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Electrochemical Characterization of Globotriose-Containing Self-Assembled Monolayers on Nanoporous Gold and their Binding of Soybean Agglutinin

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Abstract

Self-assembled monolayers (SAMs) of α -D-Gal-(1 $\rightarrow$ 4)- β -D-Gal-(1 $\rightarrow$ 4)- β -D-Glc-mercaptopentane (globotriose, Gb3-C₅-SH) were prepared both as single-component SAMs and as mixed SAMs with either octanethiol (OCT) or 8-mercaptopentane-3,6-dioxapentanol (HO-PEG₂-SH), on flat gold and on nanoporous gold (NPG) electrodes. The binding of soybean agglutinin (SBA) to the globotriose (Gb3) unit in the SAMs was then studied using electrochemical impedance spectroscopy (EIS), which is a label free method found to be quite sensitive to SAM composition and to the differences in SAM structure on NPG versus on flat Au. The affinity of SBA to the mixed SAM of HO-PEG₂-SH and Gb3-C₅-SH on NPG is found to be greater on NPG than on flat gold, and indicates a potential advantage for NPG as a substrate. The SAMs of HO-PEG₂-SH were found to resist protein adsorption on either NPG or flat gold. The non-specific adsorption of SBA to OCT SAMs on flat Au was observed in EIS by the increase in charge transfer resistance; whereas, the increase seen on the NPG surface was smaller, and suggests that EIS measurements on NPG are less affected by non-specific protein adsorption. Atomic force microscopy (AFM) images of the SBA binding to mixed SAM of HO-PEG₂-SH and Gb3-C₅-SH on NPG showed a greater number of proteins on top of the OCT containing SAMs.

There is an increasing interest in mimicking the cell surface by presenting carbohydrates in self-assembled monolayers (SAMs) for use in protein recognition studies. Such studies first involved modification of flat gold surfaces ^{1–7} and have now increasingly focused on the use of gold nanostructures ^{8–14}. Many of the reports concerning carbohydrate-lectin interactions, for example, have utilized mannose presenting SAMs and Concanavalin A ^{3, 15–18}. More complex carbohydrates have also been incorporated into SAMs, but less frequently ^{1, 13, 18–21}. An important early study found that the binding selectivity of lectins to a carbohydrate in mixed SAMs could change as a function of the fraction of carbohydrate in the SAM, and that lectin affinities for surface bound carbohydrates could differ from those in solution ²². The self-assembly of larger carbohydrates at interfaces poses more

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complex structural and steric issues than that of simpler monosaccharide bearing molecules, in addition to posing a greater synthetic challenge.

Globotriaosylceramide (Gb3) is a P^k blood group antigen, also known as CD77 antigen in B cells, in which the terminal glycan unit consisting of unique Gal α (1–4)Gal moieties²³. It is expressed in high density in kidney and brain endothelial cells and in human erythrocytes²⁴. It is an important recognition element in the attachment of Shiga toxins (stx1 or stx2) and of Shiga-like toxins (verotoxins) to the host cells²⁵. These important cytotoxins cause diarrheal disease and hemolytic uremic syndrome. Shiga toxin is produced by the pathogenic bacteria *Shigella dysenteriae* and certain enterohemorrhagic *Escherichia coli* also produce Shiga-like toxins²⁶. The Gal α (1–4)Gal unit of the globo series of glycosphingolipid is the recognition unit for most uropathogenic bacteria²⁷. *Burkholderia cepacia*, an opportunistic bacteria found in the sputa of cystic fibrosis patient has been found to bind to the unsubstituted terminal Gal α (1–4)Gal units of digalactosylceramide and globotriaosylceramide²⁸. Higher expression levels of Gb3 have been reported in human colorectal tumors and their metastases²⁹. The trisaccharide portion of Gb3 has been linked by a MUC5AC peptide epitope to keyhole limpet hemocyanin (KLH) and has been prepared for use as a vaccine for treating ovarian cancer³⁰. Gb3 is overexpressed in colorectal adenoma cells³¹, in Burkitt's lymphoma cells^{32, 33}, in breast and ovarian cancer and some other cancers³⁴. Gb3 is the glycolipid that accumulates in the lysosomes of individuals suffering from Fabry disease, a lysosomal storage disease caused by a deficiency in α -galactosidase A activity³⁵.

A limited number of derivatives of globotriose have been synthesized and used in studies on flat gold surfaces. In an early report, Bundle presented the synthesis of the P^k trisaccharide linked to mercaptohexadecanol for use in SAM-based amperometric sensors for verotoxin³⁶. SAMs of globotriose terminated alkanethiols, and of mixed SAMs with oligoethylene glycol terminated alkanethiols were prepared and properties including wetting, interaction between opposing monolayers as a function of separation using a surface forces apparatus, and surfactant adsorption using the quartz crystal microbalance with dissipation were studied³⁷. This study found that lateral interactions between globotriose groups depended on the surface density and that the interactions, primarily hydrogen bonding, could be direct or water mediated. Svedhem et al. reported synthesis of globotriose coupled to bis(16-hydroxy hexadecanyl) disulfide and also with tetra or diethylene glycol spacers, and SAM of the molecules was prepared on the gold surface²⁰. Antibody binding studies using these SAMs reported the highest amount of antibody binding at 1–10% carbohydrate on the surface²⁰. Based on IR studies, with this large spacer, it was reported that these carbohydrate SAMs on the gold surface were highly ordered and showed crystallinity; however, no electrochemical characterization has been reported so far^{20, 37–39}.

