived from the calibration curve, which averaged at 24 nmol/mg Au NPs, or 1,200 molecules per Au NP. Assuming that D-mannose ligands are close-packed on the NP, the maximal amount of D-mannose that can occupy on each 20-nm Au NP was calculated to be 72 nmol/mg Au NPs, or 3,500 molecules per Au NP (see Supporting Information for detailed calculation). This result corresponds to a surface coverage of 34% of the photocoupled D-mannose, indicating a fairly reasonable coupling efficiency.

Figure 2.4 (a) UV-vis spectra (insert: Au nanoparticle solutions) b) TEM micrographs (scale bar: 200 nm) of Au NPs (A), PFPA-disulfide-functionalized Au NPs (B), Au NPs with surface-coupled α -1,4-mannobiose (C), and subsequent treatment with Con A (D). c) Schematic illustration of the interaction of mannobiose-coupled Au NPs with Con A, and the formation of Au NP aggregates.

To further investigate the efficiency of the photocoupling reaction, various concentrations of D-mannose were used when mixing with PFPA-functionalized Au NPs during light activation. Here PFPA-disulfide **2** was used in the study. The amount of D-mannose attached to Au NPs was then determined by the anthrone/sulfuric acid assay described above, and results are summarized in Table 2.1. At lower ligand loading, the coupling yield was high but the surface coverage was low. As the amount of added ligand increased, the coupling efficiency decreased whereas the surface coverage increased drastically before saturating at around 80%. Note that even at low ligand loading concentration of 100 nmol/mg NPs, high surface coverage (80%) was obtained while relatively high coupling efficiency of 57% was achieved. The result is significant that large excess of ligand is unnecessary, which is especially beneficial to carbohydrates that are difficult or costly to obtain. Moreover, this approach provides a simple means to control the ligand density on the NPs. Nanoparticles with coupled D-mannose density varying over 3 orders of magnitude can be produced by changing the amount of the ligand initially added. Note that the amount of D-mannose coupled to Au NPs functionalized with PFPA-disulfide **2** (57.4 nmol/mg) was higher than that on NPs functionalized with **1** (31.6 nmol/mg) with the same initial ligand concentration, demonstrating that the longer spacer increased the coupling efficiency.

Table 2.1. Coupling efficiency and surface coverage of D-mannose immobilized on Au NPs using PFPA-disulfide **2** as the coupling agent.

Mannose Added (nmol /mg NPs)	Mannose Coupled (nmol /mg NPs)	Coupling Yield (%) [a]	Surface Coverage (%) [b]
0.1	0.081	85	0.11
0.5	0.40	80	0.56
1	0.78	78	1.1
5	3.1	61	4.3
10	5.3	53	7.4
50	30	59	41
80	44	56	62
100	57	57	80
120	58	N/A	80
150	57	N/A	80

[a] Coupling Yield = Mannose Coupled/Mannose Added x 100%. [b] Surface Coverage = Mannose Coupled/Max. Mannose Computed x 100%. The Max. Mannose Computed is 71.7 nmol/mg NPs for 20-nm Au NPs.

The carbohydrate-functionalized NPs were subsequently subjected to binding studies with a series of lectins, i.e. carbohydrate-binding proteins, to investigate whether the coupled carbohydrates retained their binding affinity. Concanavalin A (Con A), a mannose-binding protein, was used as a model system to test the effectiveness of this coupling chemistry. At pH>7, Con A is tetrameric, each monomer having one

saccharide binding site specific for mannose, and to a lesser extent, glucose.¹¹⁴ Upon treating the mannanose-Au NPs with Con A, rapid and drastic color change occurred (D, Figure 2.4a insert), and the UV-vis spectrum of the resulting solution showed a large SPR red-shift of ~75 nm (D, Figure 2.4a). Simultaneously observed was the cluster formation causing broadening of the SPR peak and a decrease in the absorption intensity. The aggregation of the nanoparticles is likely a result of Con A's multiple binding sites for mannose bringing together dimannose-modified nanoparticles. Indeed, TEM micrographs showed that the nanoparticles were discrete and isolated until the addition of Con A (Figure 2.4b). The tetrameric Con A acted as a crosslinking agent that agglomerated mannose strongly, forming larger sizes of nanoclusters (Figure 2.4c). A control experiment was carried out where PFPA-functionalized Au NPs were treated with Con A. No SPR peak shift was observed in the UV-vis spectrum of the resulting solution (Figure 2.11 in Supporting Information). Therefore the SPR shift and agglomeration can only be attributed to the carbohydrate ligands on the Au NPs.

Figure 2.5 UV-vis spectra of (a) monosaccharide-, and (b) disaccharide-functionalized Au NPs before and after binding with Con A. Only one set of experimental data is shown here. The experiments were, however, repeated over 5 times and the results were consistent and reproducible.

To further investigate the generality of this coupling chemistry and the specificity of surface-bound carbohydrates, monosaccharides (D-mannose, D-glucose, D-galactose) and disaccharides (maltose, sucrose, α -1,3-galactobiose) were coupled to the PFPA-functionalized Au NPs using the same experimental protocol. The resulting carbohydrate-NPs were subsequently treated with Con A and the UV-vis spectra were recorded. D-Glucose is a known ligand of Con A with a lower binding strength than that of D-mannose, and D-galactose is a non-binding ligand for Con A.^{134,135} The binding constants vary depending on the measurement methods. In the work of Mandal et al., the association constants (K_a) of D-glucose and D-mannose were reported to be $1.96 \times 10^3 \text{ M}^{-1}$ and $8.2 \times 10^3 \text{ M}^{-1}$, respectively, measured by isothermal microcalorimetry.¹³⁶ In our studies, the UV-vis spectra of the monosaccharide-modified Au NPs showed the largest SPR red-shift for D-mannose (67.7 nm) compared to D-glucose (25.2 nm), whereas almost no change was observed for D-galactose-functionalized NPs (Figure 2.5a). These results correlated well with the affinity ranking of the free monosaccharides with Con A in solution.¹³⁶ Similar results were also observed for disaccharide-functionalized NPs. Maltose, having two D-glucose units, showed a red-shift of 30.1 nm whereas sucrose, containing one D-glucose unit, gave a red-shift of 26.1 nm upon binding with Con A. Almost no change in SPR absorption was observed for galactobiose which is consisted of two non-binding D-galactose units (Figure 2.5b).

Figure 2.6 SPR peak shifts of carbohydrate-functionalized Au NPs after treating with various lectins. Each data was an average of 5 samples.

Additional cross-reactivity studies were conducted by treating Au NPs conjugated with mono- and di-saccharides (D-mannose (Man), D-glucose (Glc), D-galactose (Gal), α -1,4-mannobiose (DiMan), β -1,3-glucobioses (DiGlc), α -1,3-galactobiose (DiGal) and *N*-acetyl-D-glucosamine (GlcNAc)), with Con A and 3 other lectins (GSII, PNA and SBA). The SPR peak shifts were determined from the UV-vis spectra and are shown in Figure 2.6. The affinity ranking derived from the SPR peak shift directly correlates with reported solution binding affinity between each carbohydrate and lectin.^{68,114} For example, larger shifts were observed for the carbohydrate-lectin pairs of Gal-SBA, Gal-PNA, DiGal-SBA, DiGal-PNA, and GlcNAc-GSII, which was consistent with our previous study using a carbohydrate microarray where strong interactions were also observed for these carbohydrate-lectin pairs.¹²⁸

Conclusions

In summary, we have developed a general method for coupling carbohydrates to gold nanoparticles. The method is based on the photochemically induced CH insertion reactions of PFPAs, and it does not require chemical derivatization of the carbohydrate structures. Furthermore, the coupling reaction is fast, taking place in minutes instead of hours which is needed in most thermally-initiated conjugation reactions. The coupling efficiencies were high, and surface coverage of over 80% was obtained. The coupled carbohydrates effectively retained their recognition abilities as demonstrated by the strong interactions with their corresponding carbohydrate-binding proteins. In addition, the binding affinities of surface-bound carbohydrates with various lectins were consistent with those of the free carbohydrates with the corresponding lectins in solution. The sensitive SPR signal was conveniently used to monitor the surface chemistry occurred on the nanoparticles, especially in examining the interactions of surface-bound carbohydrates with their binding proteins where large SPR red-shifts were observed causing visible color changes of the Au NP solutions. The method developed can be readily applied to other carbohydrate structures, and we have successfully coupled oligosaccharides and polysaccharides using the same approach. This general coupling chemistry together with the convenient optical detection offers an attractive platform for label-free, rapid, and sensitive detection of carbohydrate-based molecular recognition.

Supporting Information

Characterization of PFPA-functionalized and mannobiose-conjugated Au NPs

Figure 2.7 ^1H NMR spectra of PFPA-disulfide **1** and gold nanoparticles functionalized with PFPA-disulfide **1** (Au-PFPA) in CDCl_3 with TMS (δ 0.0 ppm).

Figure 2.8 FT-IR spectra of PFPA-disulfide **1**, gold nanoparticles functionalized with PFPA-disulfide **1** (Au-PFPA), and Au NPs subsequently coupled with α -1,4-mannobiose (Au-DiMan). The azide ($-N_3$) absorption at $\sim 2125\text{ cm}^{-1}$ disappeared after light activation.

Mannose density measurement

Figure 2.9 Calibration curve obtained by treating various concentrations of D-mannose with anthrone/sulfuric acid and measuring the absorption at 620 nm.

Calculation of D-mannose density on Au NP

Figure 2.10 D- mannose molecules and Au NPs

The maximal number of D-mannose molecules on each Au NP is calculated as follows.

Assuming that D-mannose occupies in space by taking the shape of a square, each side of the square is measured to be $\sim 6 \text{ \AA}$ by Chem 3D (CambridgeSoft., Ultra, version 9.0).

The D-mannose molecule is then projected to the surface, and the surface area of each D-mannose molecule is $36 \text{ \AA}^2$.

The surface area of one 20-nm Au NP is $1.26 \times 10^5 \text{ \AA}^2 (= 4\pi \times 100^2)$.

The maximal number of D-mannose molecules occupying the surface of one Au NP in a closely packed manner is 3,491 ($=1.26 \times 10^5 \text{ \AA}^2/36 \text{ \AA}^2$).

The volume of each Au NP is $4.19 \times 10^6 \text{ \AA}^3$ ($=4/3 \pi \times 100^3$).

Assuming that the density of Au NPs equals to that of gold (19.32 g/mol), the weight of each Au NP is $8.09 \times 10^{-14} \text{ mg}$.

1 mg of Au NPs is equivalent to 0.0205 nmol ($1/8.09 \times 10^{-14} \times 6.02 \times 10^{23}$).

The moles of D-mannose on 1 mg Au NP is 71.7 nmol ($0.0205 \times 3,491$).

The D-mannose density of Au NP coated was measured as $24.1 \pm 1.7 \text{ nmol/mg Au NPs}$, and the number of D-mannose molecules is $\sim 1,200$ ($=24.1/0.0205$). And the estimated surface coverage is $\sim 34\%$ ($=1200/3491$)

Control experiments

Figure 2.11 UV-vis spectra of PFPA-functionalized Au NPs and after treating with 100 nM and 1000 nM Con A.

Chapter 3. Quantitative Analysis of Binding Affinity of Glyconanoparticles with Lectins

The work in Section 3.1 was published on “**Wang, X.**; Ramström, O.; Yan, M.,
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2010, 22, 1946-1953”

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Dynamic Light Scattering as Efficient Tool to Study Glyconanoparticle-Lectin
Interactions. *Analyst* **2011**, 136, 4174-4178”

<http://pubs.rsc.org/en/Content/ArticleLanding/2011/AN/c1an15469a>

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The work in Section 3.3 was submitted for publication.

Glyconanomaterials are synthesized under specific conditions using different chemistry and reagents. Careful evaluation of these materials must therefore be conducted to fully characterize the structure, composition, density of surface ligands as well as biological activities in order to make proper correlation with their performances. Thanks to the significantly increased specific surface areas of nanomaterials, conventional chemical analytic techniques that are insensitive to flat substrates can be readily adopted for nanomaterial characterization. In Brust's first paper on the preparation of thiol-capped Au NPs, the products were characterized by FTIR showing the presence of alkanethiol, and TEM revealing the size and shape of the nanoparticles.⁸⁹ With the rapid development of advanced analytical tools, especially sensitive surface characterization techniques, nanomaterials can now be analyzed more accurately, providing in-depth understanding of the chemical and physical properties of glyconanomaterials.¹³⁷ NMR, surface-enhanced Raman spectroscopy (SERS), and FTIR offer detailed structural analysis of nanomaterials and surface ligands. Thermogravimetric analysis (TGA) yields the amount of organic components on the nanomaterials, from which the ligand densities can be derived. Elemental analysis and XPS provide information on the elemental composition and chemical state of the bulk nanomaterials and the surface ligands. A combination of microscopy techniques of scanning probe (STM, AFM), TEM and small-angle X-ray scattering (SAXS) reveals the physical characteristics of size, shape, and assembly behaviors of the nanomaterials.^{138,139} Caution should be used when analyzing the results as the experimental conditions applied to each technique (vacuum, ambient,

solution) can significantly impact the outcome. Microscopic techniques can also be used to directly visualize the interactions of glyconanomaterials with their binding partners. In our study, when D-mannose-functionalized iron oxide nanoparticles were treated with *E. coli* strain ORN178, the nanoparticles selectively bound to the FimH lectin on the bacteria, which was clearly shown in TEM.¹⁴⁰ The surfaces can furthermore be characterized by taking advantage of the unique properties offered by the nanomaterials. A classic example is metal nanoparticles, which exhibit SPR that is highly sensitive to the surface constituents and can be conveniently monitored colorimetrically as the molecular recognition event occurs at, or close to, the surface of the nanoparticles.¹¹⁰

Biomedical imaging, therapeutics, medical diagnosis, and drug delivery are among the many areas glyconanomaterials have the potential to impact. The interaction of glyconanomaterials with biological receptors and targets is a critical process involved in these applications and the binding affinity is thus an important parameter for evaluating the performance of glyconanomaterials. When a ligand is conjugated to a solid surface, the structure of the ligand is in a sense altered. The binding affinity of the free ligand in solution can no longer be used as the substitute for the glyconanomaterial with the corresponding binding partner. In glyconanomaterials, multiple ligands are clustered on a single solid entity. Multivalency effect comes in play whereby ligands act cooperatively enhancing the overall binding affinity with the

receptor. This multivalency effect is highly sensitive to how the ligands are presented on the nanomaterial surface, i.e., the number of ligands or ligand density, the structure and length of the spacer linker, and how the ligand is attached or the coupling chemistry. Therefore the binding affinity of the glyconanomaterials must be carefully evaluated taking into consideration these parameters.

Carbohydrate-lectin interactions of free ligands in solution have been studied by many biochemical and biophysical methods including NMR spectroscopy,¹⁴¹ SPR spectroscopy,^{142,143} X-ray crystallography,^{144,145} titration microcalorimetry,¹⁴⁶ and fluorescence spectroscopy.¹⁴⁷ Quantitative analysis of glyconanomaterials is investigated to a lesser extent, and a few protocols were reported to determine the binding affinity of glyconanoparticles. Lin and coworkers used SPR to analyze the multivalent interactions between mannose, glucose, or galactose-encapsulated gold nanoparticles with Con A.¹¹ A competition binding study was carried out where equilibria were established between mannopyranoside attached on the SPR sensor, Con A, and varied concentrations of mannose-encapsulated Au NPs. The dissociation constant K_d of mannose-Au NPs with Con A was determined to be 2.3 nM, representing a binding affinity over 5 orders of magnitude higher than that of the free D-mannopyranoside with Con A in solution (K_d : 470 μ M measured by ITC¹⁴⁸). In the system developed by Wu *et al.*, magnetite-gold core/shell nanoparticles coated with proteins were allowed to interact with carbohydrate ligands on a glycan array.¹⁴⁹ A magnetic field was applied to amplify the protein-carbohydrate interactions and the

signals were visualized and quantified using a silver enhancement reagent. Apparent K_d values of 66 nM, 61 nM and 57 nM were determined for Man1, Man4 and Man9 ligands with Con A, respectively.

3.1 Fluorescence-based Competition Assays

In this part, a fluorescence-based competition assay has been developed to determine the binding affinity of glyconanoparticles to lectins. In the assay, a fixed concentration of a free ligand (for example, D-mannose) and varying amounts of ligands bound to Au NPs were incubated with fluorescein isothiocyanate (FITC)-labeled Con A. The solution was then centrifuged and the fluorescence intensity of the supernatant was measured. Two equilibria co-exist in the system: FITC-Con A with free D-mannose and FITC-Con A with D-mannose bound on nanoparticles (Figure 3.1). Since very low concentrations of Con A and free D-mannose were used, it was assumed that no agglomeration occurred. Both interactions are reversible, and steady equilibria are reached rapidly.

Figure 3.1. Equilibria involved in the competition binding assay (left). Concentration dependent fluorescence intensity curve (right). [Man] is the concentration of D-mannose on NPs determined using the anthrone/H₂SO₄ colorimetry assay described in the text. Each data point was an average of 3 assays.

In order to obtain the dissociation constant K_d , the concentration of the carbohydrate ligand on Au NPs must be determined. The colorimetric assay of anthrone-sulfuric acid was adopted to measure the ligand density on the nanoparticles.¹³³ A calibration curve was first established using the corresponding free carbohydrate, and the amount of surface-bound ligand on the Au NPs was subsequently determined. The fluorescence intensity measured from the competition studies was plotted against the concentration of bound D-mannose on the Au NPs (Figure 3.1). The resulting curve fits a typical competition assay for ligand-receptor binding, validating the assumptions made for the system. The IC_{50} value was subsequently derived and the apparent dissociation constant (K_d) calculated using the Cheng-Prusoff equation (eq. 3.1),

$$K_{d2} = \frac{IC_{50}}{1 + \frac{[M]}{K_{d1}}} \quad (3.1)$$

where IC_{50} = concentration of ligands displaying 50% of specific binding; $[M]$ = concentration of free ligand, i.e. D-mannose; K_{d1} = dissociation constant of free ligand to Con A; and K_{d2} = dissociation constant of surface bound D-mannose to Con A.

