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Lectin–carbohydrate interactions on nanoporous gold monoliths[†]

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Abstract

Monoliths of nanoporous gold (np-Au) were modified with self-assembled monolayers of octadecanethiol (C₁₈-SH), 8-mercaptooctyl α-D-mannopyranoside (αMan-C₈-SH), and 8-mercapto-3,6-dioxaoctanol (HO-PEG₂-SH), and the loading was assessed using thermogravimetric analysis (TGA). Modification with mixed SAMs containing αMan-C₈-SH (at a 0.20 mole fraction in the SAM forming solution) with either octanethiol or HO-PEG₂-SH was also investigated. The np-Au monoliths modified with αMan-C₈-SH bind the lectin Concanavalin A (Con A), and the additional mass due to bound protein was assessed using TGA analysis. A comparison of TGA traces measured before and after exposure of HO-PEG₂-SH modified np-Au to Con A showed that the non-specific binding of Con A was minimal. In contrast, np-Au modified with octanethiol showed a significant mass loss due to non-specifically adsorbed Con A. A significant mass loss was also attributed to binding of Con A to bare np-Au monoliths. TGA revealed a mass loss due to the binding of Con A to np-Au monoliths modified with pure αMan-C₈-SH. The use of mass losses determined by TGA to compare the binding of Con A to np-Au monoliths modified by mixed SAMs of αMan-C₈-SH and either octanethiol or HO-PEG₂-SH revealed that binding to mixed SAM modified 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. Minimal adsorption of immunoglobulin G (IgG) and peanut agglutinin (PNA) towards the mannoside modified np-Au monoliths was demonstrated. A greater mass loss was found for Con A bound onto the monolith than for either IgG or PNA, signifying that the mannose presenting SAMs in np-Au retain selectivity for Con A. TGA data also provide evidence that Con A bound to the αMan-C₈-SH modified np-Au can be eluted by flowing a solution of methyl α-D-mannopyranoside through the structure. The presence of Con A proteins on the modified np-Au

[†]Electronic supplementary information (ESI) available: A schematic depiction of the homebuilt flow cell used in these experiments and details of its use. Details of the modification of np-Au with 1-octadecanethiol are also provided. SEM images of nanoporous gold before and after modification with octanethiol under flow conditions, and of nanoporous gold after a TGA temperature ramp are provided, along with discussion of the data. TGA data for Con A binding to mixed SAMs on nanoporous gold under static conditions are also provided. See DOI: 10.1039/c3nj00253e

surface was also confirmed using atomic force microscopy (AFM). The results highlight the potential for application of carbohydrate modified np-Au monoliths to glycoscience and glycotecnology and demonstrate that they can be used for capture and release of carbohydrate binding proteins in significant quantities.

Introduction

Monoliths of porous materials have found many applications, including in chromatographic separations,^{1,2} and in proteomics technology.³ Among the porous materials used to create these monoliths, sol-gels^{4,5} and porous polymers are examples.^{2,6,7} Affinity chromatography using a variety of bio-molecules, including antibodies, proteins, and lectins, attached to the surface of porous monoliths has been reviewed.^{8,9} Controlled pore glass (CPG) beads have been used as stationary phases in size exclusion chromatography¹⁰ and can be surface modified with ligands for separation of biomolecules.¹¹ Immobilization of protein A or protein G in monoliths of porous silicon has been applied to the separation of immuno-globulins.^{12–15} Recently, there has been a growing interest in porous inorganic monoliths of metal oxides and of metals and their applications in separations science, and such structures and their potential applications have recently been reviewed.^{16,17} Monoliths provide a number of advantages in separations including improved mass transport and higher efficiency. Amongst these new materials, nanoporous gold (np-Au) is a new example available for research and applications. It is anticipated that surface modified np-Au monoliths will find growing application in separations, catalysis, supported synthesis, and other fields.

Nanoporous gold (np-Au) has become the subject of intense investigation in recent years. The np-Au material is prepared by the removal by a dealloying process of the less noble element(s) from an alloy containing 20% to 50% gold, with gold and silver being the most typical alloy used. The process of np-Au formation has been described in detail by Erlebacher and coworkers.^{18–22} The material presents a bicontinuous structure of interconnected ligaments and pores, with the average ligament and pore sizes for a given sample generally equivalent.^{23,24} An important characteristic of np-Au is that its pore/ligament size is tunable, either by varying the initial alloy composition, or etching time and conditions, or by employing thermal annealing after dealloying.^{19,25,26} The size and dimensions of np-Au monoliths that can be created appears in principle to be widely variable, and average pore sizes from a few tens of nanometers up to a micron can readily be accessed by adjusting the preparation and post-treatment conditions.^{23,24,26} The pores of np-Au often fall in what is classically considered the ‘macro-pore’ range of typically 50 nm and greater^{27–29} and are suitable for liquid flow-through and also leave adequate space for immobilization of ligands and subsequent binding of bio-molecules. The bicontinuous pore and ligament structure of np-Au resembles that found in controlled pore glass beads³⁰ or in silica monoliths in terms of general morphology.³¹

Applications of np-Au monoliths involving their modification with self-assembled monolayers (SAMs)^{22,26} or as supports for the immobilization of biomolecules, either by covalent conjugation³² or physical adsorption,³³ have been reported. Many researchers have

realized the potential of np-Au and have conducted studies to advance its potential for a range of applications.^{20–22,26,34–36} For example, our group has reported that monolithic structures of np-Au can be used as a support for the iterative synthesis of carbohydrates.³⁴ We have demonstrated that protein solutions can flow through free-standing np-Au monoliths oriented in a flow cell.²² The immobilization of the proteins bovine serum albumin (BSA) and rabbit immunoglobulin G (IgG) by *N*-hydroxysuccinimide/ethyl(dimethyl-aminopropyl) carbodiimide (EDC/NHS) conjugation to np-Au monoliths modified by lipoic acid was demonstrated and studied both on the exterior and interior of the samples using atomic force microscopy (AFM).²² The use of flow conditions was found to enhance the coverage of protein on the internal surfaces, whose imaging by AFM was made possible by a procedure in which the np-Au monoliths could be cleaved open.

Surface modification of porous monoliths has applications in the field of glycoscience. There is a strong interest in the separation of glycoprotein or glycoform mixtures, or capture of specific glycoproteins from biological fluids, both of which could potentially be accomplished by using lectin modified porous monoliths. The status and prognosis of a number of diseases has been found to be related to the presence of specific glycoforms of a single glycoprotein; for example, aberrant protein glycosylation patterns are prevalent in cancer^{37,38} and in rheumatoid arthritis,³⁹ and other examples of the significance of glycoform variation are being discovered. Variation in the glycosylation of immunoglobulins also affects the efficacy of antibody based therapeutics.⁴⁰ Lectin–carbohydrate interactions play many key roles in a variety of important biological processes, including cancer diagnostics, cell–cell recognition and communication, and pathogenic infection.^{41–46} The separation or capture of specific glycoforms could be accomplished by flow of glycoprotein containing solutions through porous monoliths surface modified to present lectins with the required carbohydrate binding affinities.^{47–53}

Another application of surface modified porous monoliths in glycoscience is the capture, screening or separation of lectins (or other carbohydrate binding proteins) using monoliths surface modified to present specific carbohydrates. A number of methods have been applied to immobilize ligands to achieve recognition, capture and detection of proteins.^{54–58} Immobilization of carbohydrates onto gold nanoparticles has been studied extensively by the Penadés lab,^{59–64} and others.^{65–67} Carbohydrates immobilized onto magnetic nanoparticles,^{68–70} quantum dots,^{71,72} in self-assembled monolayers (SAMs) on gold surfaces,^{73–79} or on glass slides⁸⁰ have provided new tools to investigate the specificity of lectin–carbohydrate interactions.