Globotriose encapsulated gold nanoparticles were synthesized and employed for inhibiting the toxicity of Shiga toxin⁴⁰. Gold nanoparticles encapsulated with globotriose show a size dependent effect in the inhibition of Shiga toxin, with particles of 20 nm size reported to be suitable for the multivalent binding of Stx B and globotriose^{40, 41}.

Nanoporous gold (NPG) is a nanostructured form of gold currently under intensive investigation due to its high surface area^{42, 43}. Selective dissolution of the less noble metal(s) from an alloy composed of 20–50% gold results in the bicontinuous network of pores and gold ligaments characteristic of NPG⁴². NPG provides a high gold surface area at a relatively low cost per square centimeter of geometric area⁴⁴. NPG supports a localized surface plasmon resonance response⁴⁵ that is useful for development of label free sensor and assay applications⁴⁶. NPG is a promising nanomaterial for biomolecule immobilization

for applications including biofuel cells^{47, 48} and the development of immunoassays⁴⁶. NPG has potential applications as a support for SAMs presenting biologically active ligands such as carbohydrates.

Carbohydrate presentation in cells and organelles is very likely not the same as in SAMs on flat gold surfaces, as the features of curvature and fluidity are lacking. NPG with ligament diameters closer to those of vesicles and organelles may provide a better model for the study of carbohydrate-protein interactions by introducing the features of curvature. The diameters of NPG ligaments can be in a similar size range as membrane microdomains, caveolae (55 nm diameter), nanodomains (20–30 nm) and transient lipid localization regions or confinement zones (100–300 nm), which have been reported to have high content of glycosphingolipids, and thus may be a better substrate for mimicking some membrane properties^{49, 50}. NPG can be of significance in the study of carbohydrate – protein interactions from at least two unique perspectives. The high surface area of NPG could prove valuable in developing new assays for carbohydrate binding proteins. Additionally, the curved surfaces of the NPG ligaments present an opportunity to present carbohydrate ligands in a manner which resembles their presentation at curved biological interfaces. It is known that the curvature of the surfaces of nanoparticles and nanorods influences the structure of SAMs on their surfaces⁵¹. It has also been reported that nanoparticle size affects the pK_a value for mercaptoundecanoic acid ligands⁵². The presentation of carbohydrate ligands and the effects of multivalency are likely influenced by ligand spacing that can be influenced by surface curvature. For example, Au nanoparticles of 20 nm diameter were reported to be optimal for multivalent interaction between globotriose and Shiga toxin⁴¹.

Electrochemical impedance spectroscopy (EIS) is a label-free and sensitive technique for characterizing behavior at an electrode-electrolyte interface. Quantitative information about the changes in behavior of the interface, as a result of SAM formation and subsequent protein binding can be easily obtained by EIS⁵³. In EIS, the current response of a system at equilibrium is obtained by applying a small sinusoidal potential perturbation to the system. Impedance data at the selected frequencies is then fit to an equivalent circuit model that resembles the physical interface and the system under study⁵⁴. Among the parameters extracted from this analysis, the double layer capacitance and the charge transfer resistance (R_{ct}) are the most common and are sensitive to interfacial changes. Charge transfer resistance is more sensitive to protein binding than double layer capacitance and hence it is the most widely used in analytical measurements⁵⁵.

The objective of the present study is to report the synthesis of an alkanethiol derivative bearing the globotriose moiety, and electrochemical characterization of the SAMs of the derivative and its binding behavior on NPG versus on flat gold. Herein, we report the synthesis, and electrochemical characterization of SAMs of α -D-Gal-(1 $\rightarrow$ 4)- β -D-Gal-(1 $\rightarrow$ 4)- β -D-Glc-mercaptopentane (Gb3-C₅-SH) on NPG surfaces and on flat gold, and study differences in their binding to soybean agglutinin (SBA). Both pure SAMs and 1:5 mixed SAMs of Gb3-C₅-SH with octanethiol (OCT) or with 8-mercapto-3,6-dioxyoctanol (HO-PEG₂-SH) were prepared and applied for the study of SBA binding. The general strategy used to prepare the Gb3-C₅-SH compound is shown in Scheme 1.

The synthesis of the spacer-containing trisaccharide **1** was accomplished as follows. First, an NIS/TfOH-promoted coupling between thioglycosyl donor **3**⁵⁶ and acceptor **5** afforded the corresponding disaccharide **6**⁵⁷. Glycosyl acceptor **6** was then directly glycosylated at C-4' with S-benzoxazolyl (SBox) glycosyl donor **2**^{58, 59} in the presence of AgOTf to afford fully protected trisaccharide **7**. The latter was converted in the Gb3 analogue **1** using sequential

hydrogenation (H_2 , Pd/C), nucleophilic displacement of bromine (KSac in DMF), and global deacylation (NaOMe in MeOH).