3.2 Dynamic Light Scattering

A number of analytical techniques have been used to monitor the interactions of GNPs with biological receptors, including UV-vis spectroscopy,¹⁵⁰ transmission electron microscope (TEM),¹⁵¹ surface plasmon resonance (SPR), quartz crystal microbalance (QCM),¹⁵² isothermal titration calorimetry (ITC),⁹⁴ magnetic resonance imaging (MRI),¹⁵³ and fluorescence spectroscopy.^{154,155} Each technique has its advantages and limitations, and some methods are restricted to the property of the nanomaterials. For instance, UV-vis spectroscopy only applies to metal nanoparticles that absorb light by free electron oscillations. SPR and QCM are generally performed on Au surfaces where the interactions of nanoparticles with target biomolecules immobilized on Au surfaces are monitored.

Light scattering is a powerful technique for characterizing particles in solutions. When a beam of light passes through a colloidal dispersion, the particles scatter the light in all directions. In DLS, the particles are illuminated with a monochromatic laser. The intensity of the scattered light fluctuates and the rate is dependent on the size of the particles. Analysis of the time dependence of the intensity fluctuations yields the diffusion coefficient of the particles, from which the hydrodynamic radius, or the diameter, of the particles can be calculated.¹⁵⁶ DLS has become a routine analytical tool for particle size measurement. The technique has also been used to study the interactions of nanoparticles with other species such as polymers,¹⁵⁷ DNA,¹⁵⁸⁻¹⁶⁰ and biomarkers.¹⁶¹ The advantages of DLS are: (1) excellent sensitivity, (2) low-cost, (3)

easy sample preparation, and (4) fast measurement (data can be obtained in a few minutes). In this part, we report that DLS is a highly efficient technique to study GNP – lectin interactions. GNPs of different sizes and particle composition were synthesized and their interactions with lectins were monitored by DLS. The apparent association constant (K_d) values were determined from the particle size – concentration response curves. The impact of the particle size on the affinity of the GNPs was also investigated. To the best of our knowledge, this is the first report on the quantitative analysis of GNP – lectin interactions by DLS.

Experimental details

Preparation of silica NPs

Silica NPs were synthesized following a modified Stöber protocol,¹⁶² similar to what was previously described.¹²⁵ TEOS (2 mL) was added to 200 proof absolute ethanol (34 mL) followed by NH_4OH (35%, 1 mL). The reaction was allowed to proceed at room temperature overnight with vigorous stirring to yield a white colloidal solution; the particle size was 35 nm measured by DLS. NPs of 110 nm size was synthesized following the same procedure except for the amount of reagents added: TEOS (2.8 mL), NH_4OH (2.8 mL). The 470-nm NPs were prepared using the seed-growth method.¹⁶³ TEOS (1.4 mL) was added to 200-proof absolute ethanol (34 mL) followed by the addition of NH_4OH (35%, 2.8 mL). The reaction was allowed to proceed at

room temperature overnight with vigorous stirring, after which, additional TEOS (1.4 mL) was added continuously in aliquots of 0.2 mL every 10 min.

Functionalization of silica NPs with perfluorophenyl azide (PFPA)

PFPA-silane (80 mg), synthesized following a previously reported procedure, was added directly to the Stöber solution prepared above, and the mixture was stirred at room temperature overnight. The mixture was then brought to reflux under continuous stirring for 1 h at ~78 °C to facilitate the silanization of the silica nanoparticles with PFPA-silane.¹²⁵ The mixture was centrifuged at 12,000 rpm for 30 min, and the precipitate was redispersed in the fresh solvent by sonication. This centrifugation/redispersion procedure was repeated three times with ethanol and twice with acetone.

Conjugation of carbohydrates onto PFPA-functionalized NPs

Our previously reported procedure of coupling carbohydrates onto Au NPs was followed. Briefly, the dispersion of PFPA-functionalized NPs in acetone and an aqueous solution of carbohydrate was placed in a flat-bottom dish, and the mixture was irradiated with a 450-W medium pressure Hg lamp with a 280-nm filter for 10 min. Excess carbohydrate was removed by membrane dialysis in water for 24 hours. The concentration of the resulting GNPs, ca. 18.4 mg/mL, was determined gravimetrically after drying the solution under reduced pressure for 3 hours.

Synthesis of 40-nm Au GNPs

The Turkevich method¹⁶⁴ was followed to synthesize Au NPs. An aqueous solution of sodium citrate (1 wt%, 1.2 mL) was added to a boiling HAuCl₄ solution (0.25 mM, 100 mL) under vigorous stirring for 15 min. PFPA-disulfide (Figure 3.2), was synthesized following a previously reported procedure. A solution of PFPA-disulfide (25 mg) in acetone (5 mL) was added to the Au NPs solution, and the solution was stirred for 12 hours. The resulting NPs were centrifuged at 12,000 rpm for 15 min and cleaned with acetone 3 times. Carbohydrate conjugation followed the same procedure described above for the silica NPs.

Interaction of GNPs with lectins and DLS measurements

The following general procedure was followed for all GNPs and lectins. GNPs (0.1 mg) were incubated in a pH 7.2 HEPES buffer (10 mM, 0.5 mL) containing 3% BSA for 30 min. The sample was then centrifuged and the particles rinsed 3 times with the fresh HEPES buffer. The GNPs were subsequently treated with a solution of Con A (or RCA₁₂₀) in pH 7.2 HEPES buffer at different concentrations for 2 hour while shaking. For DLS measurements, the suspension was diluted to 3 mL using the HEPES buffer. Each DLS measurement was performed at 20 scans, and was repeated 6 times.

Results and discussion

Lectin detection using silica GNPs by DLS

GNPs were synthesized using the methods developed previously in our laboratory.^{165,166} Briefly, silica NPs and Au NPs of varying sizes, synthesized by the Stöber method¹⁶² and the Turkevich method,¹⁶⁴ respectively, were functionalized with PFPA-silane or PFPA-disulfide (Figure 3.2a). Man and Gal were then coupled to the NPs by the CH insertion reaction of PFPA by UV activation.¹⁰⁶ Con A, a well-studied lectin that exhibits high affinity for the terminal α -D-mannopyranosyl group,^{114,167} was used to evaluate the DLS technique in studying GNP-lectin interactions. As demonstrated in our previous studies, Man-functionalized NPs formed aggregates upon binding with Con A,¹⁶⁶ resulting in an overall size increase as observed by TEM.¹⁵⁵

Figure 3.2 (a) Synthesis of silica and Au GNPs: **S-GNP** and **Au-GNP**. (b) GNP aggregation upon addition of lectin.

To investigate the feasibility of DLS in studying GNP-lectin interactions, Man coupled on silica NPs (35 ± 4.4 nm in diameter), **S-M-GNP**, was treated with varying concentration of Con A (1 nM – 700 nM). The hydrodynamic radius of the resulting complex was measured by DLS. At 1 nM Con A concentration, there was no obvious increase in the average particle size (Figure 3.3a). When a higher concentration of Con A was added (50, 70, and 170 nM, respectively), the median diameter of the resulting particles increased to 55.1 ± 4.5 , 64.3 ± 9.5 nm, and 94.8 ± 14.6 nm, respectively (Figure

3.3b-d). Also increased is the error margin suggesting that particles become more polydisperse with the addition of Con A.

Figure 3.3 (a-d) DLS spectra and TEM images (inserts, scale bars: 50 nm) when 35-nm **S-M-GNP** was treated with varying concentrations of Con A. (e) The particle size vs. concentration of Con A.

The sensitivity of the DLS detection was next studied. The limit of detection (LOD), defined as the lowest analyte concentration measurable against the background, was determined from Equation 3.2,

$$LOD = \frac{3\sigma}{b} \quad (3.2)$$

where σ is the standard deviation of the spectroscopic signals of the blank sample (from 12 measurements), and b is the slope of the linear calibration curve.^{168,169} Using the data from Figure 3.3e, the LOD of Con A by the 35-nm **S-M-GNP** was calculated to be 2.9 nM.

To investigate the generality of this method with respect to the nature of the particles, Au NPs (40 nm in diameter) were synthesized, and Man was then coupled onto the particles.¹⁵⁵ The resulting **Au-M-GNP** was treated with varying concentrations of Con A and the particle sizes were monitored by DLS, from which a calibration curve was established. The LOD was then calculated according to Eq. 1, which yielded 15 nM. This value was higher than that of the 35-nm **S-M-GNP** determined by the same method (2.9 nM), indicating that the Au GNPs were of approximately 5 times lower sensitivity than the silica GNPs in detecting Con A. However, the method was more sensitive than the SPR-based optical detection where the LODs were in range of 80-100 nM for 13-16 nm Au-NPs.^{150,170}

Titration experiments were next carried out to quantitatively analyze GNP-lectin interactions. A fixed amount of the 35-nm **S-M-GNP** was incubated with varying concentrations of Con A, and the DLS graphs of the resulting solutions were recorded. The increases in particle diameter, D , were computed and the results plotted against the concentration of Con A (Figure 3.4a). The saturation curve was then fitted with an overall binding model, i.e., the Hill Equation (Eq. 3.3),

$$Y = \frac{B_{\max} * X^h}{K_D^h + X^h} \quad (3.3)$$

where $B_{\max}$ is the maximum specific binding, K_D is the apparent dissociation constant, and h is the Hill coefficient. The K_D value was subsequently derived as 63 nM for **S-M-GNP** (Table 3.1). Following the same procedure, the K_D value of **Au-M-GNP** was

determined to be 86 nM (Table 3.1). These values represent more than 3 orders of magnitude increases in binding affinity than that of the free ligand Man with Con A ($K_d = 470 \mu\text{M}$).¹⁴⁸ The results are consistent with our previous studies that NPs can serve as an efficient multivalent scaffold greatly amplifying the affinity of the bound ligands with lectins.¹⁵⁵ In all cases, the h values were larger than 1, indicating a positive cooperativity of the ligands conjugated on GNPs.

Figure 3.4 The change in particle diameter (ΔD) vs. lectin concentration: experimental data (circles) and the corresponding Hill fitting curves (lines) for a) 35 nm, b) 110 nm, and 470 nm silica NPs, respectively. The scale bars in the TEM images are a) 50 nm, b) 200 nm, and c) 500 nm respectively.

Table 3.1 LOD and K_D values of GNP-lectin interactions.

GNP	Diameter (nm)	Number of ligand per NP	Lectin	LOD (nM)	K_D (μ M)	h
S-M-GNP	35	830	Con A	2.9	0.063	3.9
	110	2600		42	0.97	1.6
	470	4900		6.2×10^3	9.2	2.3
Au-M-GNP	40	950	Con A	15	0.086	2.2
S-G-GNP	35	830	Con A	<i>N/A</i>		
	35	840	RCA ₁₂₀	6.6	0.22	1.9

N/A: not applicable.

Impact of NP size on binding affinity

Size-dependency has been recognized as of high significance affecting the physical and chemical properties of NPs, including adsorption, bio-affinity and catalysis.¹⁷¹⁻¹⁷³

To study the impact of the particle size on the binding affinity of GNPs, silica NPs with an average diameter of 35, 110, and 470 nm, respectively, were synthesized. Particles of 35 nm and 110 nm in size were obtained by varying the reagent concentrations using the Stöber method.¹⁶² The 470 nm particles were prepared using a seed-growth method.¹⁷⁴ These particles were uniform in shape and size (see TEM images, Figure 3.4), and were more monodisperse than the Stöber particles as demonstrated by a narrower particle size distribution for the 470 nm nanoparticles. Man ligands were subsequently conjugated on these silica NPs following the same protocol as described above, and the ligand densities were determined by the

colorimetric method using anthrone/H₂SO₄. As the size of the NPs increases, the number of coupled Man increases (Table 3.1), which is expected since the bigger particle has a larger surface and can thus accommodate more ligands.

Figure 3.5 Percent increase in particle size (= increase in particle diameter (ΔD)/original particle diameter (D_0) x 100%) vs. concentration of Con A for various GNPs.

The GNPs were then treated with varying concentrations of Con A, and the percentage increase in particle diameter was calculated from the DLS measurements, shown in Figure 3.5. A noticeable change in the particle diameter was observed for the 35 nm **S-M-GNP** when 20 nM Con A was added. The particle size did not change for the 110 nm and 470 nm **S-M-GNP** until the Con A concentration reached 50 nM and 5 μ M,

respectively, indicating that smaller NPs were more sensitive in detecting GNP-lectin interactions. This is consistent with the LOD results determined for these GNPs, which were 2.9 nM, 42 nM, and 6.2 μ M for 35 nm, 110 nm, and 470 nm **S-M-GNPs**, respectively (Table 3.1). This size-dependent phenomenon was also observed by Huo and coworkers where Au NPs were used to detect DNA by DLS.¹⁵⁸ Titration experiments were subsequently carried out on all GNPs synthesized, and the apparent K_D values were determined from the saturation binding curves in the same manner as shown in Figure 3.4. For **S-M-GNP**, the binding affinity decreased, i.e., the K_D values increased with increasing particle size (Table 3.1). This is in agreement with our previous studies using Au GNPs where the affinity of GNPs with lectins decreased with increasing particle size.¹⁵⁵ A possible explanation for the lower LOD and binding affinity of the larger GNPs is the steric hindrance imposed by the larger particles. Although there are more ligands on the larger particles (Table 3.1), these ligands are less accessible to the lectin as compared to smaller particles with the same length of the spacer linker.

The DLS method is also highly specific. When Gal, a non-binding ligand for Con A, was conjugated to the 35-nm silica NPs and the resulting **S-G-GNP** was subsequently treated with Con A, no obvious change in particle size was observed at all Con A concentrations up to 5 μ M (Figure 3.5). **S-G-GNP** was then treated with RCA₁₂₀, an R-type lectin exhibiting broad specificity for the terminal galactose group.¹⁷⁵ RCA₁₂₀ is a dimer having one active Gal-binding site on each subunit.¹⁷⁶ The lectin can

therefore act as a crosslinker forming a complex with Gal-coated NPs.¹⁷⁷ Indeed, when **S-G-GNP** was treated with RCA₁₂₀, aggregates formed and the size of which could be monitored by DLS. Following the same procedure as described above, the LOD of RCA₁₂₀ was measured to be 6.6 nM, which is on the same order of magnitude as that of **S-M-GNP**/Con A system (2.9 nM, Table 3.1). Titration experiments were then carried out, and the particle size changes were plotted against the RCA₁₂₀ concentration. The apparent K_D was then calculated by fitting the saturation curve with the Hill equation (Eq. 3.3). The value, 0.22 μ M (Table 3.1), corresponds to an affinity enhancement of over 4 orders of magnitude in comparison to that of the free Gal with RCA₁₂₀ ($K_d = 455 \mu$ M).¹⁷⁶

Conclusions

In summary, a new method, based on DLS, was developed to study the interactions of GNPs with lectins. The method relies on the particle size growth of GNPs, resulting from the multiple binding sites on lectins that act as crosslinkers bringing GNPs together to form larger aggregates. Two GNP-lectin systems, **M-GNP** with Con A and **G-GNP** with RCA₁₂₀, were investigated and the particle size growth was observed only for the specific binding pairs. The technique is highly sensitive, and the LOD was on a par with values obtained by other techniques. Quantitative analysis is also possible from the titration experiments, from which the apparent K_D values were obtained. The results showed that the binding affinities of GNPs with lectins were 3-4

orders magnitude higher than that of the free ligands, demonstrating that NPs are an efficient scaffold amplifying the glycan-lectin interactions. The effect of particle size was also studied, and results demonstrate that smaller GNPs gave higher detection sensitivity as well as binding affinity. The method is applicable to both Au and silica NPs, and is therefore general regardless of the nature of the particles. The high sensitivity of the DLS method comes from the crosslinking ability of the lectins, which can be a limitation. However, many lectins contain more than 2 subunits and can act as crosslinkers inducing particle aggregation. The method developed here, coupled with the simplicity and fast measurement of the DLS technique, is therefore highly valuable in studying GNP-lectin interactions.

3.2 Isothermal Titration Calorimetry (ITC)

Figure 3.6 (a) Diagram of ITC cells and (b) Typical ITC data. Top: raw ITC data. Bottom: binding isotherm curve.¹⁷⁸

In this chapter, an ITC-based method has been developed for determining the binding affinity of glyconanoparticles with lectins. ITC is a sensitive and quantitative analytical technique that determines the thermodynamic parameters of binding events occurring in solution. By measuring the heat absorbed or released upon the interactions between ligands and biomacromolecules, the binding constant (K_a), enthalpy of binding (ΔH) and stoichiometry of binding (n) can be derived simultaneously.¹⁷⁹ The working principle of ITC is based on heat

measurement between a reference cell and a sample cell where the binding actually occurs (Figure 3.6a). During the experiment, the temperature of the sample and reference cell is adjusted to be the same by supplying or absorbing heat to the sample cell, and the amount of heat absorbed/released is recorded by a sensor, which is attributed to the interaction heat in sample cell. The raw data was obtained by plotting the quantity of heat versus time. Subsequently, heat was integrated as function of molar ratio between the injectant, e.g., Con A, and its binding partner, e.g. GNP-M, to give the binding curve (Figure 3.6b). Current commercial ITC instrument is capable of measuring a wide range of interactions with K_d values ranging from millimolar to nanomolar. Based on the results from literature^{11,152} and our previous studies,^{155,180} apparent dissociation constants of GNP-lectin interactions were mostly in the nanomolar range, and therefore, ITC should be a suitable method for measuring GNP-lectin interactions.¹⁷⁸

Experimental details

Preparation of gold glyconanoparticles.