A much more limited number of studies of carbohydrate modified monoliths have been reported. In one approach, amino terminated sugars were reacted with aldehyde groups on a polymeric monolith (average pore size = 89 nm) and used to present mannose for the binding of Con A.⁸¹ Bound lectin was eluted by circulating soluble methyl α -mannoside to displace the lectin from the column. Use of a fluorescently labeled Con A enabled imaging of the affinity front within the column. The affinity for Con A was found to be greater than that for lens culinaris agglutinin (LCA) or peanut agglutinin (PNA). The capacity of the monolith of dimensions 13.5 cm in length and 75 μ m in inner diameter was found to be 7.4 $\pm$ 0.6 μ g for Con A. In another study, the binding of Con A to macroporous chitosan

membranes, prepared using silica particles as the porogen, was studied under flow conditions. Adsorption isotherms for Con A, determined by spectrophotometric solution depletion methods, were found to be adequately represented by a Langmuir model.⁸² A polymeric monolith modified with sialyllactose was reported as able to efficiently separate influenza virus particles.⁸³

The complexity of the np-Au structure presents some challenges in accurately determining its loading capacity for thiolated molecules. Although it cannot be applied *in situ*, the use of the thermogravimetric analysis (TGA) method for determination of surface loading of immobilized ligands/ biomolecules onto np-Au is an attractive approach when the np-Au sample is large enough.²⁶ TGA is well-suited for assessing loading of molecules into np-Au monoliths, especially for the case of molecules that are not chromophores and thus cannot be readily followed using spectroscopic detection. Thermogravimetric methods are highly sensitive, and have been applied to assess ligand loading onto gold nanoparticles.^{84,85} The sensitivity of thermogravimetric methods can be as good as 0.1 μg .⁸⁶ TGA is a suitable method for the study of molecular loading onto np-Au monoliths due to the high melting point of gold (1064.18 °C) and the relatively low temperature range of 150 to 300 °C for the decomposition of the immobilized alkanethiols during the pyrolysis measurement.

In the present work, we demonstrate that np-Au monoliths can be loaded with SAMs of a thiolated α -mannoside derivative, 8-mercaptooctyl α -D-mannopyranoside ($\alpha\text{Man-C}_8\text{-SH}$), and that these SAMs bind the lectin Con A. We also show that the surface loading of np-Au monoliths by SAMs as a function of exposure time can be followed using TGA. Modification with the short chain polyethyleneglycol (PEG) derivative 8-mercapto-3,6-dioxaoctanol ($\text{HO-PEG}_2\text{-SH}$) alone is found to be effective at limiting non-specific lectin adsorption to the monolith, and modification with a mixture of $\text{HO-PEG}_2\text{-SH}$ and $\alpha\text{Man-C}_8\text{-SH}$ to be effective at promoting the specific binding of Con A. The capture of Con A is found to depend on the ratio of $\text{HO-PEG}_2\text{-SH}$ to $\alpha\text{Man-C}_8\text{-SH}$ in mixed SAMs on the np-Au monoliths. Selectivity of the mannose modified np-Au monoliths for binding of Con A is demonstrated by comparison of the results of exposure to Con A *versus* exposure to immunoglobulin G (IgG) or to peanut agglutinin (PNA). Both the loading of the $\alpha\text{Man-C}_8\text{-SH}$ SAM, its binding of Con A, and elution of bound Con A by free ligand are carried out under flow-through conditions using np-Au monoliths, and serve to demonstrate that these carbohydrate-presenting SAM-modified monolithic np-Au structures could potentially be used to capture lectins that bind specific carbohydrates. The successful modification of these monoliths demonstrated herein suggests their potential utility in applications involving lectin-carbohydrate and also potentially lectin-glycoprotein interactions in separations or capture of carbohydrate binding proteins or glycoproteins.

Results and discussion

Functionalization of nanoporous gold monoliths with SAMs of octadecanethiol

Np-Au is an attractive material for modification with alkane-thiolate SAMs. Imaging of the interior of np-Au monoliths, exposed by mechanically cleaving the structure open and viewing it edgewise, confirmed that the nanoporous structure is present throughout the material.^{22,33} The structure of SAMs formed on curved surfaces, such as those of np-Au

ligaments, gold nanoparticles, or gold nanorods is an active area of investigation.^{87,88} The surfaces of np-Au have been reported from X-ray diffraction data to be primarily Au(111), but with significant contributions from other faces including Au(200), Au(220), Au(222), and Au(311).^{26,89,90} Self-assembled monolayers (SAMs) of species with reactive ω -functionalized end-groups can be functionalized onto np-Au. This approach allows for the attachment of proteins using the terminal moieties.^{91,92} Conjugation to ω -functionalized organothiol SAMs allows desired functional groups to be assembled into the rigid porous framework, tethering of small molecules in a three dimensional environment, or use as a rigid support for the immobilization of biomacro-molecules on a support for use as an electrochemical biosensor.⁹³ Additional advantages of the np-Au monoliths include their chemical stability, ability to be conveniently transferred from one experiment to another, and suitability for use as a porous medium through which solutions can flow. They should have good thermal stability at the relatively low temperatures of interest for aqueous phase analytical applications. Thermo-gravimetric analysis can be used for quantitative measurement of the mass loss of the thiolated molecules functionalized onto np-Au, as a function of temperature or time. Thermogravimetric analysis is applicable in our study, due to the high melting point of gold (1064.18 °C) and the relative low temperature range (150–300 °C) over which the alkanethiol decomposes on the metal surface.⁹⁴ Considering the surface area of np-Au used for the analysis, and the mass sensitivity of the TGA instrument of 0.1 μg , TGA proves to be a reasonably sensitive technique. The TGA experiments in the current study take place on 0.08–0.10 g of np-Au, which using the surface area measured by N_2 gas adsorption isotherms and BET analysis of 4.91 $\text{m}^2 \text{g}^{-1}$ and the instrument sensitivity of 0.1 μg corresponds to a sensitivity per unit surface area of near $\sim 0.2 \text{ pg mm}^{-2}$. The typical sensitivity of SPR is reported as 0.1 pg mm^{-2} .⁹⁵ Although the experiments are different, with SPR being an *in situ* optical method, and TGA being an *ex situ* thermal method, the sensitivity of TGA for mass changes on the surfaces of the monoliths of np-Au of the size used in this study is comparable on a per unit area basis.

In a close packed crystalline monolayer of alkanethiol on gold, each thiolate occupies 21.4 $\text{\AA}^2$ equivalent to a surface coverage of *ca.* $4.67 \times 10^{14} \text{ molecules cm}^{-2}$.⁹⁶ Since the surface area of a typical np-Au monolith sample was found to be 4.91 $\text{m}^2 \text{g}^{-1}$ (based on BET surface area analysis), and the amount of the thiolate functionalized onto the surface of np-Au can be measured based on the amount of thiols decomposed using TGA, the total number of thiols functionalized onto the surface of given np-Au sample can be estimated and then expressed as a surface coverage in terms of thiolate molecules per square centimeter. The loading of 1-octadecanethiol ($\text{C}_{18}\text{-SH}$) onto np-Au monoliths was followed over time using both static and flow-through conditions (see ESI[†]). The final coverage obtained is similar, whether by static or flow assembly, of near $5.0 \times 10^{14} \text{ molecules cm}^{-2}$, calculated subject to the assumption of using the BET surface area for these np-Au samples, and is reasonably close to the expected theoretical coverage for an alkanethiol SAM of $4.7 \times 10^{14} \text{ molecules cm}^{-2}$. At earlier times in the SAM formation process, higher surface coverage is found

[†]Electronic supplementary information (ESI) available: A schematic depiction of the homebuilt flow cell used in these experiments and details of its use. Details of the modification of np-Au with 1-octadecanethiol are also provided. SEM images of nanoporous gold before and after modification with octanethiol under flow conditions, and of nanoporous gold after a TGA temperature ramp are provided, along with discussion of the data. TGA data for Con A binding to mixed SAMs on nanoporous gold under static conditions are also provided. See DOI: 10.1039/c3nj00253e

under static conditions than under flow-through conditions, possibly due to greater accumulation of physisorbed species during static assembly.

Pyrolysis of 8-mercaptooctyl α -D-mannopyranoside molecules assembled onto nanoporous gold

The interest in analyzing interactions of biomolecules with specific immobilized receptors has steadily increased in recent years. Nanoporous gold should be an attractive option as a support that allows for presentation of a large density of ligands per unit of geometric surface area. A fundamental understanding of the binding capacity of receptor modified np-Au towards specific proteins may yield useful information leading to efficient and effective coupling of ligands to np-Au for use as receptor/ligand-based sensing or separations platforms. In glycoscience, one of the main interactions of interest is carbohydrate–lectin binding.