The gold substrate surfaces were characterized by scanning electron microscopy and the true surface area of the electrodes was determined by CV scans in 0.5 M H_2SO_4 . The integrated charge under the gold oxide reduction peak was utilized to calculate true surface area of the electrodes. The surface area of the gold wire electrode itself is 0.040 cm^2 (geometric surface area of 0.032 cm^2 , roughness factor 1.25), and the surface area of NPG coated Au wire electrode was almost 300 fold higher ($\sim 12 \text{ cm}^2$). In these experiments, gold wire serves as the flat Au surface. SAMs on gold surfaces were prepared according to the standard procedure, by immersing the Au wires or NPG coated Au wires into a solution of alkanethiol in ethanol. Electrochemical characterization of the SAMs on NPG and flat gold surfaces were done using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

CV scans in solutions containing redox probes such as $\text{Fe}(\text{CN})_6^{3-/4-}$ or $\text{Ru}(\text{NH}_3)_6^{2+/3+}$ are commonly used to assess the integrity of the SAMs on surfaces since well ordered SAMs will create a barrier for electron transfer across the interface, thus greatly reducing the peak current^{60, 61}. Figure 1a shows the cyclic voltammograms for bare Au and for Au modified with Gb3-C₈-SH measured in 10 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$. The characteristic oxidation and reduction peaks of $\text{Fe}(\text{CN})_6^{3-/4-}$ are clearly observed on bare Au. The effective blocking behavior of the pure Gb3-C₈-SH SAM to the charge transfer across the interface is clearly indicated by the disappearance of the redox peaks of the probe. Cyclic voltammograms for Au wire electrodes modified with either a 1:5 mixed SAM of Gb3-C₈-SH:OCT or with a 1:5 mixed SAM of Gb3-C₈-SH: HO-PEG₂-SH are shown in Figure 1b, along with that for the pure Gb3-C₈-SH SAM as a comparison. It can be seen in Figure 1b that the charging current is very low for the pure Gb3-C₈-SH SAM and for the mixed SAM of Gb3-C₈-SH with OCT. In contrast, the charging current is somewhat larger for the mixed SAM of Gb3-C₈-SH and HO-PEG₂-SH and part of an irreversible oxidation wave is seen. HO-PEG₂-SH SAMs have been reported to be disordered due to the hydration of the terminal hydroxyl groups and solvent penetration between the oligoethylene units; this accounts for the increased permeability of the HO-PEG₂-SH and Gb3-C₈-SH mixed SAM compared to the other two SAMs in Figure 1b⁶².

A CV scan of bare NPG shows a much larger charging current compared to that of the gold wire, because of the large surface area of NPG. The redox peaks for $\text{Fe}(\text{CN})_6^{3-/4-}$ on the NPG electrode occur near the same potentials as on flat Au. The peak currents and capacitive current were observed to be almost thirty times larger on NPG than on flat Au, as seen in Figure 1c. CVs on NPG modified with pure Gb3-C₈-SH, a 1:5 mixed SAM of Gb3-C₈-SH and OCT and a 1:5 mixed SAM of Gb3-C₈-SH and HO-PEG₂-SH were then compared. Oxidation and reduction peaks are clearly seen for both the pure Gb3-C₈-SH SAM and the mixed SAM of Gb3-C₈-SH and HO-PEG₂-SH. The CV for the Gb3-C₈-SH modified NPG surface shows some reduction in the magnitude of the redox currents, and a further reduction is seen for the mixed SAM of Gb3-C₈-SH and HO-PEG₂-SH. In contrast, the magnitude of the charging current observed for the mixed SAM of Gb3-C₈-SH and OCT on NPG is greatly reduced and redox peaks are no longer observed (Figure 1d). The mixed SAM with OCT forms a highly resistive interface with very little permeability to the redox probe, most probably due to its hydrophobic nature⁴⁴.

Characterization of the SAM modified interfaces was also performed by EIS. Nyquist plots for the mixed and pure SAMs before and after incubating with $2 \mu\text{M}$ SBA are shown in Figure 2 on both flat Au and NPG surfaces. The Nyquist plot for the pure Gb3-C₈-SH SAM on flat gold shows a well-defined semicircle (Figure 2a), representing the charge transfer

resistance, characteristic of formation of a well packed monolayer on the surface, and also a small linear portion attributed to the contribution of Warburg diffusion⁵⁴. The pure Gb3-C₈-SH SAM shows little response to exposure to 2 μ M SBA, a similar observation to other reports in which pure SAMs of a carbohydrate presenting alkanethiol do not bind lectin due to excessive steric interference hindering access to the lectin binding sites⁸. In Figure 2a, the Nyquist plot for the 1:5 mixed SAM of Gb3-C₈-SH and OCT also shows a clear semicircle and a smaller linear portion. In contrast to the pure SAM, this mixed SAM does respond significantly to exposure to 2 μ M SBA, as evidenced by the large increase in the semicircle diameter indicating an increased charge transfer resistance. This increase is attributed partly to the non-specific adsorption of SBA to the hydrophobic regions of the SAM. The 1:5 mixed SAM of Gb3-C₈-SH and OCT on NPG, shown in Figure 2b, shows a defined semicircle, whose diameter increases significantly after exposure to SBA. In contrast to the result on Au wire, the Nyquist plot for Gb3-C₈-SH SAM on NPG (Figure 2b, inset) is characterized by a very small and depressed semicircle and a dominant straight line indicating that the interface is fairly ion permeable. Thus, there is a distinct difference in characteristic behavior of the Gb3-C₈-SH SAM on NPG versus on flat Au. The Gb3-C₈-SH SAM is likely a more open and disordered structure on NPG than on flat Au and hence more permeable to electron transfer of the redox probe. Mixed SAMs of Gb3-C₈-SH and HO-PEG₂-SH (Figure 2c) on Au wire show a clearly defined semicircle and smaller linear portion, and exposure to 2 μ M SBA clearly increases the semicircle diameter, attributed to an increase in charge transfer resistance due to lectin binding. In contrast, the mixed SAMs of Gb3-C₈-SH and HO-PEG₂-SH show a smaller depressed semicircle (Figure 2d); however, the response due to SBA binding can be seen in the shift of the frequency dependence of the data. It should be noted that a semicircle in the Nyquist plot was observed for NPG modified with a pure OCT SAM, but was not observed for NPG modified with a pure HO-PEG₂-SH SAM (see Supporting Information file). Consistent with the CV studies, EIS also shows that molecules with polar and bulkier end-groups such as Gb3-C₈-SH form relatively loosely packed and permeable monolayers on the surfaces of NPG, while similar data shows more closely packed and resistive monolayers of these molecules on the flat gold surface.