AuNPs, 22 nm in diameter, were prepared and functionalized with PFPA-disulfide following the same procedure as described in Chapter 2.¹⁵⁵ The subsequent carbohydrate coupling was carried out photochemically as described in Chapter 2.¹⁸¹ Briefly, a solution of PFPA-functionalized AuNPs in acetone (10 nM) mixed with an aqueous solution of carbohydrate (10 mM) was irradiated for 12 min with a 450-W medium pressure Hg lamp (Hanovia) with a 280-nm long-path optical filter. The

resulting GNPs were then dialyzed overnight to remove excess carbohydrate. Before the binding experiments, the GNPs were incubated in HEPES buffer containing 0.01% Tween 20 and 3% BSA for 1 hour, centrifuged, and placed in the buffer without BSA until further use.

ITC

ITC experiments were performed using an ITC200 microcalorimeter from Microcal, LLC. (Northampton, MA) in HEPES buffer. The concentration of lectin was 10 μM , and that of GNP was 2.5 mM. In each experiment, ~ 38 μL of lectin was injected through the computer-controlled 40- μL micro-syringe at an injection interval of 4 min into the nanoparticle solution (cell volume = 200 μL) while stirring at 1000 rpm. The experimental data were fitted to a theoretical titration curve using the software supplied by MicroCal. A standard one-site model was used with ΔH (enthalpy change, in kcal/mol), K_a (association constant, in M^{-1}), and N (number of binding sites) as the variables.

Results and discussion

GNPs were synthesized by coupling carbohydrates to Au NPs using the perfluorophenyl azide (PFPA) photocoupling chemistry developed in our laboratory.^{165,181 106,140} AuNPs of 22 nm in diameter were synthesized and functionalized with PFPA (see Supplementary Information for detailed synthesis).

D-Mannose (Man), D-galactose (Gal), and α -1,2-mannobiose (Man₂) were then conjugated to the PFPA-functionalized AuNPs by light activation.^{165,181} The density of the attached carbohydrates were determined colorimetrically using the anthrone-sulfuric acid assay (Table 3.2).¹⁸¹ Binding studies were carried out using a plant lectin Concanavalin A (Con A), a well-studied tetrameric protein having specific binding site for the terminal α -mannosyl groups.^{167,114} In the ITC experiments, the lectin was loaded into the micro-syringe and was titrated into the GNP solution placed in the calorimeter cell. When the binding occurs between GNPs and lectin, such as Man-conjugated GNP (GNP-Man) and Con A, heat will be released upon each injection. The amount of generated heat decreases with each subsequent injection as the GNPs in the cell are consumed with bound lectin. In contrast, if a non-binding lectin is used, the released heat, likely produced due to reagent mixing, will be small and will remain constant with each injection.

Figure 3.7 ITC graphs of GNP-Man with a) Con A and b) PNA. The experimental data (solid squares) were fit to theoretical titration curves (solid lines) using the software supplied by the ITC manufacturer.

Figure 3.7a is a typical ITC titration graph of GNP-Man with Con A at pH 7.6 in HEPES buffer containing 1 mM Mn^{2+} and 1 mM Ca^{2+} . As expected, heat was generated after each injection of the lectin solution, and the amount of the released heat decreased gradually with each additional injection. Because heat can also be produced just by mixing the reagents, background heat from dilutions and was subtracted from each titration experiment. Figure 3.7a (bottom) shows the

processed titration data (solid squares) and the computed integrated curve (line) which fit the data points well. The thermodynamic parameters, including dissociation constant (K_D), the enthalpy change (ΔH), the entropy change (ΔS), and the number of binding sites (n) are shown in Table 3.2. The Gibbs free energy changes (ΔG) was calculated by standard thermodynamic equation: $\Delta G = \Delta H - T\Delta S$. The enhancement factor β , defined as $\log (K_d/K_D)$, can be used to evaluate the multivalency effect when nanoparticles are used as the scaffolds.^{69,182} Compared to the binding affinity of the free α -D-mannopyranoside ligand with Con A ($K_d = 470 \mu M$),¹³⁶ the K_D value of GNP-Man with Con A ($K_D = 122 \text{ nM}$) was closed to 4 orders of magnitude smaller, i.e. the affinity was 4 orders of magnitude higher. The number of binding sites, $N (=1/n)$, represents the number of sites on the nanoparticle that were available for binding with the lectin. Note that N (306) is smaller than the number of carbohydrate ligands immobilized on the particle surface (3600), meaning that only a portion of available ligands on the GNP participated in binding the lectin. This is expected considering that the distance between each of the four monomeric binding site on Con A is 6.5 nm ,¹⁸³ which is far greater than the distance between Man ligands on the GNP surface ($2\sim 3 \text{ nm}$).¹⁵⁵ The results indicated the binding of GNP with lectin is exothermic process ($\Delta H < 0$ and $\Delta S < 0$), and an overall negative Gibbs free energy gain (ΔG) was obtained due to the slight difference between unfavorable entropy and enthalpy changes. In the control experiment where PNA lectin was titrated into GNP-Man, the released heat was small and remained unchanged throughout the titration (top, Fig. 1b).

Subsequent data fitting gave a flat titration curve (bottom, Fig. 1b). Similar results were also obtained when Con A was titrated into GNP-Gal where galactose is a non-binding ligand for Con A. These results demonstrated that there was no apparent binding between the non-interacting GNP-lectin pairs.

Figure 3.8 ITC titration graphs of Con A with (a) GNP-Man2I, (b) GNP-Man2II, and (c) GNP-Man2III. Solid squares: experimental data, lines: fitted titration curves.

To study the effect of ligand density on lectin binding, ITC experiments were carried out where the ligand density on GNP-Man2 was varied from 1400 (GNP-Man2I, Figure 3.8a), to 260 (GNP-Man2II, Figure 3.8b) and 110 (GNP-Man2III, Figure 3.8c), which were prepared following a mixed ligand strategy.¹⁵⁵ GNP-M2I, having the highest ligand density, gave the largest N value and the highest binding affinity (Table 3.2). Although GNP-M2I has 5 times and 13 times more ligands than GNP-M2III and GNP-M2III, respectively, the binding affinity was only 2

times and 5 times larger. This may be attributed to the fact that not all ligands participated in binding the lectin.¹⁵⁵ GNP-M2III had a slightly smaller N value than GNP-M2II. However, it gave a slightly more favorable thermodynamic values (ΔH and ΔG) probably due to the reduced steric hindrance at lower ligand density. The method was further tested with a bacterial lectin, cyanovirin-N (CV-N).¹⁸⁴⁻¹⁸⁷ GNP-Man2 was used since Man2 is an epitope ligand for CV-N. Mut^{DB}, a variant of CV-N and having only one active domain¹⁸⁸ (instead of two in the wide-type CV-N), was used in this study. Results from the ITC titration curves (Figure 3.9) show 4.6 orders of magnitude affinity enhancement (β) of GNP-Man2I over free Man2 to CV-N (Table 3.2), similar to the results obtained previously using a fluorescent competition assay.¹⁸⁹

Figure 3.9 ITC titration graphs of GNP-Man2 with CV-N Mut^{DB}

Table 3.2 The thermodynamic parameters of GNP-lectin interactions at 25 °C.

GNP	Ligand density (per NP)	Lectin	K _D / nM	K _d / μM	β ^a	-ΔG/ kJ mol ⁻¹	ΔH/ kJ mol ⁻¹	TΔS/ kJ mol ⁻¹	n	N ^b	
GNP-Man	3600	Con A	122	470	3.6	137	-107760	-107623	0.00327	306	
		PNA	ND ^c								
GNP-Gal	3500	Con A	ND ^c								
GNP-Man2	I	1400	Con A	34.8	24	2.8	18.3	-46356	-46338	0.00478	209
	II	260		82.6		2.5	6.5	-28407	-28401	0.0101	99
	III	110		175		2.1	59.2	-30577	-30518	0.0121	83
GNP-Man2	1400	CV-N	17.2	757 ¹⁸⁸	4.6	47.1	-6650	-6602	0.00625	160	

^aenhancement factor: $\beta = \log (K_d/K_D)$ ^bnumber of binding site on GNP: $N=1/n$ (n: number of binding sites on lectin)^cnot detectable

In conclusion, an ITC-based method has been developed to quantitatively analyze the binding affinity of glyconanoparticles with lectins. Thermodynamic parameters including dissociation constant, entropy and enthalpy changes, and the number of binding site were simultaneously determined by titrating GNPs into the lectin solution. A 3-5 orders of magnitude affinity enhancement over the free ligand was observed, which could be attributed to the multivalent ligand presentation on

nanoparticles. The results were consistent with what have been obtained previously using other analytical techniques such as fluorescence competition assays, dynamic light scattering, and microarrays. In contrast to these methods that either require a label, or rely on indirect information, ITC is a direct measurement method that gives the affinity constant, thermodynamic parameters and stoichiometry of the interactions. This technique provides a general and highly sensitive means to study nanomaterial interactions with biological entities. The method developed will benefit fundamental studies as well as the development of biosensors and theranostic tools.

Chapter 4. Impact of Ligand Presentation on the Binding Affinity of Glyconanoparticles - Multivalency Effect

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Introduction

A general feature of carbohydrate-protein interactions relates to their inherent complexity, and multivalent ligand expression is frequently used to achieve sufficiently high affinities required for sensitive recognition.^{68,69,190} In analogy to ligand clustering at cell surfaces, nanomaterials can function as efficient nano-sized scaffolds for multiple carbohydrate presentation. Strong binding enhancements resulting from ligand presentation at the surfaces of nanomaterials have been demonstrated in numerous studies.¹⁹¹⁻¹⁹³ However, the current investigations are generally short of in-depth characterization of the structures, compositions and densities of the surface ligands, as well as the biological activities of the constructs, since conventional surface analysis methods have been less optimized for nanomaterials. Owing to the increasing interest in nanomaterials research, recent development in spectroscopic and microscopic techniques has afforded detailed structural and compositional information for nanomaterials.^{137,194,195} Nevertheless, quantitative analysis of multivalent biological affinity at nanoparticle surfaces still represents a considerable challenge. Furthermore, very limited methods were available to measure the binding affinity of glyconanoparticles with proteins.^{11,196,197}

In this chapter, we present detailed quantitative analyses on nanoparticle-based multivalent carbohydrate-protein interactions. The effects resulting from the nanoparticle size was investigated, and the ligand presentation with respect to ligand density, surface environment, and linker spacer was studied. The well-established

interaction system involving the lectin Con A, together with a panel of carbohydrate species, was chosen as the target. This lectin is present as a tetramer at $\text{pH} > 7$, and possesses specific affinities to α -D-mannopyranoside, α -D-glucopyranoside and their derivatives.^{198,199} The system constitutes an excellent model for investigating the multivalency effect,¹¹⁴ and studies on mannose–Con A interactions have established that multivalent effects are highly sensitive to a range of factors, including the number of binding sites, the ligand density, the structure of the ligand linkers, and the coupling chemistry of the ligand attachments.²⁰⁰⁻²⁰³ In the present study, a recently developed photochemical carbohydrate immobilization technique was adopted,^{154,181} and an array of GNPs functionalized with different mono-, oligo-, and poly-saccharides were prepared and ligand densities determined. A fluorescence-based ligand competition assay was employed for the quantitative analysis of the binding affinities of the GNPs where the apparent K_d values of the resulting GNPs were determined. The binding affinity of GNPs with respect to the ligand density, spacer length, linker structure, as well as the nanoparticle size was furthermore investigated.

Experimental details

Synthesis of PFPA-Thiols

Figure 4.1 Synthesis of PFPA-thiols **a-d**.

3,6,9,12,37,40,43,46-Octaoxa-24,25-dithiaoctatetracontane-1,48-diylbis(4-azido-2,3,5,6-tetrafluorobenzoate) (2a) (1-Mercaptoundec-11-yl)tetra(ethylene glycol) (**1a**, 100 mg, 0.26 mmol) in absolute ethanol (10 mL) was titrated with a saturated solution of iodine in ethanol until the brown color of iodine persisted. The solution was concentrated to 2 mL and then water (10 mL) was added. The solution was extracted with diethyl ether (3 x 10 mL). The combined ethereal extracts were washed with brine, dried over Na_2SO_4 , and the solvent was removed under reduced pressure to afford the disulfide as a brown oil. A solution of 4-azido-2,3,5,6-tetrafluorobenzoic

acid¹²⁹ (61.8 mg, 0.26 mmol) in CH₂Cl₂ (10 mL) was cooled to 0 °C, and DMAP (3.7 mg, 0.03 mmol) and EDAC (57.5 mg, 0.3 mmol) were added. The disulfide obtained above was then added, and the solution was stirred for 1 hour, after which the reaction was allowed to warm up to room temperature and was stirred for 12 h under argon. The product was recovered by extracting with CH₂Cl₂ and the organic layer was washed with water, brine, and dried over Na₂SO₄. The crude product was purified by column chromatography with 1:8 v/v hexanes/ethyl acetate to afford PFPA-disulfide **2a** as a clear oil (57.1 mg, 37%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 4.52 (t, *J* = 3.2 Hz, 4H), 3.81 (t, *J* = 3.2 Hz, 4H), 3.66-3.54 (m, 24H), 3.46 (t, *J* = 5.2 Hz, 4H), 2.67 (t, *J* = 7.2 Hz, 4H), 1.61 (m, 4H), 1.57 (m, 4H), 1.28-1.23 (m, 28H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 159.4, 145.4 (d, *J* = 260 Hz), 140.2 (d, *J* = 252 Hz), 127.9, 109.5, 70.4, 69.6, 69.1, 68.4, 61.3, 37.4, 36.7, 30.4, 30.1, 29.3, 29.2, 28.4, 28.0.

23-Mercapto-3,6,9,12-tetraoxatricosyl 4-azido-2,3,5,6-tetrafluorobenzoate (a).
To a solution of PFPA-disulfide **2a** (40 mg, 0.033 mmol) in 1:1 v/v ethanol/acetonitrile (20 mL), zinc dust (50 mg) and concentrated HCl (0.05 mL) were added, and the reaction mixture was stirred under argon at room temperature for 1 hour. The reaction mixture was filtered, and the solvents were removed by rotary evaporation. The residue was dissolved in chloroform, and the resulting solution was washed twice with water followed by dilute NaHCO₃ solution, and was dried over Na₂SO₄. The crude product was purified by column chromatography with 1:8 v/v hexanes/ethyl acetate to afford PFPA-thiol **a** as a clear oil (32.4 mg, 81%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 4.40 (t, *J* = 2.8 Hz, 2H), 3.75 (t, *J* = 2.6 Hz, 2H), 3.65-

3.52 (m, 12H), 3.45 (t, $J = 5.6$ Hz, 2H), 2.40 (q, $J = 7.6$ Hz, 2H), 1.67 (m, 4H) 1.27-1.23 (m, 15H). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 159.1, 145.3 (d, $J = 255$ Hz), 140.4 (d, $J = 252$ Hz), 128.2, 108.9, 70.5, 69.9, 69.1, 68.5, 61.8, 37.7, 30.3, 29.7, 29.2, 28.7, 28.1, 23.4. IR (film) 2925, 2854, 2129, 1737, 1648, 1488, 1258, 1119, 998 cm^{-1} . HRMS (ESI) $\text{C}_{26}\text{H}_{39}\text{F}_4\text{N}_3\text{O}_6\text{S}$ $[\text{M}+\text{H}]^+$ calcd 598.2574, found 598.2535.

11,11'-Disulfanediylbi(undecane-11,1-diyl) bis(4-azido-2,3,5,6-tetrafluorobenzoate) (2b). Compound **2b** was prepared following a previously reported procedure.¹⁸¹

11-Mercaptoundecyl 4-azido-2,3,5,6-tetrafluorobenzoate (b). Compound **b** was synthesized from **2b** following the same procedure as described above for **a**. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 4.36 (t, $J = 6.6$ Hz, 2H), 2.52 (q, $J = 7.6$ Hz, 2H), 1.72 (m, 2H), 1.61 (m, 2H), 1.44-1.20 (m, 15H). ^{13}C (100 MHz, CDCl_3) δ (ppm) 159.5, 145.1 (d, $J = 252$ Hz), 140.2 (d, $J = 256$ Hz) 125.3, 108.1, 64.3, 36.1, 29.0, 28.1, 25.4, 23.7. Anal. Calcd for $\text{C}_{18}\text{H}_{23}\text{F}_4\text{N}_3\text{O}_2\text{S}$: C, 51.30; H, 5.50; N, 9.97. Found C, 51.36; H, 5.51; N, 9.99.

6,6'-Disulfanediylbis(hexane-6,1-diyl) bis(4-azido-2,3,5,6-tetrafluoro-benzoate) (2c). Compound **2c** was synthesized from **1c** following the same procedure as described above for **2a**. ^1H NMR (400 MHz, CDCl_3) δ (ppm) 4.37 (t, $J = 6.4$ Hz, 2H), 2.69 (t, $J = 7.2$ Hz, 2H), 1.81-1.65 (m, 4H), 1.53-1.38 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 159.7, 145.1(d, $J = 256$ Hz), 140.5 (d, $J = 258$ Hz), 124.4, 107.2, 64.8, 39.1, 36.4, 29.1, 28.4, 23.1. Anal. Calcd for $\text{C}_{26}\text{H}_{24}\text{F}_8\text{N}_6\text{O}_4\text{S}_2$: C, 44.57; H, 3.45; N, 12.00. Found C, 44.63; H, 3.50; N, 11.96.