Fig. 1 shows TGA data quantifying the assembly of 8-mercaptooctyl α -D-mannopyranoside (α Man-C₈-SH) onto the np-Au monolith. The α Man-C₈-SH was previously synthesized and used to form mixed SAMs on np-Au that bound Con A and studied using electrochemical impedance spectroscopy.⁹⁷ Similarly to the approach used for studying the self-assembly of C₁₈-SH onto np-Au, static and flow through methods were used to introduce the α Man-C₈-SH onto the gold surfaces of np-Au monoliths. The total amount of α Man-C₈-SH functionalized onto np-Au over time was determined from the measured mass loss determined by TGA analysis. A gradual increase in mass of α Man-C₈-SH on the np-Au occurs as expected and is shown in Fig. 1A for a representative monolith under static incubation. During the static incubation, at the 2, 4, 6, 8 and 24 hour time points, there were 0.4896 mg, 0.6486 mg, 0.7416 mg, 0.8056 mg and 0.9286 mg of α Man-C₈-SH functionalized onto the np-Au monolith, respectively. During the flow assembly, at the 2, 4, 6, 8 and 24 hour time points, there were 0.2730 mg, 0.6610 mg, 0.7850 mg, 0.7950 mg and 0.8290 mg of α Man-C₈-SH functionalized onto the np-Au monolith, respectively. The bulky man-nose end-groups could hinder the initial approach and sticking of molecules to the surface, and their insertion into the partially formed SAM. Both of these steps must occur prior to molecular rearrangement into closer packed configurations. The assembly kinetics of the mannoside under flow (Fig. 1B) are initially slower than under static conditions, suggesting that some of the mannoside species that approach the surface under flow at earlier times are swept away. The coverage then increases between the 2 hour and 4 hour time points, and a closer to maximal coverage is reached by the 6 hour time point. However, the coverages at the 24 hour time point are ultimately similar within 10% for both the static and flow self-assembly conditions, with the value under flow assembly being slightly less. The mass loss of 0.8290 mg at the 24 hour time point for flow assembly corresponds to 2.56×10^{-6} moles of α Man-C₈-SH (as compared to 2.87×10^{-6} moles for static assembly). Using an estimated specific surface area of the np-Au monolith of $4.91 \text{ m}^2 \text{ g}^{-1}$, surface coverages of $3.56 \times 10^{14} \text{ molecule cm}^{-2}$ are obtained for flow assembly and $3.99 \times 10^{14} \text{ molecule cm}^{-2}$ for static assembly, respectively. The surface coverage is less than that found for C₁₈-SH and this is expected given the steric requirements of the bulky terminal mannose group.

On the basis of the SAM formation kinetics studies reported previously⁹⁶ for alkanethiol adsorption onto flat Au(111) surfaces, we expect thiolate adsorption onto the np-Au monoliths also to involve two stages: (1) a very fast chemisorption step, during which the thiol head-groups first make contact with the gold surface and the molecules adopt a laying down conformation, and (2) a slower assembly step, during which the alkyl chains reorganize from the disordered state, transforming into close packed crystalline domains *via* chain-chain interactions (van der Waals, or dipole-dipole interaction). According to Nuzzo *et al.*, the rate of formation of a self-assembled monolayer is influenced by many factors, such as temperature, solvent, concentration and chain length of the adsorbate, and cleanliness of the substrate, and others. By using ellipsometry and contact angle measurements, the kinetics of thiolate (different chain lengths, tail groups, and concentrations) adsorption was investigated. On a typical flat Au(111) surface exposed to a 1.0 mM alkanethiol solution, the first chemisorption step may be over within minutes, and the second step may takes hundreds of minutes.⁹⁸ A similar conclusion was reached by Blanchard *et al.* in 1994, using the quartz crystal microbalance (QCM) technique to measure the adsorption kinetics of SAMs of octadecanethiol in *n*-hexane onto the surface of gold-coated quartz crystal oscillators.⁹⁹ Therefore, it is reasonable to assume that SAM formation onto np-Au may be following the same pathway as mentioned above, but with a longer incubation time required to attain the limiting thiolate packing density. The structures of SAMs on curved surfaces are just recently beginning to be investigated,^{87,88} and differences with the scenario on flat Au(111) are likely depending on the extent of surface curvature. The assembly kinetics of SAMs onto curved surfaces does not yet appear to have been studied.

For the case of static assembly onto np-Au, the diffusion of alkanethiols from solution to the outer surface of the np-Au monolith should be governed by normal linear diffusion, for which $\langle d^2 \rangle \propto 6Dt$ in three dimensions where $\langle d^2 \rangle$ is the mean-square displacement, D is the diffusion coefficient, and t is time.¹⁰⁰ A value for the diffusion coefficient of an alkanethiol in ethanol does not appear to have been reported; however, based on values reported for a range of other low molar mass organic solutes in ethanol, a value of order $10^{-5} \text{ cm}^2 \text{ s}^{-1}$ is expected.¹⁰¹ Diffusion of alkanethiol within the porous network of the np-Au monolith is most likely subject to sub-diffusion, or anomalous diffusion, for which the mean-square displacement, $\langle d^2 \rangle \propto Dt^a$, with $a < 1$.¹⁰² Studies of anomalous diffusion within np-Au have not been reported; however, it is likely that a is not very much less than 1.0, either for alkanethiol or for the proteins used later in this study. A study using fluorescence correlation spectroscopy in agarose gels of mean pore diameter $77 \pm 11 \text{ nm}$ reported a values for diffusion of particles covering a wide range of hydrodynamic radii, from rhodamine 6G dye to proteins such as ovalbumin and to latex beads up to 66 nm in radius; across this wide range of hydrodynamic radii, the value of a varied from 0.96–0.74.¹⁰³ Thus, the penetration of the alkanethiol into the np-Au will occur more slowly than expected due to its need to move through the torturous network of internal pores. The molecules may behave as if they are exhibiting a range of diffusion coefficients within the porous structure, as was reported in single molecule tracking studies of a strongly fluorescent dye followed using confocal laser fluorescence microscopy in porous silica monoliths.¹⁰⁴ If there are any dead-ends or regions of smaller pore dimension closer to a few multiples of the molecular size, molecules

may behave as if they are temporarily trapped. Given that alkanethiol is strongly binding onto the Au surfaces, during earlier times, concentration gradients will develop both within the np-Au monolith and *versus* the external solution. Using $D = 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, a molecule would on average diffuse the full thickness of the np-Au monolith (0.25 cm) within about 17 minutes. However, the magnitude of the effect of anomalous diffusion is not known and the actual root-mean-squared displacement within the np-Au could be much slower. For example, if $\alpha = 0.90$, then the time of 17 minutes is increased to 38 minutes and if $\alpha = 0.70$ it is increased to 341 minutes. However, the potential effects of anomalous diffusion do not appear to be significant enough to result in notably slower SAM formation under static incubation conditions used in the present experiments.

For the case of assembly under flow, it should also be kept in mind that the flow of solution through porous media is not necessarily uniform, and the fluid velocity profile can be quite variable and can even possess some stagnant regions within the structure;¹⁰⁵ however, the flow through the pores will reduce concentration gradients and limit the accumulation of weakly bound non-specifically adsorbed molecules. The TGA data at the earliest time point indicates that static assembly results in a transient buildup of non-specifically adsorbed molecules and weakly bound molecules that are swept away during assembly under flow. The ultimate SAM coverage within the np-Au by either method is similar, and given the similarity in final surface coverage, it should be expected, in the absence of any specific structural data to the contrary, that the ultimate SAM structure within np-Au is also similar regardless of the method of assembly for a single-component system.

Lectin-carbohydrate binding studies

Characterization of Concanavalin A adsorption onto α Man-C₈-SH SAMs on np-Au using atomic force microscopy—The interaction of lectins with α Man-C₈-SH SAMs was examined using atomic force microscopy (AFM) in order to verify the occurrence of Con A binding to mannose on the np-Au surface. Fig. 2 shows the AFM images of np-Au functionalized with α Man-C₈-SH before and after Con A exposure. To assist visualization of the immobilized proteins on the np-Au surface, both amplitude and height (topography) images are presented. The top panels (from Fig. 2A to C) represent the amplitude images; the lower panels (Fig. 2D and E) represent the topographic images of the same areas as in the top panels. The np-Au topographic features, over a scan range of $1.0 \mu\text{m} \times 1.0 \mu\text{m}$, after functionalization with α Man-C₈-SH were captured using AFM as shown in Fig. 2A and D. The small size of the mannose ligands does not affect the observed topography, and similar observations using different molecules was also previously reported by our lab.²² The corrugation of the np-Au surface appears to present rounded and interconnected ligament features with a typical lateral size ranging from 35 to 100 nm for the rounded features and the length of the ligament segments can reach 200 nm, based on the apparent dimension obtained from AFM measurement.