The structure of the molecule used as the second component in the SAM with Gb3-C₈-SH likely influences the orientation and presentation of the carbohydrate group to the lectin. OCT as a second component should promote van der Waals interaction with the octanethiol chains of Gb3-C₈-SH and in principle lead to a more regular orientation and presentation of the trisaccharide. It has been reported that shorter chain alkanethiols on flat Au remain in a primarily all trans configuration, similar to longer chain alkanethiols⁶³. The use of OCT in the SAM will invariably result in some measure of non-specific lectin binding in addition to the specific binding of lectin to the trisaccharide. In contrast, HO-PEG₂-SH molecules will have a weaker interaction with the alkyl chain regions of Gb3-C₈-SH, and should result in a less well defined presentation of the trisaccharide units. However, use of HO-PEG₂-SH should strongly limit non-specific lectin binding to the SAM. The introduction of curvature when considering the NPG surface in comparison with flat gold, should promote some measure of additional space between neighboring OCT chains, an increase in the number of gauche defects, and hence present a less ordered presentation than expected on flat gold. It has been reported from vibrational spectroscopic data that alkanethiol SAMs on nanoparticle surfaces of greater curvature present a higher fraction of gauche defects⁶⁴. It has been reported that the helical or amorphous forms of polyethylene glycol chains resist protein adsorption, while all trans configuration is not effective⁶⁵. Solvent penetration should also disorder the HO-PEG₂-SH units⁶². It is possible that the curved nature of the nanoporous gold surface promotes more effective solvent penetration amongst the HO-PEG₂-SH units and hence favors the amorphous form more than the flat gold surface. Spectroscopic

investigations of chain order and configuration of these species on flat versus nanoporous gold would be required to assess the details of the possible structural differences.

These carbohydrate modified surfaces were then used for the study of carbohydrate-lectin interaction. Soybean agglutinin (SBA) is a lectin from *Glycine max* and binds to the GalNAc/Gal carbohydrate moiety^{66–69}. The affinity of SBA to the galactoside unit in solution phase has been found to be less compared to its affinity for NAc-galactosamide unit⁶⁶. Manimala *et al.* reported a detection limit for SBA binding to globotriose of 65 pM in a microarray format with fluorescence detection, which was even lower than that reported for GalNAc⁶⁹. Binding of SBA to the enzymatically prepared form of porcine submaxillary mucin containing 2300 α -GalNAc residues was reported to bind to SBA with a dissociation constant of $K_d = 0.2$ nM. The same study also showed that the binding affinity was dependent upon the composition and carbohydrate epitope density on mucin⁷⁰. SBA was chosen as a lectin for binding to Gb3-C₈-SH in these studies. The change in charge transfer resistance after incubation with SBA for the pure Gb3-C₈-SH modified surfaces was very small for both the NPG and the flat Au surfaces (Figure 2a and 2b). This low affinity of lectins to carbohydrates at high ligand density is reported in the literature and has been attributed to the sterically unfavorable nature of the interaction, due to crowding of the neighboring carbohydrate molecules⁶. As noted earlier, the binding affinity of a lectin to a carbohydrate on a surface can be different from that in solution^{3, 22, 71}.

In order to overcome this lower affinity of the lectins for high density of carbohydrates on surfaces, carbohydrates presented on surfaces are often diluted with other molecules. Polyethylene glycols (PEG) are the molecules of choice for the creation of protein resistant surfaces, as these molecules reduce nonspecific protein adsorption to insignificant levels. Thus, the incorporation of diluting alkanethiols terminated by PEG chains has frequently been used in formation of mixed SAMs with carbohydrate bearing alkanethiols^{72–74}. Incubation of the mixed SAM modified NPG or flat gold surfaces with SBA both showed a significant increase in the charge transfer resistance indicating binding of SBA on the surface. EIS data were fitted satisfactorily, as judged by the low χ^2 value (less than 10^{-4}) and standard error (less than 20% which is the maximum acceptable value for fitting according to Zsimpwin software supplier), to the equivalent circuit model (see Supporting Information file). A significant increase in R_{ct} with protein binding was clearly evident in the Nyquist plots on either flat Au or NPG modified with the Gb3-C₈-SH and OCT mixed SAMs, and also for the mixed SAMs of Gb3-C₈-SH and HO-PEG₂-SH on flat Au. An increase in R_{ct} upon protein binding for the mixed SAMs on NPG occurs, and is seen in the resulting fit parameters, but is not prominent in the Nyquist plot. The magnitude of the increase in R_{ct} is greater on either surface for the mixed SAMs containing OCT than for those containing HO-PEG₂-SH.