6-Mercaptohexyl 4-azido-2,3,5,6-tetrafluorobenzoate (c). Compound **c** was synthesized from **2c** following the same procedure as described above for **a**. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 4.32 (t, *J* = 6.4 Hz, 2H), 2.54 (q, *J* = 7.6 Hz, 2H), 1.75 (m, 2H), 1.64 (m, 2H), 1.45 (m, 4H), (t, *J* = 7.8 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 159.3, 145.2 (d, *J* = 250 Hz), 140.4 (d, *J* = 266 Hz), 123.1, 108.1, 64.2, 36.1, 29.0, 28.1, 25.4, 23.7. Anal. Calcd for C₁₃H₁₃F₄N₃O₂S: C, 44.44; H, 3.73; N, 11.96. Found C, 44.31; H, 3.75; N, 12.04.

2,2'-Disulfanediylbis(ethane-2,1-diyl) bis(4-azido-2,3,5,6-tetrafluoro-benzoate) (2d). Compound **2d** was prepared following a previously reported procedure.²⁰⁴

2-Mercaptoethyl 4-azido-2,3,5,6-tetrafluorobenzoate (d). Compound **d** was synthesized from **2d** following the same procedure as described for **a**. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 4.45 (t, *J* = 6.4 Hz, 2H), 2.86 (q, *J* = 7.2 Hz, 2H), 1.63 (t, *J* = 8.8 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 159.1, 145.3 (d, *J* = 258 Hz), 140.5 (d, *J* = 267 Hz), 123.7, 107.2, 64.0, 23.3. Anal. Calcd for C₉H₅F₄N₃O₂S: C, 36.62; H, 1.71; N, 14.23. Found C, 36.25; H, 1.77; N, 14.02.

Preparation of carbohydrate-conjugated gold nanoparticles.

Citrate-protected Au NPs, ~22 nm in diameter, were prepared by adding sodium citrate (1 wt%, 1.8 mL) to a boiling solution of HAuCl₄ (0.25 mM, 100 mL) under vigorous stirring, and the boiling was continued for an additional 5 min when the solution became purple and finally light red.¹⁶⁴ PFPA-thiol **3a-3d** were synthesized following the procedures in Figure 4.1. In a 250-mL flask, the Au NP solution

prepared above (50 mL) was mixed with a solution of PFPA-thiol in ethanol (2.5 mM, 50 mL), and the resulting solution was stirred for 3 h. The solution was centrifuged at 10,000 rpm for 15 min, and the solid obtained was re-dispersed in ethanol (10 mL). The centrifugation and re-dispersion process was repeated for 3 times to remove excess PFPA-thiol, and the resulting PFPA-functionalized Au NPs was finally dispersed in acetone (10 mL). The concentration of the Au NP solution, about 10 nM, was determined by drying the sample under reduced pressure for 3 h and weighing. The carbohydrate coupling was carried out following the procedure reported previously.¹⁸¹ A solution of PFPA-functionalized Au NPs in acetone (10 mL) was placed in a flat-bottomed dish, and an aqueous solution of carbohydrate (1 mM, 0.5 mL) was added. The mixture was covered with a 280-nm long-path optical filter (WG-280, Schott Glass) and was irradiated with a 450-W medium pressure Hg lamp (Hanovia) for 10 min under vigorous stirring. Centrifugation of the solution at 12,000 rpm for 15 min separated the carbohydrate-attached Au NPs as precipitates. Excess carbohydrate was removed by membrane dialysis in water for 24 hours. Before binding experiments, the nanoparticles were incubated in the pH 7.4 PBS buffer solution containing 0.01% Tween 20 and 3% BSA for 30 min, centrifuged, and incubated in a pH 7.4 PBS solution without BSA for further use.²⁰⁴

Determination of carbohydrate ligand density on GNPs.

A previously-developed colorimetry method was used to determine the ligand density on Au NPs.¹⁸¹ Calibration curves were obtained for each carbohydrate where

carbohydrate solutions of various concentrations were incubated with anthrone/sulfuric acid and the absorbances at 620 nm were measured. Carbohydrates coupled on nanoparticles were subjected to the same assay where solutions of the GNPs in Milli-Q water (30–50 $\mu\text{g}/0.5\text{ mL}$) were treated with anthrone/ H_2SO_4 . Background absorption due to Au NPs themselves was accounted for by treating citrate-protected Au NPs solution of the same concentration with anthrone/ H_2SO_4 , and the absorbance at 620 nm was subtracted from that of the GNPs. The amount of surface-bound carbohydrate was then computed from the corresponding calibration curve.

Preparation of GNPs of varied carbohydrate density.

Mixed thiol solutions were prepared from PFPA-thiol and 1-hexanethiol in ethanol, with the mole percentage of PFPA-thiol varying from 10% to 98%. The functionalization of Au NPs with mixed thiols followed the general functionalization procedure described above, except that the pure thiol solution was replaced by the mixed thiol solution when treating citrate-protected Au NPs. The subsequent carbohydrate coupling was carried out following the same procedure described in detail above.

Fluorescence competition binding assay.

AuNP-a-Man solutions of various concentrations (1×10^{-8} – 5 nM) were prepared from the stock solution (10 nM). The Con A-FITC solution (190 nM) was prepared in

pH 7.4 PBS buffer containing MnCl_2 (1 mM) and CaCl_2 (1 mM). To the **AuNP-a-Man** solution (1 mL) in a 1.5-mL microcentrifuge tube, D-mannose (1.44 mM, 0.1 mL) and Con A-FITC (0.1 mL) were added. The total volume of the final solution was 1.20 mL, where the concentration of D-mannose and Con A-FITC was 120 μM and 16 nM, respectively. The solutions were shaken for 1 h, which was sufficient for reaching equilibrium as shown in a time-based study, and centrifuged at 12,000 rpm for 30 min where nanoparticles precipitated to the bottom of the tube. The supernatants were transferred to a quartz cuvette for fluorescence measurements at 480 nm excitation, and the emissions at 517 nm were recorded using Felix32 software. The incubation time was determined from a time study where GNPs were incubated with Con A-FITC for varying amount of time. The fluorescence of the supernatant was monitored, and after 1-hour incubation, the intensity no longer changed indicating that the reaction had reached equilibrium. Measurement at each concentration was repeated 5 times, and the mean value of the emission intensities was used for the analysis. For all other GNPs, the same procedure was followed except that the concentrations of the GNPs were varied.

Results and Discussion

Synthesis of GNPs and ligand density determination.

GNPs were synthesized by coupling carbohydrate ligands to PFPA-functionalized Au NPs. PFPA-thiol compounds of varying spacer lengths (**a-d**) (Figure 4.2) were synthesized following the procedure shown in Figure 4.1. PFPA-thiols **b-d** contain

varying lengths of methylene spacer linkage, whereas PFPA-thiol **a** has four ethylene oxide (EO) in addition to the 11 methylene units. These PFPA-thiols were used to investigate the impact of spacer linkage on the ligand density and binding affinity of the corresponding GNPs.

Preparation of GNPs followed the photocoupling method developed previously (Figure 4.2).¹⁸¹ Citrate-protected Au NPs were prepared from HAuCl₄ and sodium citrate. The size and uniformity were examined by DLS and TEM, which showed the spherically-shaped Au NPs of 22 ± 2.6 nm in diameter. PFPA-thiols were then introduced to the NP surface via a ligand-exchange reaction, and the presence of PFPA on Au NPs was confirmed by ¹H NMR and FTIR. Subsequent carbohydrate immobilization was carried out by a photocoupling reaction, and the unattached carbohydrates were removed by membrane dialysis. The resulting GNPs exhibited excellent solubility in water. Furthermore, the centrifuged nanoparticle pellet was easily dissolved in water, and the solution showed no change in the optical property. The GNPs dispersed well in the PBS buffer and the solutions were stable for weeks at 4 °C.

Figure 4.2 Functionalization of Au NPs with PFPA-thiol and subsequent coupling of carbohydrates.

In order to quantitatively analyze GNPs–protein interactions, it is essential to determine the carbohydrate ligand density. The anthrone-H₂SO₄ assay, a colorimetric method that was widely used to measure carbohydrate concentrations in solution²⁰⁵ and on solid surfaces¹³³ was employed to measure the carbohydrate ligand density on the GNPs.¹⁸¹ Calibration curves were obtained by treating various concentrations of each carbohydrate with anthrone/sulfuric acid, and the absorption at 620 nm was plotted against the carbohydrate concentration. Au NPs with immobilized carbohydrate ligands were then subjected to the same assay, and the amount of ligand on the nanoparticles was subsequently derived by comparing

with the calibration curve. The coupling yield was estimated from the theoretically calculated maximal amount of each ligand that can occupy the 22-nm Au NP assuming a close-packed arrangement of the ligand on the NP. Table 4.1 summarizes the coupling yields of mono-, oligo- and poly-saccharides on Au NPs functionalized with PFPA-thiol **a**. The results show that the coupling yield increased with the size of the carbohydrate, which was anticipated since the probability of attaching the ligand via CH insertion reactions increases with the number of available CH bonds on the ligand.

Table 4.1 Coupling yield of mono-, oligo-, and poly-saccharides on Au NPs.

Carbohydrates	No. of Man/NP (Theoretical)^a	No of Man/NP (Experimental)	Coupling Yield (%)
Man	6333	3991	63 ± 4.2
Glc	6333	3641	57 ± 5.8
Man2	2303	1450	67 ± 6.1
Man3	1974	937	73 ± 5.4
Glc5	1299	836	79 ± 7.2
Dex	24	19	77 ± 16

^aEach data was the average of 5 samples.

Detection limit.

The unique optical property of metal nanoparticles, plasmon resonance absorption, offers a simple and attractive means to study molecular interactions with high sensitivity.^{88,107} The binding events occurring at the surface of the Au NPs result in a red shift in the plasmon resonance band, which can be conveniently monitored by UV-vis spectroscopy.¹⁵⁰ To determine the sensitivity of the GNPs in detecting lectins, D-mannose-coupled Au NPs were titrated with Con A, and the UV-vis spectra recorded (Figure 4.3a). The absorbance at 650 nm vs. Con A concentration was then plotted (Figure 4.3b), and the dynamic linear range of each curve was used to determine the limit of detection (LOD), which was calculated to be 6.2 nM, 7.4 nM, 10 nM, and 22 nM for **AuNP-a-Man**, **AuNP-b-Man**, **AuNP-c-Man** and **AuNP-d-Man**, respectively. The results showed that the sensitivity of the GNPs increased with the spacer length, and the lowest LOD was obtained for GNPs prepared from PFPA-thiol **a** that contains the long and flexible spacer linkage.

Figure 4.3 (a) UV-vis spectra of **AuNP-a-Man** upon addition of increasing concentration of Con A. (b) Absorbance of GNPs at 650 nm vs. Con A concentration.

Determination of binding affinity by fluorescence competition assay.

We developed a fluorescence competition assay to determine the binding affinity of GNPs with lectins using a fluorescently-labeled lectin and a free competing ligand. In a typical assay, GNPs of varying concentrations and a fixed concentration of a free competing ligand were incubated with Con A-FITC (Figure 4.4a). Two equilibria, Con A with GNPs and Con A with the free ligand, were established in the system (Figure 4.4b). Because a relatively low concentration of Con A was used, no agglomeration was observed in the assay. After the solution was incubated for 1 hour, it was centrifuged, bringing down GNPs including those bound to Con A-FITC. The unbound Con A-FITC and free ligand-Con A conjugate remained in the supernatant, corresponding to the amount of Con A-FITC that did not bind GNPs. The fluorescence of the supernatant was measured (Figure 4.4d), and the intensity at 517 nm was plotted as a function of the ligand density on GNPs (Figure 4.4e). From this concentration response curve, the IC_{50} value was determined and the apparent dissociation constant K_d was computed according to the Cheng-Prusoff equation²⁰⁶ (Figure 4.4c, $K_d = K_{d2}$).

Figure 4.4 (a) The fluorescence-based competition binding assay; (b) two equilibriums established in the system; (c) Cheng-Prusoff equation, where IC_{50} = concentration of ligand displaying 50% of specific binding; $[M]$ = concentration of free ligand, K_{d1} = dissociation constant of the free ligand with Con A; and K_{d2} = dissociation constant of GNPs with Con A; (d) fluorescence spectra of the supernatant as a function of increasing concentration of **AuNP-b-Man**; (e) concentration response curve, where the IC_{50} value was obtained.

This method was furthermore validated by several control experiments. First, different concentrations of Con A-FITC, 18 nM, 40 nM and 80 nM, were used in the assay and the binding affinity determined. The results obtained were similar for all three

concentrations with less than 5% variation among the three K_d values. Secondly, different competing ligands, e.g., MeMan, Man2, and Man3, were used in addition to Man. Although the binding affinities of these ligands with Con A were significantly different from that of Man, when they were used as the competing ligand to determine the K_d value of the same GNPs, the results were consistent and did not show significant variations. Thirdly, **AuNP-a-Man** was incubated with Con A-FITC in the absence of the competing ligand. In this case the fluorescence intensity decreased with the concentration of Con A-FITC, and the typical IC_{50} concentration response curve was not observed. Lastly, Au NPs coupled with Gal, a carbohydrate that does not bind Con A, was tested to ensure that the binding was due to the specific interactions of the surface-bound ligands with the lectin. No significant changes in the fluorescence intensity were observed after varying concentrations of GNPs were incubated with Con A-FITC, demonstrating that the strong affinity of **AuNP-a-Man** with Con A was indeed due to the specific binding of surface-bound Man with Con A. This result furthermore revealed that the non-specific adsorption of lectin to non-lectin binding carbohydrates was minimal.

With the methodologies developed, we next tested the impact of nanoparticles as the scaffold on the affinity ranking of various carbohydrate ligands with lectins. We chose Man, Man2, and Man3, which have at least several-fold differences in binding affinity between each ligand with Con A (Table 4.2). These ligands were attached to Au NPs using PFPA-thiol **3a**, and the apparent K_d values of the resulting GNPs with Con A

were determined by the fluorescence competition assay. An increase in the binding affinity of 4.5-fold and 33-fold was observed for **AuNP-a-Man2** and **AuNP-a-Man3** in comparison with **AuNP-a-Man**, respectively. Since the number of ligands on each GNPs was different, when taking into consideration the ligand density, the affinity increase was 12-fold and 141-fold, respectively. This compares well with the affinity ranking of the corresponding free ligands, which was 20 and 158 times higher affinity of Man2 and Man3 than Man with Con A in solution.^{136,148}

In addition, the binding affinity of **AuNP-a-Glc** was determined, and the result showed over 4 orders of magnitude higher affinity than that of the free ligand with Con A (Table 4.2). Compared with the affinity enhancement in the case of Man, however, the binding of **AuNP-a-Man** with Con A was over 30 times higher than that of **AuNP-a-Glc**. Considering that the affinity of free Man with Con A was only 4 times higher than Glc, the affinity enhancement when the ligands were attached to NPs was significantly higher. This observation, i.e., the amplification of binding affinity difference due to multivalent interactions, has also been observed for carbohydrate ligands on neoglycopolymers²⁰⁷ and dendrimers.²⁰⁸

Table 4.2 Binding affinity of different GNPs with Con A

GNPs	Number of ligands/NP	K_d (nM)	K_D (μ M) ^a
AuNP-a-Man	3,991	0.43 ± 0.044	470^{209}
AuNP-a-Man2	1,450	0.095 ± 0.008	24.0^{136}
AuNP-a-Man3	937	0.013 ± 0.002	2.97^{136}
AuNP-a-Glc	3,641	12.7 ± 2.5	$1,786^{209}$

^a K_D : dissociation constant of the corresponding free ligand with Con A.

Binding affinity with respect to ligand presentation.

Unlike the free ligand that has the translational and rotational freedom in solution, the surface-bound ligand is no longer an un-restricted entity. Each ligand becomes a member of the nanomaterial carrier and can act cooperatively when interacting with their binding partners. The binding affinity is sensitive to a number of factors including the coupling chemistry, the size of the nanomaterial scaffold, the type and length of the spacer linkage connecting the ligand and the nanomaterial, the flexibility/rigidity of the spacer, the density of ligands and the distance between them. In the present study, the ligand density, linker length, and nanoparticle size were varied, and their impacts on the binding affinities of the resulting GNPs were investigated.

Ligand density. To control the surface ligand density, we employed the mixed SAM approach where a non-photoactive thiol together with PFPA-thiol was used to functionalize Au NPs. Solutions containing PFPA-thiol **a** or **b** and 1-hexanethiol at varying mole ratios were used to treat Au NPs. Man was subsequently coupled and the density of attached ligand measured (Table 4.3). The apparent K_d value of the resulting GNPs was then determined using the fluorescence competition assay described above. Results showed that the binding affinity generally increased with the ligand density. For **AuNP-a-Man**, however, there seems to be a maximal affinity for Au NPs treated with 98% PFPA-thiol **a** at the ligand density of 3,004 Man/NP. This maximal affinity was not observed in the case of **AuNP-b-Man**, likely due to the lower ligand density even at 100% PFPA-thiol treatment (2,824 Man/NP). To investigate the generality of this observation, Man2 and Man3 were coupled on Au NPs treated with mixed SAM of 1-hexanethiol and PFPA-thiol **a**, and the K_d values were measured with respect to the ligand density. The highest binding affinity of the resulting GNPs occurred at 50% and 30% of PFPA-thiol **a** in the mixed SAM, corresponding to the ligand density of 289 Man2/NP and 132 Man3/NP, respectively (Figure 4.5).

Table 4.3 Binding affinity as a function of ligand density.