For Con A adsorption on np-Au, we exposed the α Man-C₈-SH functionalized np-Au to 1.0 mg mL^{-1} Con A (2.0 mL protein solution in PBS (pH 7.4) supplemented with 1.0 mM calcium chloride and 0.5 mM manganese chloride) using the static incubation for a period of 3.5 hours. The sample was thoroughly rinsed with Milli-Q water, before images were

acquired using AFM. Fig. 2B and E, of scan size $1.0\ \mu\text{m} \times 1.0\ \mu\text{m}$, represent np-Au after exposure to Con A, the topographic images appear to have many small irregularly shaped features with an average dimension of $21.0 \pm 6.2\ \text{nm}$ (x -axis) and $4.2 \pm 1.8\ \text{nm}$ (y -axis), observed on the surface of the np-Au. A higher resolution topograph, of scan size $500\ \text{nm} \times 500\ \text{nm}$, is shown in Fig. 2C and F. These features are smaller than those of the np-Au ligaments of 35–100 nm and thus can generally be distinguished as topographic features. The smaller features can be seen in the line scans presented beneath panels E and F in which Con A is present on the surface. The smooth appearance of the np-Au is no longer apparent; the pores and interconnected ligament features are not as clearly prominent when compared to the np-Au not exposed to protein.

Binding of Concanavalin A to protein resistant, hydrophobic and mannose presenting self-assembled monolayers of thiols on nanoporous gold—

The structural stability of np-Au, and its high electrical and thermal conductivity, makes it a promising substrate for development of sensors, and as a support for enzyme immobilization for applications in bio-catalysis.^{22,33,34,58,106,107} The stability of np-Au monoliths to solution flow through the pores makes the material potentially attractive for applications in separations of biomacromolecules. To further examine the potential of np-Au as a platform for use in glycoscience, we applied TGA to assess the binding of Con A, a lectin with high specificity for α -D-mannosyl residues, to np-Au monoliths modified with both pure and mixed SAMs of α Man-C₈-SH. The binding of Con A was first studied to unmodified np-Au, to np-Au modified with octanethiol to represent a hydrophobic surface, and to np-Au modified with 8-mercapto-3,6-dioxaoctanol to represent a surface that should resist protein adsorption. These measurements serve as controls for comparison with the subsequent results for Con A binding to pure and mixed SAMs containing α Man-C₈-SH. Con A is a tetrameric lectin that cooperatively binds mannose or glucose containing carbohydrate structures.^{108,109} The binding of Con A to the unmodified np-Au was studied under flow-conditions, and it was estimated by TGA that 0.3200 mg of Con A was adsorbed onto the surface of bare np-Au from a solution of concentration $1.0\ \text{mg mL}^{-1}$ after 3.5 hours at $1.0\ \text{mL min}^{-1}$. The diffusion coefficient for Con A at pH 7.0 has been reported as $D_{25,w} = 5.7 \times 10^{-7}\ \text{cm}^2\ \text{s}^{-1}$, at 298 K and corrected for the viscosity of water.¹¹⁰ The static diffusion of a protein within np-Au is likely anomalous, and also subject to possible blockage or trapping in the smaller pores. Results obtained for the proteins IgG and BSA with np-Au monoliths suggested that optimal coverage can best be obtained using flow-through conditions.²² Experiments were conducted to investigate the adsorption of Con A to 8-mercapto-3,6-dioxaoctanol (HO-PEG₂-SH), octanethiol (C₈-SH), and α Man-C₈-SH functionalized np-Au monoliths. The np-Au monoliths modified with these thiolates were rinsed with ethanol and Milli-Q water follow by PBS solution, for 30 minutes per solution at a flow rate of $1.0\ \text{mL min}^{-1}$, prior to the introduction of the Con A solution. The Con A solution of concentration $9.6\ \mu\text{M}$ ($1.0\ \text{mg mL}^{-1}$) was prepared in phosphate buffer (pH 7.4, 10.0 mM) supplemented with 1.0 mM calcium chloride and 0.5 mM manganese chloride. To ensure adequate time for Con A to interact with the immobilized mannose on the surface of np-Au, the protein solution was allowed to pass through the np-Au for 3.5 hours at rate of $1.0\ \text{mL min}^{-1}$, followed by PBS and Milli-Q water for 30 minutes each at the same flow rate. Fig. 3A shows the control experiment using HO-PEG₂-SH (molar mass $165.16\ \text{g mol}^{-1}$), before and

after Con A exposure. After the 6 hour flow-through period for surface functionalization, approximately 0.5369 mg (equivalent to 3.25×10^{-6} mol or coverage of *ca.* 4.53×10^{14} molecules cm^{-2}) of thiolated HO-PEG₂-SH was present on the np-Au monolith. The PEG thiolate was reported to occupies 21.4 Å² on Au(111), corresponding to a surface coverage of 4.67×10^{14} molecules cm^{-2} .¹¹¹ Based on the TGA analysis over the range of 20 to 800 °C, using a dynamic temperature ramp of 5 °C min⁻¹, either a negligible or minute amount of Con A was subsequently adsorbed onto the HO-PEG₂-SH functionalized np-Au. Such a result is reasonable, based on the known strategy for rendering gold surfaces protein resistant that involves incorporation of terminal poly(ethylene glycol) groups in SAMs.¹¹¹ The result is promising, since despite the presentation of regions of highly irregular curvature in np-Au monoliths, modification with this short-chain PEG molecule results in a large porous structure that resists non-specific protein adsorption. Thus, the specific adsorption of Con A onto mixed SAMs on np-Au containing αMan-C₈-SH and the PEG species may be investigated with greater confidence.

Adsorption of Con A onto a np-Au monolith modified with a SAM of octanethiol was then investigated. Fig. 3B shows the TGA traces of a np-Au monolith functionalized with C₈-SH, before and after Con A adsorption. The trace for the monolith modified with C₈-SH gives a mass loss of 0.2712 mg. Using the estimated surface area of np-Au of 4.91 m² g⁻¹, a surface coverage of 2.60×10^{14} molecule cm^{-2} is obtained, representing partial surface coverage of about 56% (based on a single 8.0 mm × 8.0 mm × 0.25 mm C₈-SH functionalized nanoporous gold with mass of 88.0 mg before TGA analysis). The trace for the monolith after exposure to flow-through of the Con A solution, shows that there is a mass loss attributed to adsorbed Con A of 0.2269 mg. Thus, the hydrophobically modified np-Au monolith is seen to retain a significant amount of non-specifically adsorbed Con A. Using a molar mass of 104 kDa, the mass loss corresponds to a surface coverage of adsorbed Con A molecules of 3.04×10^{11} molecules cm^{-2} .

The TGA trace for a np-Au monolith modified with αMan-C₈-SH is shown in Fig. 3C. The mass loss due to αMan-C₈-SH (MW = 324.167 g mol⁻¹) from the np-Au surface is 0.7070 mg or 2.181 μmol (green line). This corresponds to a surface coverage of 3.04×10^{14} molecules cm^{-2} . The mass losses for a monolith after exposure of the mannoside decorated np-Au with Con A are determined to be 0.9325 and 0.9173 mg, using Con A solutions of concentration 0.75 mg mL⁻¹ and 1.00 mg mL⁻¹, respectively. The difference of approximately 0.2100 mg (equivalent to 1.84 nmol, based on the molar mass of 104 kDa for Con A) can be presumed to be the total mass of Con A adsorbed on the αMan-C₈-SH modified np-Au.

The dimensions of tetrameric Con A (6.3 nm × 8.7 nm × 8.9 nm, with a molar mass of 104 kDa) can be obtained based on the protein structure acquired from the Protein Data Bank (PDB ID: 3CNA). In theory, the maximum number of Con A molecules that may be adsorbed onto a typical flat gold substrate surface can be estimated. Assuming that Con A adopts a laying down orientation occupying a 8.7 nm × 8.9 nm surface area (the largest possible footprint on a surface), using a completely efficient packing of Con A molecules side-by-side, the maximum capacity of Con A on a typical 8.0 mm × 8.0 mm flat gold surface is 8.27×10^{11} molecules (equivalent to 1.43×10^{-4} mg). A maximum possible

amount of Con A loading onto a np-Au monolith of the same macroscopic dimensions as used in these studies can be calculated by using the total surface area of np-Au estimated *via* BET analysis of *ca.* $4.91 \text{ m}^2 \text{ g}^{-1}$ and assuming monolayer adsorption with the footprint estimated above. The maximum capacity for Con A on the np-Au monolith ($8.0 \text{ mm} \times 8.0 \text{ mm} \times 0.25 \text{ mm}$) is thus estimated to be 6.33×10^{16} molecules. If the Con A molecules arranged themselves into an ideal monolayer, 1.0 g of np-Au could load *ca.* 10.92 mg of Con A. Taking account of the mass of the np-Au plate used for TGA analysis, $\sim 88.0 \text{ mg}$, the capacity of Con A on a single np-Au monolith is *ca.* 0.96 mg. This value is larger than the experimental values of $\sim 0.2269 \text{ mg}$ in Fig. 3B ($\text{C}_8\text{-SH}$ -modified np-Au), and $\sim 0.2255 \text{ mg}$ in Fig. 3C ($\alpha\text{Man-C}_8\text{-SH}$ modified np-Au). The estimated maximum assumes that there are no blockage effects, no protein multilayer formation, and that the entire np-Au surface is modified and suitable for Con A binding. In the case of pure mannose surface exposed to Con A, when compared to the flat gold surface of equivalent geometric area ($8.0 \text{ mm} \times 8.0 \text{ mm} \times 0.25 \text{ mm}$ with 2.69×10^{12} of Con A molecules possible), the adsorbed amount of Con A on np-Au increased by 486 times. A comparison of various protein concentration and time dependent studies was carried out to determine the optimum Con A concentration and time suitable for our investigation. Con A concentrations greater than 1.25 mg mL^{-1} were observed to result in some visible precipitation of protein on the surface of np-Au; therefore, the concentration dependence was studied for Con A below 1.25 mg mL^{-1} . The surface of np-Au exposed to higher Con A concentrations typically presents traces of a fine white precipitate, which is hard to wash away even with 1% Tween 20 solution. Fig. 3C shows TGA results for two protein concentrations that were used for initially studying lectin-carbohydrate interactions (0.75 mg mL^{-1} and 1.0 mg mL^{-1}); however, we are actively pursuing protein incubation time and concentration dependent studies on single component and mixed SAMs.