Nonspecific adsorption of protein on both the HO-PEG₂-SH and OCT modified surfaces was also evaluated (See Supporting Information). The increase in diameter of the semicircle of Nyquist plot, for the OCT SAM on flat Au, showed that there is a large increase in charge transfer resistance after incubation with 0.5 μ M SBA; whereas, the increase was comparatively smaller on the NPG surface. On OCT-modified NPG the relative contribution of non-specific adsorption of lectin to the increase in charge transfer resistance is significantly diminished, as we reported previously in a study of the binding of concanavalin A to SAMs on NPG of a thiolated α -mannoside in similar mixed SAMs⁷⁵. The rough surface of NPG is potentially superhydrophobic, especially when SAM modified⁷⁶, and the reduced contact surface with protein on such a rough surface may be responsible for greater loss of nonspecifically adsorbed protein during washing, which is consistent with the literature reports on loss of adsorbed proteins on nanoscale rough surfaces during washing^{77, 78}. This explanation is plausible for the non-specifically bound protein on the

outer surface but it might not be applicable for the proteins adsorbed within the pores of NPG. The presence of surface regions within the NPG structure that present limited or defective SAM coverage, such as possibly near the ligament intersections of high negative curvature, could also be significant for apparently reducing the contribution of non-specific lectin adsorption to the increase in charge transfer resistance.

The difference in the nature of the electrode-solution interface, two-dimensional (flat gold wire electrode) vs. three-dimensional nanostructured interface (NPG), results in differences in the rate of intermolecular interaction and hence affect the kinetics of biomolecular interactions^{79, 80}. Figure 3 shows the binding kinetics of SBA to mixed SAMs of Gb3-C₈-SH and HO-PEG₂-SH (Figure 3a) and to those of Gb3-C₈-SH and OCT (Figure 3b). The kinetics of interaction is dependent on the nature of the diluting molecules in the SAM as well as on the structure of the surface. The data for change in charge transfer resistance versus time were fit to a simple exponential kinetic equation $\Delta R_{ct}(t) = \Delta R_{ct, max} (1 - \exp(-k_{eff} t))$ in order to extract effective association rate constants. For the mixed SAM of Gb3-C₈-SH and HO-PEG₂-SH, the rate constants are $k_{eff} = 0.093 \pm 0.013 \text{ min}^{-1}$ on flat Au and $k_{eff} = 0.113 \pm 0.022 \text{ min}^{-1}$ on NPG. These values are close and given the error bars suggest at most a slightly different rate of SBA binding to these two surfaces. In contrast, for the mixed SAMs with OCT, the rate of SBA binding to the surface appears to be faster on flat Au than on NPG since for the mixed SAM of Gb3-C₈-SH and OCT on flat Au, $k_{eff} = 0.135 \pm 0.019 \text{ min}^{-1}$, and on NPG a value of $k_{eff} = 0.063 \pm 0.017 \text{ min}^{-1}$ is obtained⁸⁰. It is likely that there are differences in the distribution and organization of the molecules in the mixed SAM on flat Au versus on NPG that effect the rate of association of SBA with the SAM. Since the effective rate constant was similar for the mixed SAMs with HO-PEG₂-SH on flat Au and NPG, the significant difference seen for the mixed SAMs with OCT should not be a consequence of differences in diffusion of protein to the respective surfaces or a consequence of the NPG architecture alone. On the basis of these data, a time of 120 minutes was chosen as sufficient to ensure attainment of equilibrium in the measurements presented below for assessment of lectin-Gb3 binding.

Binding affinity measurements were done for the mixed SAMs on NPG and on flat Au as a function of SBA concentration. Figure 4a shows the isotherm for SBA binding to Gb3-C₈-SH in 1:5 mixed SAMs of Gb3-C₈-SH and HO-PEG₂-SH on flat Au and on NPG. The dissociation constant (K_d) for adsorption of SBA to the 1:5 mixed SAM of Gb3-C₈-SH and HO-PEG₂-SH on flat Au is 1.57 μM and on NPG it is 0.21 μM . The binding isotherms for the 1:5 mixed SAMs with OCT are shown in Figure 4b; the value of K_d found is 0.24 μM on flat Au and 1.33 μM on NPG. These results for binding of SBA by the mixed SAMs are summarized in Table 1, shown below.