PFPA-thiol (mole%)	AuNP-a-Man		AuNP-b-Man	
	Number of Man/NP	K_d (nM)^a	Number of Man/NP	K_d (nM)^a
10%	107	27.4 ± 2.3	99	92 ± 12
30%	283	8.67 ± 0.87	260	16.1 ± 5.5
50%	544	1.93 ± 0.25	549	12.3 ± 4.1
70%	1,444	1.33 ± 0.31	1,196	10.8 ± 1.8
90%	2,275	1.01 ± 0.22	1,726	9.6 ± 3.0
95%	2,756	0.88 ± 0.14	2,090	7.1 ± 1.7
98%	3,004	0.39 ± 0.09	2,474	5.0 ± 1.0
100%	3,991	0.43 ± 0.044	2,824	4.0 ± 0.7

^aEach data was the average of 3 measurements each on 5 samples.

Figure 4.5. Binding affinity vs. ligand density for Man (a), Man2 (b), and Man3 (c) with Con A. Each data point was the average of 3 measurements each on 5 samples.

A few general observations can be drawn from the ligand density studies. For all three ligands, there was a sudden increase in binding affinity, and after which, the K_d values remained more or less constant. The ligand density at which the drastic change in binding affinity occurred was estimated from the intercept of the two relatively linear curves in each graph, which was 370 Man/NP, 166 Man2/NP and 138 Man3/NP, respectively. Assuming that the ligands were evenly distributed on the GNPs, the footprint occupied by each ligand on the nanoparticle surface was calculated to be 4.1 nm², 9.2 nm² and 11.0 nm², and the distance between neighboring ligands were

approximately 2.0 nm, 3.0 nm and 3.3 nm for Man, Man2 and Man3, respectively (see detailed calculation in Supporting Information). Note that the distance between each binding site on Con A is 6.5 nm as determined by X-ray structural analysis.¹⁸³ This distance is larger than the ligand spacing on NPs indicating that the binding is not monovalent and the higher ligand density is necessary for the enhanced affinity. The internal diffusion mechanism, which states that a lectin molecule “binds and jumps” from ligand to ligand along the scaffold,¹⁹⁰ can be applied to explain the results. According to the mechanism, the more ligands there are on the scaffold, the longer the dwelling time of the lectin on the ligands, and the slower the lectin would dissociate. This affinity increase was observed for all three ligands where the K_d values decreased drastically at lower ligand density (Figure 4.5). The affinity started to decrease after the maximal affinity was reached, and this decrease in affinity was more pronounced as the size of the ligand increased. This was likely due to the steric effect where the larger ligand hinders the lectin binding.²¹⁰

Spacer. PFPA-thiols **a**, **b**, **c** and **d** were used as the coupling agent to evaluate the effect of spacer linker on the binding affinity of the resulting GNPs. PFPA-thiols **c**, **d** contain a shorter spacer of six and two methylene units, and the K_d values of the corresponding GNPs were 15 nM and 19 nM, respectively (Table 4.4). When Au NPs were functionalized with PFPA-thiol **b** having 11 methylene units, K_d decreased to 4.0 nM, which represents 4-5 times increase in binding affinity as compared to the shorter spacer **c** and **d**. When PFPA-thiol **a** was used, the binding affinity increased an

additional order of magnitude in comparison to PFPA-thiol **b**. Because the ligand density increases with the spacer length, it could also contribute to the observed enhancement in the binding affinity. However, the density increase alone could not account for the magnitude of the affinity enhancement. At the similar ligand density, for example, 544 and 549 Man/NP for **AuNP-a-Man** and **AuNP-b-Man**, respectively, the binding affinity was 6 times higher for **AuNP-a-Man** than **AuNP-b-Man** (Table 4.4). This affinity increase was therefore solely caused by the difference in the spacer linkage between the two GNPs. In order to quantitatively assess the binding affinity enhancement of different GNPs, an affinity enhancement factor (EF) was used, as defined in Eq. 4.1,

$$EF = K_D/[K_d \times N] \quad (4.1)$$

where K_D is the dissociation constant of free ligand with Con A, which is 470 μM for Man,²⁰⁹ K_d is the apparent dissociation constant of GNPs with Con A, and N is the number of ligands on each GNP. Here, the ligand density is taken into consideration, and the EF value can therefore be used to rank binding affinity with respect to the difference in the spacer linkage. Results in Table 4.4 show that the EF values for **AuNP-d-Man** and **AuNP-c-Man** were similar, ~ 16 , whereas that of **AuNP-b-Man** was 2.6 times higher at 42, and that of **AuNP-a-Man** was in addition 17 times higher at 274. These results clearly demonstrate that longer spacers led to enhanced binding affinity of GNPs with lectins. The spacer elevates the ligand further from the solid substrates. On curved surfaces such as nanoparticles, a longer spacer would also result in a larger distance between the ligands at the same ligand density. Both events would

Table 4.4 Binding affinity of GNPs with Con A.

GNPs	Number of Man/NP	K_d (nM)^a	EF
AuNP-a-Man	3,991	0.43 ± 0.044	274
AuNP-b-Man	2,824	4.0 ± 0.72	42
AuNP-c-Man	1,959	15 ± 2.0	16
AuNP-d-Man	1,590	19 ± 2.2	16

^aEach data was the average of 5 samples, 3 measurements each.

reduce the steric hindrance when the lectin approaches the ligands, making the ligands more accessible for interacting with the lectin.

Nanoparticle size. It is well-established that the physical and chemical absorption, and the catalytic property of gold nanoparticles are highly size-dependent.¹⁷¹⁻¹⁷³ In this study, the effect of nanoparticle size on binding affinity was investigated by varying the diameter of Au NPs. In addition to the 22-nm nanoparticles, Au NPs of 7 nm, 14 nm, and 30 nm in diameter were synthesized using the same protocol (Figures 4.6), and Man was subsequently conjugated using PFPA-thiol **b**. The ligand densities of the resulting GNPs and their binding affinities were measured (Table 4.5). As expected, the number of ligands attached on Au NPs increased with the size of the nanoparticles. The binding affinity of GNPs with particle sizes of 7 nm, 14 nm, and 22 nm were similar, whereas a decrease of about 6-fold in binding affinity was observed for the

30-nm GNPs. The EF values calculated for each GNPs showed a considerable size-dependent effect, with the EF value increasing with decreasing particle size. Smaller nanoparticles yielded the highest affinity enhancement, likely due to their large surface-to-volume ratio and higher mobility in solution.

Figure 4.6 TEM images of gold nanoparticles in 4 different sizes: 7 nm (a), 14 nm (b), 22 nm (c) and 30 nm (d).

Table 4.5 Binding affinity of GNPs of varying sizes

Au NP Diameter (nm)	Number of Man/NP	K_d (nM)	EF
7.2 ± 1.8	297	3.38 ± 0. 67	468
14 ± 2.6	1,127	3.14 ± 0.49	132
22 ± 3.3	2,824	3.99 ± 0. 81	42
30 ± 4.0	4,486	24.8 ± 3.1	4.2

Conclusion

In conclusion, we developed a fluorescence-based competition assay to determine the apparent dissociation constants of GNPs with lectin. The assay was successfully used to determine the K_d values and to evaluate the binding affinity of GNPs. When carbohydrate ligands were attached on the Au NP scaffold, their interactions with lectins were drastically enhanced, and several orders of magnitude increases in binding affinity were observed between GNPs and lectin. Systematic studies were conducted to investigate the impacts of nanoparticle size, spacer length, ligand size and density on the binding affinity of GNPs. Results show that the lectin binding to ligands on GNPs is profoundly affected by how the ligands are displayed on the NP surface. Findings from this study are important that GNPs with controlled binding affinity can be readily synthesized by varying ligand density, spacer linker, and scaffold configuration. This will open up immense opportunities for tailor-made glyconanomaterials where the ligand display can be exploited to tune their bioaffinities.

Supporting Information

Calculations of ligand density and distance

Figure 4.7 Schematic illustration of Con A and GNP.

Assuming that carbohydrate occupies in space by taking the shape of a square, the ligand molecule is then projected to the surface using Chem 3D (CambridgeSoft, Ultra, version 9.0), and the surface area of each Man, Man2 and Man3 molecule were calculated to be 0.24, 0.66, and 0.77 nm², respectively.

The ligand-ligand distance (L) can be calculated from the following:

$$L = \sqrt{\frac{S_{NP}}{N}}$$

where S_{NP} is the surface area of Au NP, and N is the number of ligands attached. At the point where there was a sudden increase in binding affinity, the number of ligands was determined from the intercept of the two relatively linear curves of each graph which was 370, 166 and 138 ligands per NP for Man, Man2, and Man3, respectively. Therefore, the ligand distance at the deflection point is 2.0, 3.0 and 3.3 nm for Man, Man2 and Man3, respectively.

Chapter 5. Applications of Glyconanomaterials in Bioanalysis

The work in Section 5.1 was published on “**Wang, X.**; Matei, E.; Deng, L. Q.; Ramström, O.; Gronenborn, A. M.; Yan, M., Multivalent Glyconanoparticles with Enhanced Affinity to Anti-Viral Lectin Cyanovirin-N *Chem. Commun.* **2011**, 47, 8620-8622”

<http://pubs.rsc.org/en/Content/ArticleLanding/2011/CC/C1CC12981C>

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The work in Section 5.2 was published on “**Wang, X.**; Ramström, O.; Yan, M., Dye-doped silica nanoparticles as efficient labels for glycans. *Chem. Commun.* **2011**, 47 (14), 4261-4263” (Section 3.2)

<http://pubs.rsc.org/en/Content/ArticleLanding/2011/CC/c0cc05299j>

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The work in Section 5.3 was submitted for publication.

5.1 Signal Enhancement Study Glycan-lectin Interactions using Anti-HIV Lectins

In this chapter, a specific platform was presented for the binding signal enhancement of glyconanoparticle with biologically important anti-HIV lectin Cyanovirin-N in a rigorous and coherent way. Our method enables sensitive recognition of different type of lectin mutants with various binding sites using high-mannose conjugated gold nanoparticles, and more significantly visualize the difference using UV-vis and TEM techniques. No other known methods can efficiently accomplish this. This fact alone results in a platform, where the effect of ligand display on multivalency can be efficiently monitored, as well as quantitatively analyzed. The present coupling method is novel, using only unprotected high mannose oligosaccharides structures. We have demonstrated that our method is general, versatile, and has been validated with other solution- and surface-based assays. It is of high importance that our results have shown that the glyconanoparticles system is highly valid in distinguishing multivalency effects based on nanoparticle presentation. The binding affinities of both Man2 and Man3-conjugated nanoparticles were found several orders of magnitude higher than those of free Man2 and Man3 in solution, based on ITC results.

Lectins, carbohydrate-binding proteins, play critical roles in a plethora of biological processes.^[1] An in-depth understanding of carbohydrate-lectin interactions is not only fundamentally important for elucidating their biological functions, but also of

outstanding practical value in the design and development of therapeutics and diagnostic tools. Cyanovirin-N is an 11 kDa cyanobacterial lectin that exhibits inhibitory activity against a number of viruses, including HIV, at concentrations as low as nanomolar. Its anti-HIV activity is mediated by binding to the high-mannose (HM) structures on the envelope glycoprotein gp120.^[2] Previous studies established that the binding epitope(s) on *N*-linked high oligomannosides for CV-N comprised α -D-Manp-(1 $\rightarrow$ 2)- α -D-Manp moieties on the glycan's D1 and D3 arms.^[3] Multivalency, resulting in cooperative interactions of multiple ligands with multiple receptors, is a general phenomenon that occurs in many biological processes involving molecular recognition. Multivalent interactions are often significantly stronger than the corresponding monovalent interactions, and, as such, the design and creation of multivalent reagents is an important strategy for generating diagnostic and therapeutic tools.^[4] In glycobiology, these kinds of approaches are especially relevant given the commonly observed weak affinities between glycans and lectins.^[5] On the other hand, high glycan structures exhibit drastically enhanced apparent affinities, compared to the monovalent ligands. However, the synthesis of high glycans is tremendously demanding, involving multiple protection/ deprotection steps and complex stereochemistry control. As such, their availability is limited. An alternative approach for creating multivalency is to use a scaffold, such as polymers, lipids or nanomaterials, on which multiple copies of a ligand can be presented, thereby generating a multivalent ligand.^[6] For example, Melander and coworkers prepared small molecule-coated gold nanoparticles (AuNPs) as effective inhibitors for HIV

fusion,^[7] and Gervay-Hague's group reported that galactosyl- and glucosyl-functionalized AuNPs exhibited 300 times better binding to gp120.^[8] In previous studies from our group, we showed that carbohydrate ligands conjugated to AuNPs exhibited affinities up to five orders of magnitude higher than those of the corresponding monomeric ligands with lectins.^[9]

Here, we conjugated two low-mannoses, Man2 and Man3, to the AuNP scaffold, and investigated the binding affinity of the resulting GNPs with CV-N lectins (Figure 5.1). In order to derive quantitative numbers for the affinity enhancement caused by AuNPs, we developed a fluorescence competition assay and determined the apparent dissociation constant of GNP binding to CV-N (K_D). The results from this assay were compared with the K_d values of monomeric glycan binding to CV-N using isothermal titration calorimetry (ITC).

Experimental Section

Preparation of gold glyconanoparticles

AuNPs, 22 nm in diameter, were prepared and functionalized with PFPA-disulfide following the same procedure as described in a previous chapter.^[9] The subsequent carbohydrate coupling was carried out photochemically as reported previously.^[10]

Before the binding experiments, the GNPs were incubated in 20 mM sodium phosphate buffer solution (pH 6.0), containing 0.01% NaN₃, 0.01% Tween 20 and 3% BSA for 1 hour, centrifuged, and placed in buffer without BSA until further use.

CVN binding assays

GNP-M3 (5 nM, 1.0 mL) was incubated in a solution of CVN^{Q50C} or CVN^{MutDB} in 20 mM sodium phosphate buffer solution (10 μ M, 1.0 mL), pH 6.0, containing 0.01% NaN₃ for 1 hour with constant shaking. UV-vis spectra of the resulting solutions were recorded on a Perkin Elmer Lambda 45 UV-vis spectrophotometer, and each measurement was performed at least 3 times.

Isothermal Titration Calorimetry (ITC)

ITC experiments of Man3 binding to CVN were performed using an ITC200 Microcalorimeter from Microcal, LLC. (Northampton, MA) in 50 mM sodium phosphate buffer, pH 7.5, 200mM NaCl, 0.02% NaN₃. The concentration of CV-N was 50 μ M, and that of Man3 was 0.64 mM. In each individual experiment, ~38 μ L of Man3 was injected through the computer-controlled 40- μ L micro-syringe at an interval of 4 min into the protein solution in the same buffer (cell volume = 200 μ L) while stirring at 350 rpm. Calorimetric titrations of Man2 binding to CVN were performed using a VP-ITC isothermal titration calorimeter (MicroCal, LLC; Northampton, MA). Titrations were carried out at 30°C in the same buffer as described above for Man3. A 35 μ M CV-N solution was placed in the calorimeter cell (~1.44 mL active volume), stirred at 310 rpm, and 9- μ L aliquots of 1.5 mM Man2 solution were added at 2 min intervals from a 295- μ L stirring syringe. A total of 30 injections

were performed. The experimental data were fitted to a theoretical titration curve using the software supplied by MicroCal. A standard two-site model was used with ΔH (enthalpy change, in kcal/mol), K_a (association constant, in M^{-1}), and N (number of binding sites) as the variables.

Fluorescence competition binding assays

The previously reported protocol was adapted,^[9] as described below. A series of **GNP-M2** solutions were prepared by diluting a stock solution (10 nM) to concentrations between 5 nM and 1×10^{-8} nM. A stock solution of Cy5-CVN (1.20 μM) was prepared in pH 6.0 sodium phosphate buffer (20 mM). To a solution of **GNP-M2** (1 mL) in a 1.5-mL microcentrifuge tube, Man2 (0.48 mM, 0.1 mL) and Cy5-CVN (1.2 μM , 0.1 mL) were added. The total volume of the final solution was 1.20 mL, and the concentrations of Man2 and Cy5-CVN were 40 μM and 100 nM, respectively. The solutions were shaken for 1 hour, and then centrifuged at 12,000 rpm for 30 min until all nanoparticles were completely pelleted at the bottom of the tube. The supernatants were taken out for fluorescence measurement using a PTI spectrofluorimeter (Photon Technology International). Excitation was at 649 nm and emission was recorded at 666 nm for analysis. The same procedure was followed for **GNP-M3**, except that Man3 was used as the competing ligand. Measurement for each concentration was repeated 5 times, and values were averaged.

K_{D1} and K_{D2} , apparent dissociation constants for GNP binding to the glycan-binding site on Domain A and Domain B of CVN^{Q50C}, respectively, were obtained from best-fitting the response curves, using a two-site competitive binding model with the equation in KaleidaGraph software.

Figure 5.1 Synthesis of Man2- and Man3-conjugated AuNPs **GNP-M2** and **GNP-M3**.

Results and discussion

GNP-M2 and **GNP-M3** were prepared following a previously established procedure,^[10] outlined in Figure 5.1. Uniform, ~22 nm AuNPs were synthesized by the Turkevich method^[11] and were subsequently functionalized with PFPA-disulfide (Figure 5.1). Man2 and Man3 were then conjugated to the PFPA-functionalized AuNPs using a photocoupling method^[10] by way of a CH insertion reaction of the photogenerated perfluorophenylnitrene.^[12] The ligand density was determined using a

colorimetric approach with anthrone/sulfuric acid. Values of $1,516 \pm 232$ Man2 and $1,037 \pm 148$ Man3 were obtained for **GNP-M2** and **GNP-M3**, respectively.