Interactions of Concanavalin A with mixed SAM modified np-Au monoliths containing 8-mercaptooctyl α -D-mannopyranoside—Having demonstrated that the flow system can be conveniently used to modify np-Au monoliths with SAMs, including those of $\alpha\text{Man-C}_8\text{-SH}$ and $\text{HO-PEG}_2\text{-SH}$, and for estimating Con A adsorption, we then proceeded to modify the monoliths with mixed solutions of these two compounds. The np-Au monoliths was modified with a solution of $\alpha\text{Man-C}_8\text{-SH}$ + $\text{HO-PEG}_2\text{-SH}$ of 0.20 mole fraction $\alpha\text{Man-C}_8\text{-SH}$ and total concentration 5.0 mM. Con A was introduced as described in the prior procedure and then its adsorption was estimated using TGA. The np-Au with adsorbed Con A was allowed to air dry for 24 hours prior to TGA analysis. Fig. 4A is a TGA trace showing the mass loss *versus* temperature of the np-Au monolith modified with $\alpha\text{Man-C}_8\text{-SH}$: $\text{HO-PEG}_2\text{-SH}$ (1 mM $\alpha\text{Man-C}_8\text{-SH}$, 4 mM $\text{HO-PEG}_2\text{-SH}$) over the range 10°C to 800°C at a temperature scan rate of 5°C min^{-1} . The trace is shown for the mixed SAM modified monolith and for a monolith from the same batch that was exposed to a 1.0 mg mL^{-1} solution of Con A (3.5 hours, 1.0 mL min^{-1}). From the TGA analysis, the mass loss of the mixed SAM from the np-Au surface is 0.5624 mg (red line). Exposure to Con A gives a sample that undergoes an additional mass loss of 0.1210 mg attributed to adsorbed Con A (green line). The difference of approximately 0.1210 mg (equivalent to 1.056 nmol, based on the molar mass of 104 kDa for Con A) can be presumed to be the total mass of Con A adsorbed on the mixed SAM modified np-Au. The amount of Con A adsorbed is smaller

than that on bare np-Au (0.2816 mg) or on the np-Au modified with the thiolated α -mannoside alone (0.2255 mg) treated under these same conditions. This can be attributed to the smaller mole fraction of mannose and the greater spacing between mannose units that can reduce the amount of cooperative binding of Con A to the surface. Similarly, a np-Au monolith modified with the same α Man-C₈-SH + HO-PEG₂-SH mixed SAM using the flow through approach was subsequently exposed to a 1.0 mg mL⁻¹ Con A solution under static conditions. Under the static incubation conditions, only 0.04 mg of Con A was bound to the modified np-Au, an observation we attribute to the slower penetration of Con A into the porous gold environment in the absence of flow (Fig. S5, see ESI[†]). A three-fold increase in Con A bound to the np-Au monolith was observed when Con A solution was introduced using flow at 1.0 mL min⁻¹, as shown in Fig. 4. It should be noted that the mole fraction of mannose on the surface is likely different from that in the solution from which the mixed SAM is formed, as is known from other studies. Methods such as X-ray photo-electron spectroscopy (XPS) can be applied to estimate relative surface coverages of the two components,¹¹² by examining the ratio of the C/O signals, for example. In the case of carbohydrate containing SAMs, the unique signal of the acetal functionality from the high-resolution carbon peak can be used to more clearly identify the mole fraction of the carbohydrate component.¹¹² In this study of mixed SAMs of a trimannoside alkanethiol and diluting PEG alkanethiol derivatives, the fraction of trimannoside was 20–30% less on the surface than in the solution from which the SAMs were formed. It is thus likely in our present study that the fraction of α Man-C₈-SH is also lower on the surface than in solution.

The interaction of Con A with np-Au monoliths modified with mixed SAMs of α Man-C₈-SH and C₈-SH was also investigated. The presence of the C₈-SH as a hydrophobic diluent is expected to attract additional Con A due to non-specific adsorption. Octanethiol was chosen simply because the spacer group of the α Man-C₈-SH was synthesized with an eight carbon alkyl chain, and thus the use of octanethiol will not obstruct the mannose moiety and will still allow the mannose to be presented above the C₈-SH SAMs and available to interact with Con A. The preparation of the mixed SAMs containing C₈-SH and thiolated HO-PEG₂-SH on np-Au was carried out using the same flow methods noted above. Since Con A can be adsorbed onto the surface in this case either by specific adsorption to the mannose units, or by non-specific adsorption on the C₈-SH regions, a higher Con A loading is expected. Fig. 4B shows the TGA trace of α Man-C₈-SH and thiolated HO-PEG₂-SH mixed SAMs (prepared from solution of concentration 1 mM α Man-C₈-SH and 4 mM HO-PEG₂-SH) on np-Au monoliths, before and after Con A exposure. A total mass loss of 0.6184 mg is attributed to the mixed SAM of α Man-C₈-SH and HO-PEG₂-SH. An additional mass loss of 0.4254 mg is seen after exposure to flowing Con A solution and is attributed to adsorbed Con A. A time dependent study of Con A adsorption after 3.5 and 24 hour periods, suggested that an exposure time of 3.5 hours is appropriate to achieve the limiting amount of protein binding in our investigation using the flow conditions. The total amount of Con A adsorbed was 0.4254 mg (black line) and 0.4196 mg (blue line), at 3.5 and 24 hour time points, respectively. An increase of 0.3044 mg of adsorbed Con A (equivalent to 1.93×10^{15} molecules or 2.909 nmol of Con A) was observed between the mixed SAM of α Man-C₈-SH + C₈-SH and α Man-C₈-SH + HO-PEG₂-SH. Thus, the total amount of Con A adsorbed is ~3.5 times higher on the np-Au that was modified with the mixed SAM containing α Man-

C₈-SH and the C₈-SH diluent. The additional amount of adsorbed Con A is consistent with additional non-specific adsorption onto the hydrophobic C₈-SH regions in addition to binding to the presented mannose units. Many other variables with respect to np-Au structure, SAM structure or composition, formation time, protein concentration, and exposure time to the lectin solution, as well as comparisons of static *versus* flow conditions remain to be explored and we are actively investigating the influence of these parameters on protein coverage inside np-Au.