The higher measured affinity of SBA to the mixed SAM with OCT on flat Au as compared to the mixed SAM with HO-PEG₂-SH on flat Au is likely due to additional non-specific adsorption of SBA onto the OCT regions. The lower affinity of SBA to the mixed SAM with OCT on NPG points to a possible advantage of the NPG surface in reducing the contribution from non-specific protein adsorption, and is consistent with our prior observations on binding of Concanavalin A to SAMs of OCT and a thiolated mannoside⁷⁵. In contrast, on NPG the affinity is higher for the mixed SAM with HO-PEG₂-SH than for the mixed SAM with OCT. Differences in presentation of the Gb3 ligand, extent of non-specific SBA adsorption, and possible differences in the mixed SAM structure can all be factors influencing the measured binding affinity. It has been reported that on curved Au surfaces such as nanoparticles, phase separation can occur in some cases if a mixture of alkanethiols are used as ligands^{51, 81, 82}. Changes in the extent of clustering of the Gb3 ligands could alter the affinity of the mixed SAM modified surface for SBA, most likely such that increased clustering would result in an increase in steric interference between neighboring

Gb3 units and a decrease in apparent affinity. A more open presentation of the Gb3 ligand, as could be provided by surface curvature, would be expected to potentially increase the apparent affinity. The mixed SAMs with HO-PEG₂-SH should have minimal non-specific binding, and the enhanced affinity observed to the mixed SAM on NPG as compared to flat Au is intriguing and suggests a possible difference in Gb3 ligand presentation. The higher affinity observed for binding of SBA to the HO-PEG₂-SH containing mixed SAMs on NPG than to those on flat Au suggests that NPG may have the advantage of presenting mixed SAMs of higher lectin affinity that are also fully resistant to non-specific protein adsorption. The nature of the underlying surface thus comes into play for influencing the presentation of molecules at the interface and the binding affinity.

Atomic force microscopy (AFM) was employed with the goal of imaging the binding of SBA to the Gb3-C₈-SH-containing SAMs in order to verify the lectin binding and to compare the distribution of individual bound protein on the mixed SAMs of Gb3-C₈-SH with either OCT or with HO-PEG₂-SH. NPG was prepared on the surface of gold sputtered mica sheets for use in the AFM studies. The NPG surface was then modified with mixed SAMs of Gb3-C₈-SH/OCT or Gb3-C₈-SH/HO-PEG₂-SH (1:5, 6 mM, respectively) for 24 hours in room temperature, before exposure to SBA as described above. Figure 5a and 5c show NPG pre-assembled with the Gb3-C₈-SH/HO-PEG₂-SH, Gb3-C₈-SH/OCT SAMs prior to exposure to SBA, respectively. After the adsorption of SBA, the typical smooth hilly appearance of the NPG is no longer apparent, and the pores and interconnected ligament features are not as clearly prominent (Figure 5b and 5d) when compared to the NPG not exposed to protein (Figure 5a and 5c). On the basis of the AFM topographic images shown in Figure 5b and 5d, NPG modified with Gb3-C₈-SH/OCT shows a significantly higher protein density when compare to the NPG modified with Gb3-C₈-SH/HO-PEG₂-SH. This observation is consistent with the EIS results, where a greater increase in charge transfer resistance, as indicated by the increase in diameter of the semicircle in the Nyquist plots, is seen for SBA adsorbed onto Gb3-C₈-SH/OCT than for those of Gb3-C₈-SH/HO-PEG₂-SH. Furthermore, the coverage of SBA also reveals that the binding of SBA to the surfaces is specific for the mixed SAMs with HO-PEG₂-SH but shows a significant contribution from non-specific adsorption for the mixed SAMs with octanethiol. These observations are consistence with our prior studies using a different lectin-carbohydrate pair, 8-mercaptooctyl α -D-mannopyranoside/HO-PEG₂-SH and concanavalin A ⁷⁵.

These experiments indicate that the differences in lectin – carbohydrate binding on flat Au versus on NPG are significant and also suggest some potential advantages of NPG and for use of EIS as a label-free screening method. The mixed SAMs of Gb3-C₈-SH with the hydrophobic OCT diluent show a reduced contribution from non-specific binding on NPG, which is a potential advantage for use of NPG as a surface. Additionally, the mixed SAMs containing the commonly used protein resistant diluent species in the form of HO-PEG₂-SH indicate stronger binding to the Gb3-C₈-SH containing SAMs on NPG than on flat Au. EIS is a method that is sensitive to the variations of gold substrate and monolayer structure and their combined effects on protein binding affinity. Detailed characterization of the SAMs on NPG and on flat Au by vibrational spectroscopic methods could help reveal differences in conformational order. NPG in an electrode array format may prove to be of future interest for lectin-carbohydrate interactions.

1. Experimental

1.1. Materials

Gold wire, 0.2 mm diameter (99.99%) was from Electron Microscopy Sciences, Fort Washington, PA). Sodium carbonate (99%), sulfuric acid (certified ACS plus), nitric acid (trace metal grade), and 30% hydrogen peroxide were all from Fisher Scientific (Pittsburg,

PA). K[Ag(CN)₂] (99.96%), K[Au(CN)₂] (99.98%), n-octanethiol (98%), calcium chloride dihydrate (99.5%), manganese chloride tetrahydrate (99%), soybean agglutinin (from *Glycine max*, lyophilized powder), potassium ferrocyanide (99%), potassium ferricyanide (99%), ethanol (HPLC/spectrophotometric grade), and phosphate buffered saline were all from Sigma-Aldrich (St. Louis, MO). All reagents were used without further purification. Milli-Q water, 18.2 MΩ, was used for preparation of all aqueous solutions.

The synthesis of α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc-mercaptooctane (Gb3-C₈-SH) is described in detail in the supporting information file. The synthesis of 8-mercapto-3,6-dioxaoctanol (HO-PEG₂-SH) was performed as previously reported ⁷⁵.