Binding affinities of **GNP-M3** to CV-N was evaluated using two CV-N variants: CVN^{MutDB} and CVN^{Q50C}. CVN^{Q50C} is essentially a wild-type variant, comprising two separate glycan binding sites one on Domain A and one on Domain B.^[3a, 3c] Domain A exhibits a slight preference for the Man3 units and domain B for the Man2 units.^[13, 3c] The Cys substitution at position 50 was introduced to allow for specific fluorescence labeling of CV-N without interfering with glycan binding. In the CVN^{MutDB} variant on the other hand, the glycan binding site on domain B is completely eliminated, while the site on domain A still can bind glycan ligands. Since this variant no longer can cross-link glycans on gp120, it has lost its anti-HIV activity.^[14] Therefore, in interactions with GNPs, we would expect that CVN^{Q50C} can act as a crosslinker and form a complex with **GNP-M3**, while no such crosslinking should be possible between **GNP-M3** and CVN^{MutDB}. Indeed, **GNP-M3** treatment with CVN^{Q50C} caused a red shift from 529 nm to 542 nm in the surface plasmon resonance (SPR) band of AuNPs (Figure 5.2a), indicative of particle size growth.^[15] Such an increase in particle size was further confirmed by TEM which revealed the presence of clusters of aggregated particles (Figure 5.2c). When **GNP-M3** was treated with CVN^{MutDB}, however, no SPR shift was observed (Figure 5.2b). TEM images were devoid of aggregates and only isolated single particles were observed in this case (Figure 5.2c). Dynamic light scattering (DLS) measurements of CV-N treated **GNP-M3** particles yielded average particle sizes of 25.9 ± 3.5 nm and 38.3 ± 4.6 nm for CVN^{MutDB} and

CVN^{Q50C}, respectively. These results are all consistent with our previous structural studies on CVN^{MutDB} that revealed a single glycan binding site.^[14]

Figure 5.2 UV-vis spectra of **GNP-M3** before (solid line) and after (dotted line) treatment with a) CVN^{Q50C} and b) CVN^{MutDB}. TEM micrographs of **GNP-M3** treated with c) CVN^{Q50C} and d) CVN^{MutDB}. Scale bars: 100 nm.

The binding affinities of the GNPs to the CV-N variants were evaluated using a recently developed fluorescence-based competition assay.^[9] In the experiment, free ligand competitor (Man2 for **GNP-M2**, Man3 for **GNP-M3**) together with varying concentrations of **GNP-M2** or **GNP-M3** was incubated at a fixed concentration of Cy5-CVN^{Q50C}, specifically Cy5-labeled CVN^{Q50C} (Figure 5.3a, see experimental

section for details). The solution was centrifuged to remove all GNPs and the fluorescence intensity of the supernatant was measured. The difference in fluorescence intensity of Cy5-CVN^{Q50C} before and after incubation with GNPs corresponds to the amount of the bound CVN^{Q50C}. Concentration response curves for **GNP-M2** or **GNP-M3** permit the determination of IC₅₀ values (Figure 5.3c).

Figure 5.3 Fluorescence competition assay. a) Schematic representation of binding scenario. b) Modified Cheng-Prusoff equation based on a competitive two site binding model, where [M] is the concentration of the free ligand, and K_{d1} and K_{d2} are the dissociation constants of the free ligand for the glycan-binding sites on Domains A and B of CVN^{Q50C}, respectively. The data were fitted using the maximum bound fractions, f_{Bmax1} and f_{Bmax2}, corresponding to the two binding sites, and dissociation constants K_{D1} and K_{D2} as adjustable parameters. c) Concentration response curves of **GNP-M2** and **GNP-M3**.

In order to extract the binding constants of GNPs with CVN^{Q50C}, it is necessary to know the K_d values of the monomeric ligands, Man2 and Man3, with CVN^{Q50C}. These values were determined by ITC and the dissociation constants, K_{d1} (glycan-binding site on Domain A) and K_{d2} (glycan-binding site on Domain B), were calculated based on a two-site binding model (see Figure 5.4). Values for K_{d1} and K_{d2} of 700 μ M and 64 μ M for Man2, and 3.4 μ M and 43 μ M for Man3, respectively, were calculated (Table 5.1). These values agree well with our previous observation that slightly stronger binding of Man2 to the site on Domain B than to that on Domain A occurs, while the opposite is true for Man3.^[3a, 3c, 14] These data, together with the IC_{50} values determined from the data shown in Figure 5.3c, were then used to calculate the apparent dissociation constants for the site on Domain B, K_{D1} and K_{D2} , based on a two binding site model (Figure 5.3b).

Figure 5.4 Calorimetric titration of a) CVN^{Q50C} (35 μ M) with Man2 (1.5 mM), and b) CVN^{Q50C} (50 μ M) with Man3 (0.64 mM) at 30 $^{\circ}$ C. The raw data were obtained for 30 and 20 automatic injections, respectively. The integrated curves show experimental points (■) and the best fit (-). The buffer was 50 mM sodium phosphate, 200 mM NaCl, pH 7.5, containing 0.02% NaN₃.

The data summarized in Table 5.1 demonstrate that both GNPs, **GNP-M2** and **GNP-M3**, exhibit an affinity enhancement by several orders of magnitude compared to the affinities measured for the isolated, monomeric sugars interacting with CVN^{Q50C}.

Taking into account the number of ligands on the particles, i.e. considering the affinity/ligand, an increase up to several hundred times is still present for the AuNP-bound glycan (Table 5.1). In addition, **GNP-M3** exhibited a higher affinity than

Table 5.1 Affinities for Man2/3 (K_d) and **GNP-M2/3** (K_D) binding to CVN^{Q50C}.

Numbers in parentheses correspond to EF ($=K_{d1}/K_{D1} \cdot \text{Number of ligands on GNP}$).

Ligand	K_{d1} or K_{D1} (Domain A)	K_{d2} or K_{D2} (Domain B)
Man2	$700 \pm 50 \mu\text{M}$	$64 \pm 4 \mu\text{M}$
GNP-M2	$56.4 \pm 7 \text{ nM}$ (8.2)	$0.24 \pm 0.1 \text{ nM}$ (176)
Man3	$3.4 \pm 0.2 \mu\text{M}$	$43 \pm 2 \mu\text{M}$
GNP-M3	$0.011 \pm 0.007 \text{ nM}$ (309)	$11.8 \pm 2.3 \text{ nM}$ (3.6)

GNP-M2 for both domains. These results correlate well with the general affinity ranking of the free ligands Man2 and Man3, and are consistent with observations in our previous study with a different GNP-lectin system.^[9] Interestingly, for both **GNP-M2** and **GNP-M3**, the affinity enhancement is more pronounced for the better binding domain. For example, Man2 exhibits a higher affinity for the binding site on Domain B, and with **GNP-M2**, the affinity enhancement factor (EF) is 178 for the Domain B site vs. 8.3 for the Domain A site (Table 5.1). For **GNP-M3**, on the other hand, the opposite was observed that the EF is higher for the Domain A site (340) than for the Domain B site (3.8).

Conclusion

In conclusion, we have successfully grafted low-mannose ligands onto AuNPs via an efficient photocoupling reaction. The resulting GNPs interacted with the CV-N

variants CVN^{MutDB} and CVN^{Q50C} in a manner that is consistent with the expected behavior of one- and two-site binders. Crosslinked complexes and aggregates were observed when **GNP-M3** was treated with the two-site CVN^{Q50C} while only single particles were seen after treatment with the single-site variant CVN^{MutDB}. Furthermore, these GNPs exhibited significantly higher affinity towards the CV-N lectins, compared to the free glycan ligands, demonstrating that AuNPs serve as an efficient multivalent scaffold that significantly enhances the apparent affinity. This affinity enhancement compares well with that of other synthetic multivalent ligands. Therefore, a general strategy can be envisioned which uses simple glycans, rather than large and complex sugars, for grafting onto a multivalent scaffold for affinity amplification. These types of approaches will aid in development of effective new glyconanomaterials for diagnostic and therapeutic applications.

5.2 Labeling Native Glycans with Dye-doped Silica Nanoparticles

Introduction

A major challenge in bioanalysis, including high-throughput screening, is the means to effectively display the outcome of the ligand-receptor interactions. Fluorescence is by far the most commonly used detection method, which requires the conjugation of a fluorescent tag to the ligand to be studied.²¹¹⁻²¹³ Among the fluorescent tags, organic dyes continue to be the label of choice due to the availability of a wide variety of commercial products of diverse structures, functionalities, solubility, and spectral properties. A major drawback of organic dyes, however, is their relatively poor photostability. When exposed to light, organic dyes can photobleach and lead to a decrease in fluorescence intensity. A clever solution to this problem is to entrap the fluorescent dye inside a solid matrix, for example silica nanoparticles.²¹⁴ The nanoparticle protects the dye molecules from being directly exposed to the environmental oxygen, and thus greatly enhances the photostability of the entrapped dye.²¹⁵ Furthermore, because a large number of dye molecules can be embedded inside a nanoparticle, high fluorescence emission can be obtained, the intensity of which exceeds the dye molecule itself or even quantum dots.²¹⁶⁻²¹⁹ In addition, silica nanoparticles are biocompatible and of low toxicity.^{3,84} Uniformly sized silica nanoparticles can be readily prepared from inexpensive starting materials following simple synthetic procedures.¹⁶²

Ligand labeling often requires a robust conjugation method where the labeling agent can be covalently attached to the ligand. For many biomolecules, this can be conveniently accomplished, for example, by using a commercial kit containing chemically-derivatized labeling agents. For ligands that lack functional groups or are difficult to derivatize, the task of labeling can be complex and challenging. We have developed a general coupling chemistry, based on perfluorophenyl azides (PFPAs), that can conjugate a variety of molecules regardless of their chemical structures.^{126,140,181,220} Upon photochemical or thermal activation, PFPA is converted to a highly reactive singlet perfluorophenyl nitrene which forms covalent adducts with neighboring molecules by way of CH insertion and/or C=C addition reactions. Therefore, by functionalizing FSNPs with PFPA, ligands can be conjugated to FSNPs via the photocoupling reaction of the surface PFPA. In this study, we present that PFPA-functionalized FSNPs can be used as highly efficient fluorescence labels for carbohydrate ligands. Carbohydrates are an important class of biomolecules involved in many important biorecognition processes including, for example, cell communication, immune responses, fertilization, and infections.^{57,61,64,178} Studies of these processes are, however, hampered by the high complexity of glycan structures and the lack of efficient bioanalytical tools.^{74,221-223} The present study seeks to address some of these challenges by developing a new method to efficiently label underivatized carbohydrates with FSNPs. We evaluate FSNP-labeled carbohydrates for their affinities with lectins. The synthesized glyco

FSNPs are furthermore applied to image bacteria, and to probe carbohydrate-protein interactions on a lectin microarray.

Experimental details

Synthesis of FITC-doped silica nanoparticles FSNPs.

Fluorescein isothiocyanate (39 mg, 0.10 mmol) was mixed with APTMS (23 μ L, 0.10 mmol) in 100 mL of absolute ethanol, and was stirred at 42 °C for 24 h to yield the FITC-silane precursor as a bright yellow solution. The fluorescent nanoparticles were synthesized following a modified protocol from the classic Stöber protocol,¹⁶² similar to what was previously described.¹²⁵ The dye precursor solution (5 mL) was mixed with TEOS (2.8 mL), and the mixture was added to 200 proof absolute ethanol (34 mL) followed by NH₄OH (35%, 2.8 mL). The reaction was allowed to proceed at room temperature for at least 8 h with vigorous stirring to yield a bright yellow colloidal solution. The particle diameters were determined by TEM and DLS (Figure 5.9 and 5.10 in Supporting Information)

Functionalization of silica nanoparticles with PFPA.

PFPA-silane (80 mg), synthesized following a previously reported procedure,¹²³ was added directly to the Stöber solution prepared above, and the mixture was stirred at room temperature overnight. The next day the mixture was brought to reflux while continuing stirring for 1 h at ~78 °C to facilitate the covalent bond formation between PFPA-silane and the silica nanoparticles.¹²⁵ The mixture was centrifuged at 8,000 rpm

for 10 min, and the precipitate was redispersed in the fresh solvent by sonication. This centrifugation/redispersion procedure was repeated three times with ethanol and twice with acetone.

Conjugation of carbohydrates onto FSNPs.

Our previous reported procedure of coupling carbohydrates on gold nanoparticles was followed.¹⁸¹ The solution of PFPA-functionalized FSNPs in acetone (20 mg/mL, 5 mL) was placed in a flat-bottom dish, and an aqueous solution of carbohydrate (10 mg/mL, 1 mL) was added. The mixture was covered with a 280-nm long-path optical filter (WG-280, Schott Glass) and was irradiated with a 450-W medium pressure Hg lamp (Hanovia) for 10 min under vigorous stirring. Centrifugation of the solution at 8,000 rpm for 10 min separated the carbohydrate-attached FSNPs as precipitates. Excess carbohydrate was removed by membrane dialysis in water for 24 hours. The concentration of FSNP, ~17.2 mg/mL, was determined by drying the solution under reduced pressure for 3 hours and weighing.

Determination of carbohydrate density on glyco-FSNPs.

A previously developed colorimetric method was followed to determine the density of carbohydrates immobilized on FSNPs.¹⁸¹ Calibration curves were first obtained for each carbohydrate where carbohydrate solutions of various concentrations were incubated with anthrone/sulfuric acid and the absorbances at 620 nm were measured.¹⁵⁵ A freshly-prepared anthrone solution in concentrated H₂SO₄ (0.5 wt%, 1

mL) was added to a carbohydrate solution in water (0.5 mL) in an ice bath under stirring. The solution was then heated to 100 °C and stirred for 10 min. After cooled to room temperature, the UV-vis spectra of the resulting solutions were recorded on a Perkin-Elmer Lambda 45 UV-vis spectrometer.

Carbohydrates coupled on nanoparticles were subjected to the same assay where solutions of the glyco-FSNPs in Milli-Q water (30–50 µg/0.5 mL) were treated with anthrone/H₂SO₄. Background absorption due to FSNPs themselves was accounted for by treating FSNPs solution of the same concentration with anthrone/H₂SO₄, and the absorbance at 620 nm was subtracted from that of the glyco-FSNPs. The amount of surface-bound carbohydrate was then computed from the corresponding calibration curve.

Binding with Con A and E. coli.

The binding affinity of FSNP-labeled Man, FSNP-Man, was evaluated using Con A and *E. coli* strain ORN178 and ORN208 according to the following procedure. FSNP-Man (2.5 mg) were incubated in a pH 7.2 HEPES buffer solution (1 mL, 10 mM) containing 3% BSA for 30 min, centrifuged, and the particles were incubated in the pH 7.2 HEPES solution without BSA for another 20 min. The nanoparticles were subsequently treated with a solution of Con A in HEPES buffer (1 mL, 10 µg/mL) containing MnCl₂ (1 mM) and CaCl₂ (1 mM), or *E. coli* solution for 1 hour while shaking. In cases where aggregation was induced after binding with Con A, the

suspension was transferred to a centrifuge tube and was centrifuged at 8,000 rpm for 10 min.

Fabrication of lectin microarrays.

Aldehyde-coated glass slides were prepared following a reported procedure.²²⁴ Piranha-cleaned glass slides were treated with a solution of 3-(trimethoxysilyl)butyl aldehyde in toluene (2 mM) for 4 hours, rinsed with toluene and dried with N₂. Solutions of lectins were prepared in pH 7.4 phosphate-buffered saline (PBS) at varying concentrations of 0.1-1 mg/mL with 40% glycerol added to prevent evaporation of the liquid droplets after printing. Con A and SBA were then printed onto the aldehyde-functionalized glass slide using a robotic printer (BioOdyssey Calligrapher miniarrayer; Bio-Rad Laboratories, Inc.). The glass slides were then incubated in a humid chamber (80% humidity) at 25 °C for 3 h to facilitate the immobilization of the lectins. After incubation, the blocking solution of BSA in pH 7.4 PBS buffer (1%) was added and the slides were incubated for 1 h, rinsed with the PBS buffer and was dried with N₂.

Microarray assay and fluorescence imaging.

The lectin microarrays were incubated in the solution of glyco FSNPs in HEPES (2.5 mg/mL) for 2 h, and were then gently rinsed with the HEPES buffer 3 times and dried. The slides were scanned under a microarray scanner (GenePix 4000B, Molecular

Devices, Inc) at excitation of 532 nm. The fluorescent images were recorded and the data were analyzed using the supplied software (Axon GenePix Pro 5.1).

Results and discussion

Figure 5.5 (a) Synthesis of PFPA-functionalized FSNPs, and (b) high-throughput synthesis of glyco FSNPs.

Fluorescein (FITC)-doped silica nanoparticles were synthesized using a modified Stöber method.¹⁶² Fluorescein isothiocyanate was silanized with 3-aminopropyltriethoxysilane yielded FITC-silane (Figure 5.5a, which was then copolymerized with tetraethyl orthosilicate.^{214,217} The resulting FSNPs, ~100 nm in diameter, showed intense fluorescence even at low particle concentrations. To test whether the entrapped FITC can withstand the UV irradiation condition used for

the photocoupling reaction, a solution of FSNPs was irradiated with a 450-W medium-pressure Hg lamp for 10 min. The resulting solution remained highly fluorescent, and the fluorescence intensity decreased only to a small extent. On the other hand, when a solution of FITC was irradiated under the same condition, the fluorescence intensity was reduced to about 50% of the original value (Figure 5.11 and 5.12 in Supporting Information) These results are consistent with the observations by others that the photostability is significantly improved when fluorescent dyes are embedded in silica nanoparticles.^{219,225}

Next, FSNPs were functionalized with PFPA by treating the as-prepared FSNPs with PFPA-silane (Figure 5.5a).¹²⁵ To covalently label carbohydrates with FSNPs, our previously established procedure for coupling carbohydrates on gold nanoparticles was followed.^{155,181} In the process, a solution containing the carbohydrate and FSNPs was irradiated with a medium-pressure Hg lamp for 10 min (Figure 5.5b). Excess reagents were removed by dialysis, and the resulting glyco FSNPs showed excellent water solubility and high fluorescence emission intensity. The density of the immobilized carbohydrate was determined using a previously developed colorimetric assay,¹⁸¹ from which the coupling yields were calculated. The results showed that the photocoupling reaction was highly efficient. The coupling yield, ranging from 36% to 54% (Table 5.2 in Supporting Information), increased with the size of the carbohydrate, a result that is consistent with our previous study using gold nanoparticles.¹⁸¹

The labeling process is well adaptable for high-throughput where the photocoupling step can be performed in parallel. A pilot study was carried out where four micro-vials containing PFPA-FSNPs and four different carbohydrates were photoactivated simultaneously. The products showed successful conjugation of each carbohydrate on the FSNPs. The number of reaction wells can be further increased by using the microarray technology to enable a rapid parallel synthesis of larger libraries of FSNP-labeled ligands.