Variation of Con A binding with SAM composition within np-Au monoliths—In order to study the adsorption behavior of Con A on surfaces composed of mixed SAMs of α Man-C₈-SH and HO-PEG₂-SH, various molar ratios of these two alkanethiolates were prepared. Fig. 5A represents the mass losses determined from TGA for Con A bound on np-Au modified with mixed SAMs of HO-PEG₂-SH + α Man-C₈-SH prepared from solutions (5 mM total concentration in each case) with mole fraction of α Man-C₈-SH equal to 0.05, 0.10, 0.20, 0.33, 0.50, 0.66, and 0.75, respectively. Amounts of Con A from 0.24–0.28 mg adsorbed onto the surfaces of np-Au were observed when the mole fraction of α Man-C₈-SH was more than 0.20. An increased amount of Con A (0.39 mg) was observed bound to mixed SAMs at the 0.10 α Man-C₈-SH solution mole fraction. The lesser extent of Con A bound onto the np-Au surface modified with the mixed SAMs of 0.05 α Man-C₈-SH solution mole fraction is likely due to the decreasing amount of mannoside and increasing amount of protein resistant HO-PEG₂-SH on the surface of np-Au. The data suggest that lowering the fraction of mannose in the SAM reduces the steric crowding in the range near 0.10 solution mole fraction of α Man-C₈-SH and provides average spacing between mannose units on the surface that is more conducive to Con A binding, but then as the mannose fraction is reduced further binding diminishes. The variation of the binding response of lectins to composition of carbohydrate presenting SAMs has been studied in some cases, and the variation with SAM composition is quite sensitive to molecular structure in ways that are not fully understood.^{77,79,112} It is thought that for SAMs where the carbohydrate units are too closely arranged or have too high of a surface density, steric hindrance results in less than optimal or even quite limited binding of lectin. It has long been known that interactions of lectins with carbohydrate SAMs involve polyvalency.^{73,75} For example, in studies of the binding of galectins (Gal-3, Gal-4, and Gal-8) to lactose presenting mixed SAMs,¹¹³ a significantly enhanced binding was observed for 4% lactose (for binding of Gal-3 or Gal-4) or 2.7% lactose (for binding of Gal-8) in the mixed SAM with a diluting triethylene glycol terminated short alkanethiol. In this study, it was proposed that the SAMs with high fraction of lactose were too sterically crowded for good lectin binding, and that there was an optimal low fraction of lactose at which the average spacing between lactose units was close to that between carbohydrate recognition domains in the galectins. Clustering of carbohydrates can also occur in such SAMs,¹¹⁴ and this should also affect the variation in binding response with composition. The results of Fig. 5A suggest that Con A binding is enhanced to the np-Au modified from the solution with 0.10 mole fraction of α Man-C₈-SH, possibly due to achieving a surface density of mannose on the np-Au surface that is more conducive to polyvalent interactions and enhanced lectin binding. As the mannose fraction is reduced further (mole fraction 0.05), binding is diminished. On the surfaces bearing the higher fractions of α Man-C₈-SH (solution mole fraction 0.20 and greater), the amount of Con A

bound varies little suggesting that there may be a tradeoff between steric hindrance to binding and the presence of more mannose units on the Au surface.

Fig. 5B represents the time dependence of Con A binding onto np-Au modified with the HO-PEG₂-SH + α Man-C₈-SH mixed SAM of 0.10 solution mole fraction α Man-C₈-SH. Five different time points (1, 3, 6, 15 and 24 h) were investigated to determine the loading of Con A onto the np-Au monolith. As expected, an increase in the amount of Con A was observed at the 1, 3 and 6 hour time points. At longer protein exposure times, 15 and 24 hours, the amount of Con A was found to be 0.59 and 0.66 mg, respectively. The increase in Con A binding to the modified np-Au can be attributed to increasing exposure of Con A protein binding sites to the immobilized α Man-C₈-SH molecules and ultimately saturation appears to be reached at longer times near 24 hours or greater.

Lectin and protein selectivity of the mannoside modified np-Au monoliths—

The binding of Con A and IgG (rabbit polyclonal) by np-Au monoliths modified by HO-PEG₂-SH + α Man-C₈-SH mixed SAMs of 0.10 α Man-C₈-SH solution mole fraction was compared (Fig. 5C). The protein solutions, 1.0 mg mL⁻¹, in PBS buffer (pH 7.4, 10 mM) were flowed through the monoliths for a period of 3.5 hours and then removed for TGA analysis. The greatest mass loss was found for Con A (0.39 mg) bound into the monolith, as expected given the preference of mannose for binding to Con A. The binding of IgG under these conditions was not negligible, but was less than 10% of the amount of Con A bound. No specific interaction is expected between IgG and mannose, except for some possible weak carbohydrate-carbohydrate interaction. Non-specific adsorption or some trapping of IgG into the np-Au monoliths likely occurs onto some bare Au regions. A similar experiment was repeated using peanut agglutinin (PNA), which is also not known to form specific interactions with the α -D-mannosyl moiety. A mass loss of 0.1 mg of PNA non-specifically adsorbed onto the surface of modified np-Au was observed, as seen in Fig. 5C. A higher extent of possible weak carbohydrate-carbohydrate interaction may be a plausible reason for the increased amount of PNA bound to the surface of np-Au when compared to IgG.

When exposing the mixed SAMs to a 1.0 mg mL⁻¹ Con A solution, a mass loss of 0.39 mg was observed after the TGA analysis. Since the PEG component was previously demonstrated to resist Con A adsorption (see Fig. 3A), the mass loss observed in this study should be solely attributed to Con A bound to the α -D-mannosyl moiety in the mixed SAMs. To further investigate the specificity of Con A toward the mannoside moiety, the 0.10 α Man-C₈-SH solution mole fraction mixed SAM sample was exposed to a protein solution of 0.5 mg mL⁻¹ of PNA and 0.5 mg mL⁻¹ Con A. A mass loss of 0.29 mg was observed. The smaller mass loss of protein observed in this case is reasonable, because the initial concentration of Con A is one half the amount used in the single component Con A solution study mentioned earlier. Even with the presence of a non-specifically binding protein, the 0.29 mg of protein observed demonstrates the selectivity of Con A towards the thiolated α -mannosides. Furthermore, the protein loaded in this study should dominantly be Con A because the concentration of the PNA used in this mixed proteins solution is 50% of that used for single protein (1.0 mg mL⁻¹ PNA) component study; therefore, non-specific adsorption of PNA onto the mixed SAMs should be in a smaller fraction. These results

indicate that the selectivity of the mannose–lectin interaction is preserved in these flow experiments and this is promising for future applications of carbohydrate modified np-Au monoliths for screening of carbohydrate-binding proteins.

Elution of Con A from np-Au monoliths by methyl α -D-mannopyranoside—The above results demonstrate the capacity of np-Au for SAM modification and lectin binding. As noted in studies on other materials, immobilized lectins may be useful for glycoprotein, polysaccharide and glyco-peptide purification with intact immunoglobulins.^{115,116} Thus, we were interested in whether the captured Con A on the gold surface of np-Au can be released. As with other chromatography techniques, many approaches can be used to elute the bound lectin from the np-Au. For example, the bound Con A on the surface of np-Au can be released by using an elution buffer containing methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, or sodium dodecyl sulfate.^{115–117} Further, specific separation of the dimeric Con A from its native tetrameric form is also possible by using an elution buffer of a different pH. At neutral and alkaline pH, Con A is known to exist as a tetramer (104 kDa) of four identical subunits; below pH 5.6, Con A dissociates into active dimers of mass 52 kDa.^{118–120} Given that Con A binds to α Man-C₈-SH with weak affinity ($K_a = 0.82 \times 10^4$ M⁻¹), we elected to elute the immobilized Con A using commercially available methyl α -D-mannopyranoside in phosphate buffer (pH 7.4, 10.0 mM) supplemented with 1.0 mM calcium chloride and 0.5 mM manganese chloride.

Fig. 6 shows the TGA analysis of the Con A bound to the surface of np-Au, pre-formed either with mixed SAMs of α Man-C₈-SH + HO-PEG₂-SH (5.0 mM, 0.20 α Man-C₈-SH solution mole fraction) or α Man-C₈-SH + C₈-SH (5.0 mM, 0.20 α Man-C₈-SH solution mole fraction), and the elution study using methyl α -D-mannopyranoside. In the elution study, the elution buffer solution was eluted at 1.0 mL min⁻¹ for 3 hours and the elution buffer was passed through to a waste container, rather than recycling through the monoliths. To minimize retention of residual buffer solution in the monoliths, Milli-Q water was introduced to wash the monoliths at 1.0 mL min⁻¹ for another 30 min. Based on the TGA measurement, after the bound Con A was washed with the methyl α -D-mannopyranoside elution buffer, a total of 0.1216 mg (at 400 °C) of Con A was released from the monoliths modified by mixed SAMs of α Man-C₈-SH + HO-PEG₂-SH. The green line shown in Fig. 6A, indicates that the elution buffer successfully reversed the binding of Con A, and had minimum to no effect on the mixed SAMs. Fig. 6C shows a bar chart at various chosen upper temperatures in the TGA analysis to indicate that the mass loss of Con A was similar at the three temperatures shown, and thus it is reasonable to use 400 °C as the temperature at which to determine the mass changes arising from the decomposition of thiolate molecules or the lectin Con A. Similar observations were observed for the elution study for the bound Con A on the mixed SAMs of α Man-C₈-SH + C₈-SH. The TGA analysis shows a loss of 0.4414 mg of Con A, and minimum perturbation of the mixed SAMs during the elution procedure. The bar charts in Fig. 6D, also show a similar trend as in Fig. 6C. It is noteworthy to point out that the amount of Con A (either on α Man-C₈-SH + HO-PEG₂-SH or α Man-C₈-SH + C₈-SH) lost from the elution study is comparable to the amount of bound Con A removed in Fig. 5, indicating robustness of our experimental approaches and

confirming high reproducibility, and authenticity of the generated data. The results for the mixed SAMs and Con A binding from Fig. 5 were overlaid in Fig. 6 for comparison.