1.2. Preparation of nanoporous gold

An alloy of gold and silver was electrodeposited on gold wire of 0.2 mm diameter and 0.5 cm length. The solution used for electrodeposition was prepared by combining 4.9 ml of 50 mM K[Ag(CN)₂] and 2.1 ml of 50 mM K[Au(CN)₂] (each in 0.25 M sodium carbonate) to give a composition of 70% Ag and 30% Au in the 7.0 mL deposition solution used in the electrochemical cell. The deposition was carried out at a potential of −1.0 V (versus Ag|AgCl) for 10 minutes. Thus prepared alloys were dealloyed by submerging alloy coated gold wires into concentrated nitric acid solution (trace metal grade) for 24 hours in a sealed container. Gold wires were cleaned by dipping them in piranha solution (H₂SO₄: H₂O₂ (30%), 4:1 for 10 minutes and rinsed with plenty of water and then with HPLC grade ethanol (*Piranha solution reacts vigorously with organic substances so caution should be taken while preparing and handling*). The surface morphology and general structure of NPG was studied by scanning electron microscopy (JEOL JSM-6320F field emission SEM). Electrochemical surface area was determined by the gold oxide stripping method by scanning between 0 and 1.5 V at a scan rate of 100 mV sec^{−1}. The charge under the oxide reduction peak was integrated to estimate the surface area of the gold wires used in this study, using the reported conversion factor of 450 μC cm^{−2} ^{83–85}.

1.3. Preparation of self-assembled monolayers

The SAM preparation used in this study follows established procedures^{1, 50, 51}. Nanoporous gold covered gold wires or gold wires alone were immersed in either single component solutions of Gb3-C₈-SH (5 mM) or mixed component thiol solution of Gb3-C₈-SH-C₈-SH/OCT or Gb3-C₈-SH/HO-PEG₂-SH in ethanol for 17 hours at room temperature. The resulting SAMs on the gold surfaces were copiously rinsed with ethanol, water and PBS buffer prior to use in electrochemical experiments.

1.4. Electrochemical impedance spectroscopic measurement: EIS measurements

EIS measurements were done in a three-electrode cell containing 5 mM K₃[Fe(CN)₆] and 5 mM K₄[Fe(CN)₆] at the formal potential of the redox probe, and at frequencies from 10⁵ Hz to 100 mHz, at 12 points per decade. A PARSTAT 2273 potentiostat/galvanostat/FRA was used for the EIS measurements, and the Powersine software was used for data collection. Data analysis was done by using Zsimpwin 3.21 software (Princeton Applied Research, Oak Ridge, TN), by fitting to the modified Randles equivalent circuit shown in the supporting information file, in which the double layer capacitor has been replaced by the constant phase element (Q) to account for the non-ideal behavior associated with the roughness of the electrode. This circuit has been applied for fitting EIS data obtained on both modified NPG and gold wire surfaces. Charge transfer resistance was used as a measure of the resistance at the interface created by the addition of a layer of molecules due to lectin-carbohydrate binding. EIS measurements to assess binding kinetics were performed by incubating the electrode in the protein solution for the desired time followed by removal and acquisition of EIS data in the Fe(CN)₆^{3−/4−} redox probe solution.

1.5. Cyclic voltammetry

The surface area of the gold electrodes was determined using the oxide stripping method. Cyclic voltammetry (CV) scans between 0 V and 1.5 V (vs. Ag|AgCl) were conducted at a scan rate of 100 mV sec⁻¹ in 0.5 M H₂SO₄ that was degassed with argon. Additional CV scans to probe SAM integrity on gold wire and on NPG coated gold wire electrodes were conducted between 0 and 0.5 V (vs. Ag|AgCl) in 10 mM K₃[Fe(CN)₆] at a scan rate of 100 mV sec⁻¹. Each determination of surface area was repeated ten times.

1.6. Atomic force microscopy imaging and analysis

The AFM imaging was performed in Tapping Mode™ with a Multimode™ AFM (Veeco, Santa Barbara, California, USA) operating with a NanoscopeIIIa™ controller. We used TAP300G (Budget Sensors, Sofia, Bulgaria) silicon cantilevers with a resonance frequency of about 300 kHz. The scan frequency was typically between 1.0 to 1.5 Hz per line and the modulation amplitude was a few nanometers. We used a first or second order polynomial function to remove the background slope. The AFM scanner was calibrated (under contact mode condition) laterally via the periodicity of a mica (0001) surface (0.518 nm) and vertically using mica etch pits as a height calibration source. The etch pits are produced by wet etching the mica substrates in concentrated hydrofluoric acid and are formed with a specific geometry. Along the long axis of the etch pits, steps of approximately 1 nm are observed which correspond to the molecular planes of mica and permit for height calibration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Synthesis of α -D-Gal-(1 $\rightarrow$ 4)- β -D-Gal-(1 $\rightarrow$ 4)- β -D-Glc-mercaptooctane (Gb3-C₈-SH) for self-assembled monolayer (SAM) formation is reported
- Electrochemical impedance spectroscopy reveals differences in binding affinity of Gb3-C₈-SH containing mixed SAMs to soybean agglutinin on nanoporous versus flat gold in a convenient and label-free manner
- Mixed SAMs of Gb3-C₈-SH with 8-mercapto-3,6-dioxaoctanol (1:5 solution molar ratio) show higher affinity for soybean agglutinin on nanoporous gold
- Electrochemical impedance spectroscopy shows that the contribution of non-specific protein binding is diminished when using nanoporous gold as a supporting surface

Figure 1.