Figure 5.6 Fluorescence spectra of (a) FSNP-Man and (b) FSNP-Gal before (black lines) and after (red lines) incubating with Con A; (c) DLS of Man-FSNPs before (red) and after (green) binding with Con A; (d) TEM image of Man-FSNPs after treating with Con A.

The bioactivity of FSNP-labeled carbohydrates was tested in the following studies. D-Mannose labeled with FSNP, FSNP-Man, was treated with *Concanavalin A* (Con A), a well-studied lectin which exhibits specific affinity to α -D-mannopyranoside, α -D-glucopyranoside, and their derivatives.^{114,183} At pH>7, Con A exists as a tetramer, inducing significant nanoparticle agglomeration upon binding to Man-containing ligands.¹⁸¹ When FSNP-Man was incubated with Con A for 1 hour, the fluorescence intensity of the solution decreased drastically (Figure 5.6a). Precipitates were observed where the nanoparticles agglomerated into clusters (Figure 5.6d). In contrast, when FSNP-labeled D-galactose, FSNP-Gal, was treated with Con A following the same procedure, the fluorescence intensity decreased only slightly (Figure 5.6b). The small intensity decrease is likely due to the nonspecific adsorption of Con A on the nanoparticles. The FSNP-Man-Con A aggregates were additionally examined by dynamic light scattering (DLS), which showed, on the average, a 10-time increase in the particle size in comparison to FSNP-Man (Figure 5.6c).

Figure 5.7 TEM images after FSNP-Man was treated with *E. coli* strain ORN178 (a), or ORN208 (b). Insert: fluorescence image of the corresponding sample. Scale bars: 500 nm.

The utility of the FSNP labeling technique was next investigated by applying glyco FSNPs in imaging, and for studying glycan-lectin interactions on a lectin microarray. In the first experiment, FSNP-Man was treated with *E. coli* bacteria strain ORN 178. This particular strain contains a Man-specific binding domain, i.e., the FimH lectin, on type 1 pili.^{226,227} The TEM image of the resulting solution displays a large number of FSNP-Man on the *E. coli* (Figure 5.7a), which can be attributed to the multivalent interactions between Man ligands on the FSNP-Man with the FimH lectin on the bacteria.¹⁴⁰ The strong interaction was further confirmed by fluorescence imaging where intense fluorescence was observed on the bacteria (Figure 5.7a insert). In contrast, when FSNP-Man was treated with *E. coli* strain ORN208 that is deficient of the Man-binding FimH protein, no fluorescence was observed on the bacteria. In fact, almost no nanoparticles were attached to the bacteria surface (Figure 5.7b).

Figure 5.8 (a) Preparation of lectin microarray, incubation with FSNP-Man, and fluorescence imaging. (b) Fluorescence image and (c) fluorescence intensities of lectin microarray after treating with FSNP-Man. Each data point was the average of the 7 spots on the microarray.

The FSNP-labeled carbohydrates were next employed to study carbohydrate-lectin interactions on a lectin microarray. The lectin microarray was fabricated on aldehyde-functionalized glass slides following the established literature procedure.²²⁴ Solutions of Con A and soybean agglutinin (SBA, a non-Man-binding lectin) in pH 7.4 phosphate-buffered saline (PBS) containing 40% glycerol were then printed on the aldehyde slides using a robotic printer.²²⁸ The lectin array slide, after blocking with bovine serum albumin (BSA), was incubated in the FSNP-Man solution for 2 hours (Figure 5.8a). As anticipated, fluorescence was

observed on all Con A spots (Figure 5.8b). The relative fluorescence intensity on Con A were ~10 times higher than those on SBA at the printing concentration of 1 mg/mL (Figure 5.8c). Even at 0.1 mg/mL, the fluorescence intensity from Con A was still noticeably higher than that from SBA, although the spot quality deteriorated at lower printing concentrations. These results clearly demonstrate that the FSNP-labeled carbohydrates are highly suited for interrogating carbohydrate-lectin interactions on microarray. Due to the irreversible agglomeration upon the interactions, the binded nanoparticles were found difficult to be displaced by other ligands. However, in order to extensively evaluate the specificity and apparent affinity of glyco FSNPs to lectin microarray, we currently developed an alternative competition-based assay, with unlabelled ligands added with FSNPs during the incubation. The preliminary results showed the fluorescence intensity was highly affected by concentration of free ligand competitors, and calculated affinities of FSNPs were several orders of magnitudes higher than that of monovalent binding in solution.

Conclusion

In conclusion, a simple and general method was developed to label carbohydrates with dye-doped silica nanoparticles. The strategy applies to underivatized carbohydrate structures, thus avoiding complex synthesis and purification steps that are often involved in the chemical derivatization of these ligands. The labeling is highly efficient, and the resulting FSNP-labeled carbohydrates retained their binding affinity

and selectivity towards lectins. The utility of this labeling technique was successfully demonstrated where FSNP-labeled carbohydrates were applied in bacteria detection and imaging, and in probing glycan-lectin interactions on microarray. These results illustrate that, although the labeling chemistry, i.e., the CH insertion reaction of PFPA, yields a random orientation of the attached ligands, the labeled ligands retain their binding selectivities nonetheless. Further, the labeling reaction may allow the exposure of all epitopes on the ligands, and thus a biased epitope selection can be avoided. The technique developed can in principle be readily applied to other biologically significant molecules including pharmaceuticals and metabolites. The advantage of this method resides in its generality and simplicity, where the labeling process employs a single labeling agent, PFPA-FSNP, and a uniform coupling chemistry. Ligands are labeled in their native forms without undergoing prior chemical derivatization. These features, combined with the straightforward preparation of dye-doped silica nanoparticles and the low material cost, may open up a myriad of opportunities for this technique in bioanalysis and diagnostic applications.

Supporting Information

TEM and DLS characterization of FITC-doped SNPs

Figure 5.9 TEM image of FITC-doped SNPs. (Scale bar: 0.2 μm)

Figure 5.10 Size distribution of FITC-doped SNPs measured by DLS.

Photo-stability of FITC-doped silica nanoparticles and FITC

Figure 5.11 Fluorescence spectra of FITC-doped silica nanoparticles before and after UV irradiation for 10 min.

Figure 5.12 Fluorescence spectra of FITC before and after UV irradiation for 10 min.

Determination of immobilized carbohydrate density and coupling yield

Surface area of each FSNP: $S = \pi \cdot d^2 = 36625 \text{ nm}^2$

Volume of each FSNP: $V = \pi \cdot d^3 / 6 = 6.59 \times 10^5 \text{ nm}^3 = 6.59 \times 10^{-16} \text{ cm}^3$

Density of FSNP = 2.3 g/cm^3

Concentration of FSNP = 20.61 mg/mL

So in 1 mL, No. of FSNP = $(0.02061 / 2.3) / (6.59 \times 10^{-16}) = 1.36 \times 10^{13}$

By assuming the projection area of carbohydrate molecules, such as D-mannose 0.24 nm^2 , the number of maximal ligands occupied on each FSNP is:

$$N_{\text{Max}} = 36625 / 0.24 = 1.53 \times 10^5$$

The experimental number of ligands (N_{exp}) was determined by anthrone- H_2SO_4 colorimetric measurements (using FSNP as background). The coupling yield was calculated as $N_{\text{exp}} / N_{\text{Max}} \times 100\%$

Table 5.2 Ligand density and coupling yield of glyco FSNPs

Carbohydrate ligand	No. of ligand per FSNP ($\times 10^4$, experimental)	No. of ligand per FSNP ($\times 10^4$, calculated)	Coupling yield (%)
D-Mannose (Man)	5.52	15.3	36
α -1,2-Mannobiose (Man2)	2.72	5.56	49
α -1,3-1,6-Mannotriose (Man3)	2.48	4.76	52
D -Glucose (Glc)	5.64	15.3	37
Maltopentaose (Glc5)	1.72	3.16	54

5.3 High-throughput Glycan Screening on Lectin Microarray

Microarrays have become increasingly used in biological research for large-scale ligand profiling, owing to its features of high-throughput signal output and fast data analysis. Frequently, glycan microarrays are fabricated which are subsequently employed to study glycan-lectin interactions using fluorescently-labeled lectins.^{51,80} An alternative configuration is to construct lectin microarrays which can then be probed with labeled glycans. Protein microarrays are more developed than glycan microarrays, and they can be fabricated using established procedures^{224,229} or acquired from commercial sources.²³⁰ Glycan labeling, however, can be challenging. Unlike proteins that can be readily labeled using commercially available kits, labeled glycans, on the other hand, are not easy to obtain. One strategy is to label glycoproteins. In the study done by Hirabayashi and coworkers, an glycan profiling technique was described based evanescent-field fluorescence detection strategy. The interactions between lectin microarrays fabricated on epoxy-derivatized slides and fluorescent Cy3-tagged glycoproteins, glycopeptides and tetramethylrhodamine (TMR)-labeled oligosaccharides were monitored, and the dissociation constants were also determined by competition assays.²¹³

We have demonstrated that dye-doped silica nanoparticles provided an attractive platform to label glycans for the recognition of carbohydrate-binding proteins, lectins. Because multiple dye molecules are trapped inside the NPs, the resulting fluorescent nanoparticles are brighter than single dye molecule, and the fluorescent intensity is

more stable towards photobleaching and environmental degradation.^{215,228} In the previous chapter, we demonstrate that the FSNP-labeled glycans are effective in probing lectin microarrays. In this chapter, a novel “super-microarray” design will be adapted where FSNP-labeled glycans will be used to study glycan-lectin interactions in high throughput. The super-microarray differs from the conventional microarray that it consists of multiple microarrays (Figure 5.13). By applying a poly(dimethyl siloxane) (PDMS) isolator, each microarray can be isolated and therefore different assays can be conducted in each individual microarray (Figure 5.13).

Figure 5.13 Schematics of lectin super-microarray fabrication, and the subsequent assays with glyco-FSNPs.

The super-microarrays can be used for high throughput screening of ligands (Figure 5.14a) and for high throughput competition assays to determine binding affinity of glycan-lectin interactions (Figure 5.14b). Both applications will be demonstrated in this Chapter where FSNP-labeled glycans will be used on lectin supermicroarrays. Competition assays will be carried out simultaneously on a single super-microarray, from which IC_{50} values will be derived and the binding affinity calculated. This provides a high throughput method to determine binding affinity of glycan-lectin interactions.

Figure 5.14 Schematics of (a) high-throughput screening, and (b) competition assay on super microarrays.

Experimental details

Preparation of FITC-doped glyconanoparticles.

See section 5.2¹⁶⁶

Fabrication of lectin super-microarrays.

The lectin super-microarray was prepared on an epoxy-coated glass slide. Glass slides were firstly cleaned in Piranha solution (conc. $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$, 1:1, v:v), followed by soaking in a toluene solution of 3-glycidyloxypropyltrimethoxysilane (12.6 mM, >95%, TCI America; Portland, OR) for 4 hours, rinsed thoroughly with toluene and dried with nitrogen. Con A (*Concanavalin A*), BSA (*Bovine serum albumin*), SBA (*Soybean Agglutinin*), PNA (*Peanut Agglutinin*), BS-I (*Bandeiraea simplicifolia*) (Sigma-Aldrich; St. Louis, MO), DBA (*Dolichos Biflorus Agglutinin*), UEA (*Ulex Europaeus Agglutinin I*), WGA (*Wheat Germ Agglutinin*) and RCA (*Ricinus Communis Agglutinin I*) (Vector Laboratories; Burlingame, CA) were used as received. The concentration of Con A was determined spectrophotometrically at 280 nm using $A^{1\%, 1\text{cm}} = 13.7$ at pH 7.2.¹³⁶ CV-N (Cyanovirin-N) and OAA (*Oscillatoria Agardhii Agglutinin*) wild-type lectins and CN-V mutants CVN^{Q50C} and $\text{CVN}^{\text{MutDB}}$ were supplied by Professor Angela M. Gronenborn's research laboratory at University of Pittsburgh. Lectin solutions were prepared in phosphate-buffered saline at concentrations of 1 mg/mL (except PNA and RCA at 0.25 mg/mL) with 40% glycerol added to prevent complete drying of the liquid droplets after printing. A volume of 0.3mL lectin solution was added to the microtiter plate well and the solutions were spotted onto the epoxy-coated glass slides using a robotic printer (BioOdyssey Calligrapher Mini-arrayer; Bio-Rad Laboratories, Inc.) and capillary pin of $\sim 360\mu\text{m}$ size. Each spot was printed 5 times. Each lectin was printed with 5 duplicate spots. The glass slides were then incubated in a humid chamber (80% humidity) at 25 °C for

3 h to facilitate the immobilization of the lectins. A SecureSeal™ hybridization chambers sheet (Grace Bio-lab, Bend, OR) was carefully placed on the glass slide to create 16 individual wells.²¹³ A blocking solution of BSA in pH 7.4 PBS buffer (1%) was then added to each well and the slide was incubated at room temperature for 1 h, rinsed with the PBS buffer and was dried with N₂.

Treating supermicroarray with glyco-FSNP.

The lectin supermicroarrays were incubated in the solution of glyco FSNPs in HEPES (1.5 mg/mL) for 2 h; for the competition assays, 1nM to 1mM free ligand Man2 was added in each well with FSNP-Man2 (1.5mg/mL); and were then gently rinsed with the HEPES buffer containing 0.1% Tween 20 for 3 times and dried with N₂. The slides were scanned under a microarray scanner (GenePix 4100A, Molecular Devices, Inc) at excitation of 532 nm. The fluorescent images were recorded and the data were analyzed using the supplied software (Axon GenePix Pro 5.1).

Results and discussion

Optimization of super-microarrays and assay conditions

Lectin microarrays were prepared following a literature procedure by immobilizing lectins on epoxy-functionalized surfaces.²²⁴ Piranha-cleaned glass slides were treated with an epoxy-silane, GPTMS (Figure 5.13). Lectin solutions in PBS containing glycerol were then printed onto GPTMS-modified glass slides using a microarray printer. Multiple lectin microarrays were printed on the same glass slide where each

microarray was separated by a 16-well PDMS isolator to give a super-microarray with 16 individual microarray on a single slide (Figure 5.13).

The lectin super-microarrays were tested for their ability for screening carbohydrate ligands. The carbohydrates were labeled with FITC-doped silica nanoparticles. We have demonstrated previously that dye-doped silica nanoparticles can efficiently label glycans.¹⁶⁶ Because multiple dye molecules are trapped inside silica nanoparticles, the resulting FSNPs are brighter and the fluorescence is more stable than the free dye molecules as they are protected by the nanoparticles.²¹⁵ FSNPs of 110 nm diameter were synthesized,¹⁶⁶ and carbohydrate ligands were conjugated onto the fluorescent nanoparticles using the previously developed PFPA photocoupling chemistry to yield FSNP-labeled carbohydrates, glyco-FSNPs (Figure 5.13).^{125,166,181}

The binding experiments were conducted by incubating buffer solutions containing glyco-FSNPs in each well on the lectin super-microarray. After rinsing with the fresh buffer and drying, the slide was subjected to a microarray scanner and the fluorescence image was recorded. The lectin printing concentration, the concentration and incubation time of glyco-FSNPs were varied to study the impact of these experimental conditions on the binding results. Figure 5.15a shows that when Man2-FSNPs were incubated with Con A, the fluorescence intensity increased linearly with the Con A printing concentration from 0.1 to 2.5 mg/mL. Similarly, the fluorescence intensity increased with the concentrations of Man2-FSNPs from 0.05 to 1.0 mg/mL

(Figure 5.15b). The incubation time was then varied while keeping concentrations of Con A and Man2-FSNPs at 1 mg/mL, and 1.5 mg/mL, respectively. As shown in Figure 5.15c, the intensity increased until 75 min and reached saturation after 2 hours. The incubated lectin spots were characterized by AFM. Images show that the spot was fairly uniformly covered with FSNPs (Figure 5.16). More than single layer of FSNPs was observed in some areas, most likely due to nanoparticle agglomeration before/during incubation. In a control experiment where a non-binding lectin, SBA, was treated with Man2-FSNPs, the signals remained low until after 2 hours, and increased afterwards because of the non-specific adsorption (Figure 5.15c). To minimize particle agglomeration and non-specific adsorption, the glyco-FSNP concentration and the incubation time were kept at 1.5 mg/mL and 2 hours, respectively, for subsequent studies.

Figure 5.15 Interactions of Man2-FSNPs with super-microarray: **(a)** fluorescence intensity vs. the printing concentration of Con A ($\square$); **(b)** fluorescence intensity vs. the concentration of Man2 -FSNPs ($\circ$); **(c)** fluorescence intensity vs. Man2-FSNPs (1.5 mg/mL) incubation time (inserts: fluorescence images of Con A spots (top panel) and SBA spots (bottom panel)). The concentration of Man2-FSNPs in (a) was 1.5 mg/mL, and the printing concentration of Con A was 1 mg/mL in (b). Each data point was the average of five duplicate spots on the array. The lines were drawn to aid visualization

Figure 5.16 Fluorescence (a) and AFM images (b-d) of a printed Con A spot (1 mg/mL) after incubation with Man2-FSNP (1.5 mg/mL) for 2 h.