The elution study confirms the effectiveness of methyl α -D-mannopyranoside for removal of the bound Con A from the mannoside modified np-Au surfaces. This result was expected, as the single mannoside alkanethiol derivative possesses weak affinity toward lectin; therefore, for stronger binding of lectin Con A onto the gold surface of np-Au, multivalent dimannoside and trimannoside moieties are necessary in order to increase the binding strength of Con A on surface. In 1993, Brewer and coworkers presented a series of branched chain oligomannosides, and based on their study using titration microcalorimetry analysis, a branched chain trisaccharide moiety possesses an ~60 fold increase in affinity compared to methyl α -D-mannopyranoside.¹²¹ We are actively pursuing this area of work using thiolated biantennary complex type oligosaccharide or branch chain disaccharide, and trisaccharide moiety of mannose on the gold surface of np-Au to immobilize Con A. The results and related works should be reported in the future.

Conclusion

Monoliths of SAM-modified np-Au have been demonstrated to exhibit the key features needed for their further development and application in glycotecchnology and related fields. Functionalization, resistance to non-specific adsorption, stability under flow conditions, selectivity in protein binding, and successful elution of protein have been demonstrated. The present study uses the establish mannose – Concanavalin A system, but can be readily expanded to other carbohydrate –protein interactions. We have recently verified specific binding of soybean agglutinin to a globotriose derivative on np-Au.¹²² Alkanethiols presenting mannose can be functionalized with good coverage onto the surface of np-Au monoliths. Using static or flow-through conditions, SAMs containing α Man-C₈-SH and either octanethiol or HO-PEG₂-SH were formed on the surfaces of np-Au monoliths and the immobilized α Man-C₈-SH derivative remained effective in binding Concanavalin A. The short HO-PEG₂-SH molecule on np-Au was found effective at making the gold surfaces protein resistant. Because of the inertness of np-Au and its high melting point, TGA was successfully applied to evaluate the loading of adsorbed thiulates on np-Au, just as it has previously been applied to evaluate loading on Au nano-particles.²⁶ TGA was successfully applied to estimate the amount of Con A bound onto the surfaces of modified np-Au monoliths. The surface coverage of the α Man-C₈-SH or C₁₈-SH on np-Au monoliths, regardless of whether static or flow-through methods are used, is shown to be time dependent but to reach similar values after a sufficient length of time. The coverages reached suggest thorough functionalization of the np-Au structure. The surface coverage estimated by TGA is close to the limit expected for a well-organized SAM of C₁₈-SH on an equivalent area of flat Au (111). This result clearly indicates the potential for modifying np-Au monoliths with alkanethiols.

The binding of Con A on np-Au was reversed by using a methyl α -D-mannopyranoside elution buffer solution. Such reversibility is essential for affinity based applications that could involve protein capture and release. In the present study, the total amount of Con A dissociated from the np-Au was not directly determined, rather the difference of the Con A

mass loss from the np-Au from one experiment and that from the remaining mixed SAMs after the elution study from another experiment were used to estimate the mass of dissociated Con A. For the case of elution studies, it would clearly be of interest to follow protein concentration in solution in real-time throughout the experiment using, for example, a UV-visible absorbance detector at 280 nm. Integrated approaches, such as coupling TGA with mass spectrometry and/or gas chromatography to identify the decomposed fragments would clearly be of interest. The collected data from such an integrated approach could provide insight into thermodynamic and mechanistic aspects of the thermal decomposition behavior of molecules bound within np-Au.

The portable nature of np-Au monoliths, their size and shape flexibility, and the fact that monoliths bearing different surface modifications can be arranged in sequences within flow systems, suggests the possibility of their use in modular systems for biomolecule separation, controlled release, or selective capture. Further investigation of the packing density, the degree of chain ordering, quantification of the surface compositions of mixed SAMs, and the molecular orientation and the presence of defects in SAMs on np-Au is required and will involve a range of spectroscopic techniques. Our observation that np-Au monoliths can be cleaved open means that such studies will not be restricted to probing only the external surface.²⁶ For much longer term applications involving flow, it will also be important to conduct further studies of SAM stability. Np-Au in the form of monoliths of almost any desired size/geometry and adaptable for integration with flow technologies, appears to be promising for development for applications involving lectin or glycoprotein capture, or for separation/identification of glycoproteins or glycoforms.

Experimental section

Preparation of nanoporous gold monoliths

Preparation of nanoporous gold plates (monoliths) was performed according to literature procedures, with a few modifications.^{22,33,58} In brief, 10 carat yellow gold sheets (4.0 inch $\times$ 2.0 inch $\times$ 0.0098 inch, L $\times$ W $\times$ H, respectively) were purchased from Hoover and Strong (Richmond, Virginia, USA). The stated atomic composition of this commercial alloy is 41.7% Au, 20.3% Ag, and 38% Cu. Depend on the experiment protocols, the 10 carat sheet was cut into pieces of size of 8.0 mm $\times$ 8.0 mm $\times$ 0.25 mm, L $\times$ W $\times$ H, respectively, and placed in a concentrated nitric acid bath for 48 hours (the acid solution was refreshed at the 24 hour time point). Trace metal grade nitric acid was purchased from Fisher Scientific (Pittsburgh, Philadelphia, USA). After the nitric acid de-alloying treatment, samples were rinsed thoroughly with Milli-Q water (18.2 M Ω , Millipore Corporation, Boston, USA) to neutral pH, followed by rinsing with HPLC grade ethanol (Sigma Aldrich, St. Louis, Missouri, USA). The microstructure of np-Au was characterized using scanning electron microscopy using a JEOL JSM-6320F field emission SEM (JEOL USA, Inc., California, USA).

Solvents and compounds for self-assembled monolayer formation

1-Octadecanethiol (referred to as C₁₈-SH) with a purity of more than 98.0% was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). HPLC grade ethanol was also purchased

from Sigma-Aldrich (St. Louis, Missouri, USA). All chemicals and reagents were used as received. Modification of np-Au with SAMs of C₁₈-SH was accomplished by immersing the np-Au monolith in a solution of C₁₈-SH in ethanol (6 mM) for periods of 2, 4, 6, 8 and 24 hours, followed by rinsing and repeated immersion and removal from pure solvent. Modification of np-Au with SAMs of 8-mercaptooctyl α -D-mannopyranoside (α Man-C₈-SH) was performed with the same procedures and conditions. Modifications of np-Au samples with mixed SAMs of 0.20 solution mole fraction of the mannoside derivative (α Man-C₈-SH) and with either octanethiol or HO-PEG₂-SH were prepared in absolute ethanol. The np-Au surfaces were also modified with the desired thiolates using an enclosed flow cell system made of Teflon[®], a brief description is available in the ESI,[†] Fig. S1.

Synthesis of 8-mercaptooctyl α -D-mannopyranoside, and of 8-mercapto-3,6-dioxaoctanol

The syntheses of 8-mercaptooctyl α -D-mannopyranoside⁹⁷ and 8-mercapto-3,6-dioxaoctanol have been reported.¹²² The structures of these two species are shown below:

8-Mercaptooctyl α -D-mannopyranoside

8-mercapto-3,6-dioxaoctanol

Protein solutions

The lyophilized powder of Concanavalin A (Con A) from *Canavalia ensiformis* (Jack Bean), of greater than 95.0% purity was purchased from Sigma Aldrich (St. Louis, Missouri, USA) and used as received. The Con A protein solution, 1.0 mg mL⁻¹, was prepared in phosphate buffer (pH 7.4, 10.0 mM) supplemented with 1.0 mM calcium chloride and 0.5 mM manganese chloride. Peanut agglutinin (PNA) from *Arachis hypogaea* was also purchased from Sigma-Aldrich and solutions were prepared. Methyl α -D-mannopyranoside (99.0%) and rabbit polyclonal immunoglobulin G (IgG) were also purchased from Sigma-Aldrich.

To overcome the apparent slow penetration of protein into the interior of the np-Au monolith samples under static conditions, a homemade flow cell system was used in order to achieve protein binding to the interior of the np-Au monoliths.²² The protein solution was kept in an ice bath and passed through the flow cell at a flow rate of 1.0 mL min⁻¹ for the various times used as described below. Following washing with Milli-Q water (18.2 M Ω) by passage through the flow cell, thermogravimetric analysis (TGA) was performed. We anticipate that the flow cell system permits the protein solution to be forced to flow through the nanoporous structure and hence protein can then be bound more efficiently to mannose units on the surfaces throughout the interior of the np-Au sample. Comparison of the np-Au

structure by SEM before and after flow cell experiments revealed no evident effect of exposure to the flow conditions on the np-Au nanostructure (Fig. S4, see ESI[†]).