Cyclic voltammogram of the bare and modified electrodes in 10 mM $K_3[Fe(CN)_6]$. CV scans were performed in 10 mM $K_3[Fe(CN)_6]$ at a scan rate of 100 mV sec^{-1} in a three electrode cell arrangement, using gold wires as working electrode, Ag|AgCl as a reference electrode and platinum wire as a counter electrode. Figure 1a shows CVs on a bare gold wire electrode (GW, solid line), and also for a gold wire electrode modified with an SAM of Gb3-C₈-SH (dotted line). Figure 1b shows CV data on gold wire electrodes modified with mixed SAMs of 1:5 Gb3-C₈-SH:HO-PEG₂-SH (long dash line), 1:5 Gb3-C₈-SH:OCT (dash dot dash dot line), and the SAM of Gb3-C₈-SH (dotted line). The ratio referred to is the solution molar ratio from which the SAM was prepared. Figure 1c shows CV data for bare NPG (solid line), SAM of Gb3-C₈-SH (dotted line), and 1:5 mixed SAMs of Gb3-C₈-SH:HO-PEG₂-SH (long dash line), and SAM of 1:5 Gb3-C₈-SH:OCT (dash dot dash dot line). Figure 1d is an expanded view of the CV for the mixed SAM of 1:5 Gb3-C₈-SH:OCT on NPG.

Figure 2.

Nyquist plots for the modified gold wire and NPG electrodes before and after exposure to soybean agglutinin (SBA). Figure 2a shows data for SAM of Gb3-C₈-SH (solid line), Gb3-C₈-SH after incubation with 2 μM SBA (dotted line), 1:5 mixed SAM of Gb3-C₈-SH:OCT before (long dash line), and after (short dashed line) incubation with 2 μM SBA on gold wire. Figure 2b shows data for the 1:5 mixed SAM of Gb3-C₈-SH:OCT SAM on NPG before (long dashed line), and after (dotted line) incubation with 2 μM SBA. The analogous Nyquist plots for the Gb3-C₈-SH SAMs on NPG before (solid line) and after (dotted line) incubation with 2 μM SBA are shown in the inset of Figure 2b. Figure 2c shows data for the 1:5 mixed SAM of Gb3-C₈-SH: HO-PEG₂-SH SAM on gold wire before (solid line) and after (dotted line) incubation with 2 μM SBA. Figure 2d shows data for the 1:5 mixed SAM of Gb3-C₈-SH: HO-PEG₂-SH SAM on NPG before (solid line) and after (dotted line) incubation with 2 μM SBA.

Figure 3.

Binding kinetics for soybean agglutinin (SBA) to mixed SAMs containing Gb3-C₈-SH as measured by the increase in charge transfer resistance. The binding kinetics study was performed by incubating modified electrodes with 0.5 μ M SBA for the given time interval, rinsing and then conducting EIS measurements. Figure 3a shows data for binding of SBA to Gb3-C₈-SH in mixed SAMs of 1:5 Gb3-C₈-SH: HO-PEG₂-SH on gold wire electrodes (circles) and on NPG electrodes (triangles). Similar data for mixed SAMs of 1:5 Gb3-C₈-SH:OCT are shown on gold wire (circles) and on NPG (triangles) in Figure 3b.

Figure 4.

Binding isotherms for the interaction of soybean agglutinin (SBA) with Gb3-C₈-SH in mixed SAMs as measured using the increase in charge transfer resistance. Figure 4a shows data for mixed SAMs of 1:5 Gb3-C₈-SH: HO-PEG₂-SH on gold wire electrodes (circles) and on NPG electrodes (triangles). Figure 4b shows data for mixed SAMs of 1:5 Gb3-C₈-SH:OCT on gold wire (circles) and on NPG (triangles).

Figure 5.

Soybean agglutinin (SBA) binding to the SAM surfaces. 5(a) and 5(c) show AFM topographic images of NPG modified with mixed SAMs of 1:5 Gb3-C₈-SH:HO-PEG₂-SH and 1:5 Gb3-C₈-SH:OCT, respectively. A higher SBA coverage was observed on the mixed component SAMs surface containing OCT (5d), as compared to the SAM containing HO-PEG₂-SH (5b). The AFM topographic images were taken over a 500 nm × 500 nm area.

Scheme 1.

Retrosynthetic analysis for the synthesis of α -D-Gal-(1 $\rightarrow$ 4)- β -D-Gal-(1 $\rightarrow$ 4)-D-Glc-mercaptooctane (Gb3-C₈-SH) (compound **1**).

Table 1

Summary of binding affinities (K_d values) for soybean agglutinin to mixed self-assembled monolayers containing α -D-Gal-(1 $\rightarrow$ 4)- β -D-Gal-(1 $\rightarrow$ 4)-D-Glc-mercaptopentane (Gb3-C₅-SH) and either 8-mercapto-3,6-dioxapentanol (HO-PEG₂-SH) or octanethiol (OCT).

Substrate	1:5 Gb3-C ₅ -SH/HO-PEG ₂ -SH	1:5 Gb3-C ₅ -SH/OCT
Flat gold	1.57 μ M	0.24 μ M
Nanoporous Gold	0.21 μ M	1.33 μ M