Super-microarrays for high-throughput ligand screening

In this study, we evaluate the application of lectin super-microarrays in screening carbohydrate ligands using glyco-FSNPs. A total of 14 lectins were used; their binding carbohydrate ligands are listed in Table 5.3. The lectin microarrays were fabricated following the same protocols and parameters developed in the section described above. Due to the size limitation of the PDMD isolator and printing spot size, only 7 lectins could fit in one isolator well, and therefore, the 14 lectins were divided into two groups. The lectins were printed on GPTMS-functionalized glass slides in 7 x 5 array, and the array was repeated in 2 x 8 array format to create 16 microarrays on each chip (Figure 5.13). Figure 5.17 lists the carbohydrate ligands used in this study. Each ligand was coupled on FSNPs following the protocol described above, and the surface density was similar to what was reported in our previous study.¹⁶⁶ In the binding experiments, a 2 x 8 PDMS isolator was placed on the lectin super-microarray slide to create 16 wells, and different glyco-FSNPs were incubated in each individual well containing the 7 x 5 lectin microarray.

Table 5.3 Lectins and their corresponding carbohydrate binding ligands.

Lectin		Origin	Binding ligands
CVN-Q	Cyanovirin-N Q ^{50C}	<i>Cyanobacteria</i>	α -1,2-Man
CVN-M	Cyanovirin-N Mut ^{DB}	<i>Cyanobacteria</i>	α -1,2-Man
SBA	Soybean Agglutinin	<i>Glycine max</i> (soybean)	GalNAc
BSA	Bovine serum albumin	<i>Bovine serum</i>	N/A
Con A	Concanavalin A	<i>Canavalia ensiformis</i>	Man
PNA	Peanut Agglutinin	<i>Arachis hypogaea</i> (peanut)	Gal(β -1,3) GalNAc /Gal
BS-I	Bandeiraea simplicifolia	<i>Griffonia</i> (<i>Bandeiraea</i>) <i>simplicifolia</i> seeds	GalNAc, and Gal
OAA	Oscillatoria Agardhii Agglutinin	<i>Cyanobacteria</i>	α -1,6-Man
PFA	Homolog of OAA	N/A	α -1,6-Man
W77	Mutant of OAA	<i>Cyanobacteria</i>	α -1,6-Man
DBA	Dolichos Biflorus Agglutinin	<i>Dolichos biflorus</i> (horse gram) seeds	GalNAc
UEA	Ulex Europaeus Agglutinin I	<i>Ulex europaeus</i> (Furze gorse) seed	L-fucose
WGA	Wheat Germ Agglutinin	<i>Triticum vulgaris</i> (wheat germ)	GlcNAc
RCA	Ricinus Communis Agglutinin I	<i>Ricinus communis</i> (castor bean) seeds	Gal/ GalNAc/ Lac

Figure 5.17 Carbohydrate structures used in this study.

Results of the microarray analysis are shown in Figure 5.18. The binding pairs such as 2 α -Man2/2 α , 2 α -Man3 with CVNs/Con A, Man with Con A, Gal with PNA/BS-I, as well as 6 α -Man2/3 α , 6 α -Man3 with OAA/PFA, Fuc with UEA and Glc with WGA

show noticeably higher intensities than those non-binding pairs. The results were consistent with the affinity of these glycan-lectin pairs (see association constants shown in Table 5.4), demonstrating the high selectivity of the super-microarrays. The signal intensities also revealed the relative binding affinities of glycan ligands with lectins. For instance, Con A that is a tetrameric protein having saccharides binding sites to α -Man and α -Glc in the affinity order of 2α , 2α -Man₃ ($3.79 \times 10^5 \text{ M}^{-1}$) > 2α -Man₂ ($4.17 \times 10^4 \text{ M}^{-1}$) > Man ($8.2 \times 10^3 \text{ M}^{-1}$) > Glc ($1.96 \times 10^3 \text{ M}^{-1}$) (Table 5.4). In this study, Con A spots showed the highest intensity for 2α , 2α -Man₃ and the lowest for Glc and derivatives (GlcNAc and Lac), and minimal signal was detected for the non-binding ligand Gal (Figure 5.18). Another group of lectins studied were CV-N mutants Q^{50C} and Mut^{DB}. These are anti-HIV lectins that recognize α -1,2 linked Man structures such as 2α -Man₂ and 2α , 2α -Man₃ (Table 5.4). The natural ligand is Man₉, an oligosaccharide that is difficult and expensive to obtain.¹⁸⁴ We have shown in Chapter 5.1 that by conjugating the epitope structure of lower Man structures such as 2α -Man₂ and 2α , 2α -Man₃ to gold nanoparticles, the affinity of the resulting glyconanoparticles were increased several orders of magnitude.¹⁸⁹ These epitope ligands labeled with SFNPs were therefore used in the present studies to probe lectin microarrays. Results in Figure 5.18 shows that 2α , 2α -Man₃ conjugated on FSNP exhibited higher fluorescent density than Man₂-FSNP, which correlated well with the affinity ranking of the free ligands (Table 5.4) as well as the ITC¹⁸⁸ and gold GNP¹⁸⁹ studies. Similar results were also observed for other lectin-glycan binding pairs that the array results were consistent with the affinity rank order of the corresponding free ligands.

Table 5.4 Association constants (K_a) of lectins and glycans.

Lectin	Glycan	$K_a (M^{-1})$
CVN-Q ¹⁸⁹	2 α -Man2	1.43x10 ³ (Domain A); 1.56x10 ⁴ (Domain B)
	2 α , 2 α -Man3	2.94x10 ⁵ (Domain A); 2.33x10 ⁴ (Domain B)
CVN-M ¹⁸⁸	2 α -Man2	1.32x10 ³
	2 α , 2 α -Man3	2.94x10 ⁵
Con A ¹³⁶	Man	8.2x10 ³
	2 α -Man2	4.17x10 ⁴
	2 α , 2 α -Man3	3.79x10 ⁵
	Glc	1.96x10 ³
SBA ²³¹	GalNAc	1.51x10 ⁶
PNA ²³²	Gal	0.98x10 ³
	GalNAc	2.43x10 ³
BS-I ¹⁴⁷	Gal	2.1x10 ⁴
	GalNAc	1.87 x10 ⁵
DBA ²³³	GalNAc	4.2x10 ³
WGA ²³⁴	GlcNAc	0.4x10 ³
RCA ¹⁷⁶	Gal	2.2x10 ³
	GalNAc	4.84x10 ⁴

Figure 5.18 Fluorescence image (a, c) and fluorescence intensity (b, d) of lectin super-microarrays interacting with glyco-FSNPs.

Super-microarrays for high throughput binding affinity determination

The 2 x 8 PDMS isolator creates 16 individual microarrays, and therefore, 16 different assays can be carried out simultaneously on a single super-microarray. When 16 ligand competition assays are performed, a dose-response curve can be plotted, from which the IC₅₀ value can be derived and the dissociation constant subsequently calculated. This provides a high throughput means to determine binding affinity from a single super-microarray.

Competition binding assays were performed by mixing free competing ligands of varying concentrations with a fixed concentration of glyco-FSNPs and the resulting solutions were transferred to the wells on the super-microarray. Figure 5.19 showed a typical fluorescence image of the lectin super-microarray after incubating with Man2-FSNP and varying concentration of 2 α -Man2. It can be seen that only the binding lectins for 2 α -Man2 (CVN-Q, CVN-M and Con A) exhibited strong and different signals with varying concentrations of 2 α -Man2. Further, the fluorescence intensity decreased with increasing 2 α -Man2 concentration, which was anticipated since 2 α -Man2 competed with Man2-FSNP for binding the lectins on the array. By plotting the fluorescence intensity against the concentration of 2 α -Man2, a dose response curve was obtained, from which the IC₅₀ value can be derived. Figure 5.19b-d show the results from the three binding lectins CVN-Q, CVN-M and Con A. Following a revised Cheng-Prusoff equation

$$K_D = IC_{50} / (1 + [M] / K_d)$$

where K_d is the dissociation constant of the free ligand with the corresponding lectin, K_D , the apparent dissociation constant of immobilized lectin with glyco-FSNP was calculated,^{154,206} and results are shown in Table 5.5.

Figure 5.19 (a) Fluorescence image of a lectin super-microarray after incubating with Man2-FSNP and varying concentrations of 2α -Man2. (b-d) Fluorescence intensities vs. free 2α -Man2 concentration for CVN-Q CVN-M (c) and Con A (d). Insert in (b) shows the fluorescence images of the corresponding spots.

Table 5.5 Apparent K_D values of glyco-FSNPs with lectins obtained from super-microarrays.

	Ligand density (ligand/nm²)¹	CVN-Q	CVN-M	Con A	PNA
FSNP- Man3	1.3	1.66 nM (15.5 μ M)	0.43 nM (3.4 μ M)	0.0083 nM (2.97 μ M)	N/D ³
FSNP- Man2	1.5	16.6 nM (89.8 μ M)	589 nM (757 μ M)	5.40 nM (24 μ M)	N/D
FSNP- Man	4.2	N/D	N/D	13.7 nM (470 μ M)	N/D
FSNP- Gal	4.3	N/D	N/D	N/D	114 nM (1050 μ M)

¹Ligand density was measured by anthrone-H₂SO₄ colorimetric method.

²The data in brackets are dissociation constants of the free ligand with the lectin.

³N/D= not detectable.

A dramatic increase in affinity was observed when glycans attached on nanoparticle surface. Results showed that the multivalent presentation of carbohydrate ligands significantly enhanced the binding affinity of FSNPs by 4~6 orders of magnitude in comparison to the free ligands in solution. In addition, the apparent affinity of FSNP-Gal with its binding lectin PNA was determined as well, and similarly K_D was 7 orders of magnitude lower than K_d of free galactose ligand. The apparent K_D values (Table 5.5) were comparable to those obtained using other microarray platforms, such as surface plasmon resonance^{235,236} and evanescent-field fluorescence.²¹³

Conclusion

A new platform has been developed to study glycan-lectin interactions. It is based on lectin microarrays which are probed with glycans labeled with dye-doped nanoparticles. This labeling technique has several key advantages. First, the coupling chemistry is highly general and can be applied to a wide range of glycans including higher glycan structures, underivatized carbohydrates, as well as reducing sugars.²³⁷ Secondly, compared with fluorescent dyes, dye-doped nanoparticles provide higher fluorescence intensity and much improved photo-stability. The labeled glycans can be readily purified by dialysis thus avoiding the potential complex solution-phase purification procedure when fluorescent dyes are used. Thirdly, multiple copies of the glycan can be conjugated on one nanoparticle introducing multivalency effect that significantly enhances their interactions with lectins.

The lectin super-microarrays further increase the throughput of microarrays by creating multiple microarrays on the same chip. Thus different assays can be performed simultaneously permitting large-scale glycan profiling. Furthermore, ligand competition assays can be conducted on a single super-microarray to afford the dose response curve, from which the IC_{50} and apparent K_D values can be readily obtained. This represents a new analytical method for affinity measurement. Compared with the reported methods of nuclear magnetic resonance (NMR),²³⁸ enzyme-linked immunosorbent assay (ELISA),²³⁹ isothermal titration calorimetry (ITC),²⁴⁰ precipitation inhibition,²⁴¹ equilibrium dialysis,²⁴² spectrophotometry,²⁴³ fluorescence

competition assay,¹⁵⁵ surface plasmon resonance (SPR),^{236,244} carbohydrate microarrays,²⁰⁴ and quartz-crystal microbalance (QCM),^{245,246} our method allows for high-throughput data outputs and much reduced glycan consumption (only ~3 µg of glycans consumed for each assay). The high throughput nature of lectin super-microarrays in conjunction with the versatility of nanoparticle-based glycan labeling technique provides a robust and unique approach ideally suited for quantitative glycan profiling in a large library. The protocol could also be applied to other biological entities such as DNA, antibodies, and cells. The technology presented herein thus opens new opportunities facilitating the development of glycomics as well as application in disease diagnosis and therapeutics.

Chapter 6. Conclusion

Merging nanotechnology with biology has seen exponential growth of research activities in functional bio-nanomaterials. Nanomaterials in general, and glyconanoparticles in particular, are witnessing a tremendous interest for a wide range of applications in chemistry, biology and medicine, and their merits are well known in comparison to other formats. Essential to glyconanoparticle preparation is the conjugation chemistry that can efficiently attach carbohydrates to the nanomaterials. We developed a simple and versatile photocoupling chemistry that allows the covalent immobilization of a wide range of carbohydrate structures to nanomaterials, including gold and fluorescent silica nanoparticles. The coupling reaction was efficient and high yielding, and the resulting surface-bound carbohydrate ligands retained their binding affinities and selectivities. The development of new analytical methods to characterize glyconanoparticle-protein interactions, including fluorescence competition assays, dynamic light scattering and isothermal calorimetry, highlight the importance of quantitative analysis of structural and functional properties of glyconanomaterials, especially the biorecognition properties that must be carefully analyzed in the context of ligand presentation and display. Results from my studies have shown that glyconanoparticles are highly potent - potentially superior - vehicles for targeting carbohydrate-mediated biological effects due to the multivalency effects commonly associated with carbohydrate-protein interactions. Nanoparticles as carriers can greatly enhance the binding affinity of the carbohydrate ligands, and in addition, the affinity is

significantly impacted by the carbohydrate presentation on nanoparticle surfaces, which could be applied for enhanced recognition of biologically significant lectins, such as anti-HIV CV-N in our study. The new glycan label, dye-doped silica nanoparticles, greatly facilitated the high-throughput labeling using nanomaterials as the scaffold, and FSNP-labeled glycans have shown potential applications in protein recognition, bacteria imaging, and high-throughput glycan screening on lectin microarrays. With the wealth of existing results on glyconanomaterials and their demonstrated multivalent interactions with proteins, advanced developments in therapeutic and diagnostic applications based on biologically-functionalized nanomaterials are poised to evolve.

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Appendices

Appendix A. List of Publications

- **Wang, X.;** Ramström, O.; Yan, M., Thermodynamic analysis of interactions between glyco-nanoparticles and binding lectins *via* isothermal titration calorimetry. *submitted for publication*.
- **Wang, X.;** Matei, E.; Deng, L. Q.; Ramström, O.; Gronenborn, A. M.; Yan, M., Interaction of fluorescent silica glyconanoparticles with lectin “super-microarray”: a high-throughput glycan screening strategy. *submitted for publication*.
- **Wang, X.;** Ramström, O.; Yan, M., Dynamic Light Scattering as Efficient Tool to Study Glyconanoparticle-Lectin Interactions. *Analyst* **2011**, *136*, 4174-4178.
- **Wang, X.;** Matei, E.; Deng, L. Q.; Ramström, O.; Gronenborn, A. M.; Yan, M., Multivalent Glyconanoparticles with Enhanced Affinity to Anti-Viral Lectin Cyanovirin-N *Chem. Commun.* **2011**, *47*, 8620-8622.
- **Wang, X.;** Ramström, O.; Yan, M., Dye-doped silica nanoparticles as efficient labels for glycans. *Chem. Commun.* **2011**, *47* (14), 4261-4263.
- **Wang, X.;** Ramström, O.; Yan, M., Quantitative analysis of Multivalent Ligands Presentation on Gold Glyconanoparticles and Their Effects on Protein Binding. *Anal. Chem.* **2010**, *82* (21), 9082-9089.
- **Wang, X.;** Ramström, O.; Yan, M., Glyconanomaterials: Synthesis, Characterization, and Ligand Presentation. *Adv. Mater.* **2010**, *22*, 1946-1953.
- **Wang, X.;** Ramström, O.; Yan, M., A Photochemically Initiated Chemistry for Coupling Underivatized Carbohydrates to Gold Nanoparticles. *J. Mater. Chem.* **2009**, *19* (47), 8944-8949.

- **Wang, X.;** Liu, L. H.; Ramström, O.; Yan, M. D., Engineering Nanomaterial Surfaces for Biomedical Applications. *Exp. Biol. Med.* **2009**, 234 (10), 1128-1139.

Papers not included in this dissertation:

- Deng, L. Q.; **Wang, X.;** Norberg, O.; Dong, H.; Joliton, A.; Yan, M.; Ramstrom, O., Stereocontrolled 1-S-Glycosylation and Comparative Binding Studies of Photoprobe-Thiosaccharide Conjugates with Their O-Linked Analogs. *submitted for publication.*
- Tong, Q.; **Wang, X.;** Wang, H.; Yan, M., Glyco-nanoparticle microarray for detection of carbohydrate-protein interactions. *submitted for publication.*
- Wang, X. H.; **Wang, X.;** Fernández, R.; Yan, M.; La Rosa, A., Nanolithography on Responsive Surfaces. *J. Nanoscience Lett.* **2011**, *in press.*
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- Wang, X. H.; **Wang, X.;** Fernández, R.; Ocola, L.; Yan, M.; La Rosa, A., Electric Field-Assisted Dip-pen Nanolithography on poly(4-vinylpyridine) (P4VP) Thin Films. *ACS Appl. Mater. Interfaces* **2010**, 2 (10), 2904-2909.
- Tyagi, A.; **Wang, X.;** Deng, L. Q.; Ramström, O.; Yan, M., Photogenerated Carbohydrate Microarrays to Study Carbohydrate-Protein Interactions using Surface Plasmon Resonance Imaging. *Biosens. Bioelectron.* **2010**, 26, 344-350.
- Surangi, J.; **Wang, X.;** Yan, M., Linear Discriminant Analysis and Statistical Pattern Lectin Recognition Using Gold Glyconanoparticle. *in preparation.*
- Zorn, G.; Tyagi, A.; **Wang, X.;** Wang, H.; Castner, D. G.; Yan, M., Chemical Composition and Presentation of Perfluorophenylazide Surfaces: Analysis and Impact on Ligand Immobilization. *in preparation.*

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