Flow-cell for surface modification of np-Au monoliths

The np-Au surfaces were also modified with the desired thio-lates, such as, C₁₈-SH or α Man-C₈-SH and/or HO-PEG₂-SH, using an enclosed flow cell system made of Teflon[®]. The homemade flow cell system was designed and used in conjunction with a peristaltic pump (Model 77390-00, Cole-Parmer Instrument Company, Illinois, USA) equipped with polytetra-fluoroethylene (PTFE) tubing. Fig. S1 (see ESI[†]) shows a schematic representation of the flow cell system. The flow cell system contains 8.0 mL of solution. The design allows a typical de-alloyed np-Au monolith, 8.0 mm $\times$ 8.0 mm $\times$ 0.25 mm, to be inserted into a sealed chamber, oriented perpendicular to the flow and closely fit around the edges. To minimize the chances of damaging or contaminating the np-Au plate, the setup allows *in situ* functionalization of np-Au with thiolated molecules as mentioned above (6.0 mM in ethanol, for 2, 4, 6, 8 and 24 hours). At the end of each flow experiment with the appropriate thiolated molecules, any residual thiolates were flushed away using ethanol at the same flow rate for 30 minutes.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) was performed using a Q500 Thermogravimetric Analyzer (TA Instruments, Delaware, USA). The mass of each np-Au sample with a typical dimension of 8 mm $\times$ 8 mm $\times$ 0.25 mm was approximately 0.08 to 0.1 g. Samples were first air dried for 24 hours. The carrier gas used was nitrogen at a flow rate of 40 mL min⁻¹. The np-Au samples were split in half using a clean razor blade so that they could fit in a platinum weighing boat and be heated from room temperature to 800 °C at ramping rate of 5 °C min⁻¹. Prior to initiating the temperature ramp, N₂ gas was allowed to flow through the sample for 5–10 minutes. After the end of this thermal ramp, there is evidence that the np-Au (at least in unmodified form) has undergone some extent of thermal annealing (Fig. S3, see ESI[†]).

For systematic comparison of the amount of material decomposed in TGA, the mass loss was measured between two plateaus in the thermograms at 20 °C and 400 °C (T_i and T_f regions, respectively), throughout our study. The interval of $T_i - T_f$ is arbitrarily set; T_i has no fundamental significance, but the invariance of the mass in the temperature range above T_i and up to at least 100 °C is a good indication that no water or any volatile solvent remains trapped in the porous environment that would contribute to the mass loss and give a false reading. However, the T_f region is set as a final temperature, at which the decomposition of thiolate molecules appears to be complete. In the previously reported applications of TGA to estimate coverage of tricyclohexylphosphine-stabilized gold nano-particles, the mass loss (~24.5%) was noted as occurring over the range of 120–300 °C and to be completed by 350 °C.⁸⁵

Atomic force microscopy imaging and analysis

The AFM imaging was performed in Tapping Mode[™] with a Multimode[™] AFM (Veeco, Santa Barbara, California, USA) operated with a Nanoscope IIIa[™] 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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Fig. 1.

Thermogravimetric analysis of adsorption and assembly of 8-mercaptooctyl α -D-mannopyranoside onto representative np-Au monoliths of dimensions 8.0 mm $\times$ 8.0 mm $\times$ 0.25 mm. (A) Under static immobilization conditions, and (B) under flow-through conditions (6 mM in ethanol, 1.0 mL min⁻¹) at 2 (red), 4 (pink), 6 (blue), 8 (green) and 24 hour (orange) time points. TGA analysis was carried out by ramping the temperature at 5 °C min⁻¹ from room temperature to 600 °C. (C) Coverage *versus* time for static and (D) flow-through conditions, showing the adsorption kinetics of 8-mercaptooctyl α -D-mannopyranoside, in terms of molecules cm⁻² vs. time, onto np-Au. See text for mass loss at each time point in mg.

AFM topographs of Concanavalin A immobilized on nanoporous gold functionalized with thiolated α -mannosides

Fig. 2.

Tapping mode-AFM topographs of the α Man-C₈-SH functionalized np-Au surface after adsorption of Concanavalin A. Top panels (A, B, and C) represent the amplitude images; lower panels represent the topographic images of the same areas as in top panels. The scan size is 1000 nm $\times$ 1000 nm for mannose passivated np-Au without exposure to Con A. AFM topographic images of mannose functionalized gold surface after exposure to 1.0 mg mL⁻¹ Con A (B and E, 1000 nm $\times$ 1000 nm scan size). Higher resolution AFM topographic images, 500 nm $\times$ 500 nm scan size, of the areas indicated by the boxes in C and F. Line profiles for the segments identified are shown in the panels underneath panels D, E, and F.

Fig. 3.

Thermogravimetric analysis of Con A immobilized on np-Au monoliths modified with SAMs of 8-mercapto-3,6-dioxaoctanol (A), octanethiol (B), or 8-mercaptooctyl α -D-mannopyranoside (C). (A) A very small amount of Con A (red curve) was adsorbed onto the HO-PEG₂-SH (black curve) modified np-Au monoliths. (B) Non-specific adsorption of Con A (green curve) was observed on the hydrophobic C₈-SH SAM surface (blue curve), (C) a higher amount of Con A (pink curve is for 0.75 mg mL⁻¹, blue curve is for 1.00 mg mL⁻¹ of Con A) adsorbed onto the α -mannoside modified np-Au monolith (green curve) as expected due to the specific interactions between the lectin and carbohydrate. Experiments were carried out under flow-through conditions, the Con A solution (1.0 mg mL⁻¹) was allowed to pass through the np-Au for 3.5 hours at rate of 1.0 mL min⁻¹.

Fig. 4.

Thermogravimetric analysis of Con A bound onto np-Au monolith modified with mixed SAMs of 8-mercapto-3,6-dioxaoctanol, octanethiol, and 8-mercaptooctyl α -D-mannopyranoside. (A) Specific interaction of Con A (green curve) with α Man-C₈-SH was observed for np-Au monoliths modified with mixed SAMs of α Man-C₈-SH and HO-PEG₂-SH (red curve). (B) A significant amount of Con A (black and blue curves) was non-specifically adsorbed onto np-Au modified with mixed SAMs of α Man-C₈-SH and octanethiol (orange curve).

Fig. 5.

Specific protein adsorption onto mixed SAMs of HO-PEG₂-SH + α Man-C₈-SH. (A) The amount of Con A bound onto mixed SAMs of HO-PEG₂-SH + α Man-C₈-SH SAMs of solution mole fraction of α Man-C₈-SH of 0.05, 0.10, 0.20, 0.33, 0.50, 0.66 and 0.75, respectively, was investigated. The highest protein adsorption was observed for the 0.10 solution mole fraction. (B) Time dependent study of Con A adsorption onto mixed SAM (HO-PEG₂-SH + α Man-C₈-SH, 0.10 solution mole fraction of α Man-C₈-SH, 2.5 mM, ethanol) on np-Au. A gradual increase of Con A loading and saturation at longer incubation time was observed. The total Con A adsorbed on the surface on modified np-Au is 0.59 mg

at 15 hours, and 0.66 mg at 24 hours. (C) Specific and non-specific interaction of IgG, PNA and Con A towards the HO-PEG₂-SH + α Man-C₈-SH mixed SAMs of 0.10 solution mole fraction α Man-C₈-SH. Blue bars represent the mass of the mixed SAMs before exposure to proteins. Red bars represent the additional mass of adsorbed protein.

Fig. 6.

Elution of bound Con A from np-Au monoliths. (A) Con A immobilized *via* specific interactions with the mannoside moieties in the mixed SAMs of α Man-C₈-SH and HO-PEG₂-SH, was washed with methyl α -D-mannopyranoside. According to the TGA mass loss, approximately 0.1216 mg (at 400 °C) of bound Con A was eluted out from the np-Au monolith (red line). (B) A similar strategy was used to elute Con A bound to the mixed SAMs of α Man-C₈-SH and C₈-SH. Based on TGA analysis, 0.4414 mg (at 400 °C) of Con A were eluted off the np-Au monoliths. (C) and (D) Show bar charts, reflecting the amount of mixed SAM formed on np-Au monoliths, amount of Con A loss after TGA analysis, and amount of Con A eluted off the np-Au, at the indicated temperature.