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Electrochemical Impedance Spectroscopy Study of Concanavalin A Binding to Self-Assembled Monolayers of Mannosides on Gold Wire Electrodes

Jay K. Bhattarai, Yih Horng Tan, Binod Pandey, Kohki Fujikawa, Alexei V. Demchenko, and Keith J. Stine

Department of Chemistry and Biochemistry, University of Missouri–St. Louis, Saint Louis, MO 63121, USA

Abstract

The interactions of the lectin Concanavalin A (Con A) with self-assembled monolayers (SAMs) of thiolated mono-, di-, and tri-mannosides were studied on the surface of gold wires using electrochemical impedance spectroscopy (EIS). The SAMs of mannosides were prepared either pure or along with thiolated triethylene glycol (TEG) at different molar ratios (1:1, 1:2, 1:4, 1:9, and 1:19) to better understand and optimize the interaction conditions. The charge-transfer resistance of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe was compared before and after the interaction at different concentrations of Con A to determine the equilibrium dissociation constant (K_d) and limit of detection (LOD). Values of K_d were found in the nanomolar range showing multivalent interactions between mannosides and Con A, and LOD was found ranging from 4–13 nM depending on the type of mannoside SAM used. Analysis using the Hill equation suggests negative cooperativity in the binding behavior. Peanut agglutinin was used as a negative control, and cyclic voltammetry was used to further support the experiments. We have found that neither the pure nor the widely dispersed monolayers of mannosides provide the conditions for optimal binding of Con A. The binding of Con A to these SAMs is sensitive to the molar ratio of the mannoside used to prepare the SAM and to the structure of the mannoside. A simple cleaning method has also been shown to regenerate the used gold wire electrodes. The results from these experiments contribute to the development of simple, cheap, selective, and sensitive EIS-based bioassays, especially for lectin–carbohydrate interactions.

Graphical abstract

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Keywords

Mannoside; Regenerated gold wire; Self-assembled monolayer; Concanavalin A; Electrochemical impedance spectroscopy

1. Introduction

Recent advances in analytical techniques have enabled scientists to continue exploring and probing a variety of carbohydrate–protein interactions, often in attempts to identify potential carbohydrate biomarkers [1–3]. It is well known that carbohydrate–lectin interactions, with typical dissociation constants in the micromolar range for monosaccharides, play key roles in a variety of important biological and physiological processes. These processes, amongst many, include cell-surface recognition, adhesion, cell–cell communications, cancer metastasis, and host–pathogen infection [4, 5].

The ubiquity of carbohydrate–lectin interactions poses enormous promise for their exploitation in medical diagnosis and treatment. Therefore, many efforts across different scientific disciplines have been devoted to identifying new lectins as well as to gaining an in-depth understanding of their functions and precise mechanism of their association with specific carbohydrate ligands. Concanavalin A (Con A) is a plant lectin that is the most extensively studied member of the lectin family, as it was the first legume lectin isolated and sequenced [6]. It was not until 1989 that the 2.9 Å resolution structure of the complex between Con A and methyl α -D-mannopyranoside was determined [7]. The remarkable biological properties of Con A stem from its abilities to agglutinate erythrocytes [8], interact with carbohydrates or glycan parts of glycoproteins non-covalently in a manner that is usually reversible and highly specific [9, 10], and precipitate glycogen and starch from solution [8, 9]. Studies have shown van der Waals and direct hydrogen bonding to be the key forces binding the carbohydrate to the lectin [11, 12].

While the isolation of glycans from natural sources is challenging, advances in carbohydrate synthesis have allowed scientists to synthesize structurally diverse and complex carbohydrate analogs for investigation of glycoconjugate behavior [12–15]. The existing techniques commonly used to investigate carbohydrate–protein interactions reported in the literature, including nuclear magnetic resonance spectroscopy, mass spectrometry, X-ray photoelectron spectroscopy, and X-ray diffraction, have permitted researchers to gain insight into the geometry of carbohydrate–protein interactions at the atomic level [12, 16, 17]. However, in most cases these methods do not investigate the carbohydrate–protein interaction with the lectin solvated and in a native conformational state, and the

carbohydrate is presented as a part of an assembly. The thermodynamics of carbohydrate–lectin interactions may be investigated in solution using isothermal titration calorimetry (ITC), as has been used to examine the binding of Con A to a series of 15 mannosides including methyl α -mannoside, the α (1-3) and α (1-6) dimannosides, and α (1-3,1-6) trimannoside, each with and without methylation of the free primary hydroxyl [16]. In these studies, the binding affinity determined from ITC data was highest for the O-methylated α (1-3,1-6) trimannoside and reported as $K_a = 4.90 \times 10^5$ ($K_d = 2.0 \mu\text{M}$), slightly higher than $K_a = 3.37 \times 10^5$ ($K_d = 3.0 \mu\text{M}$) reported for the free trimannoside. Lower K_a values of 0.81×10^4 ($K_d = 123 \mu\text{M}$) and 1.34×10^4 ($K_d = 75 \mu\text{M}$) were reported for the O-methylated and free forms of the α (1-6)-linked dimannoside, and values of 3.35×10^4 ($K_d = 30 \mu\text{M}$) and 1.41×10^4 ($K_d = 71 \mu\text{M}$) were reported for the O-methylated and free forms of the α (1-3)-linked dimannoside. The K_a value for methyl α -D-mannopyranoside reported in the study was 0.82×10^4 ($K_d = 122 \mu\text{M}$). More recently localized surface plasmon resonance [18], surface plasmon resonance (SPR) [19], quartz crystal microbalance (QCM) [20], and electrochemical techniques [21] have been used to investigate biomolecular interactions and have proven to play an important role in investigating carbohydrate–lectin interactions [22, 23].

Electrochemical impedance spectroscopy (EIS) is a label-free and powerful electrochemical/analytical technique frequently used for the study of the interactions of glycans with lectins [24, 25]. This technique studies the interactions of glycans with lectins on a solid support, most commonly on gold, either by immobilizing the glycan or lectin first followed by the corresponding lectin or glycan as an analyte, respectively [24]. The charge-transfer resistance (R_{ct}) of the electrode using a redox probe is compared before and after the interactions, and the data are most commonly represented in the form of Nyquist plot [26]. The increase in R_{ct} value signifies the interaction between the glycan and its corresponding lectin.

Hu and co-worker used a lectin Con A-based EIS biosensor for the selective detection of human liver cancer cell Bel-7404 having high-mannose oligosaccharides chains on their surface [27]. The limit of detection of this biosensor was found to be 234 cells/mL. The Tkac group used a glycan-based EIS biosensor for the detection of different types of lectins and influenza viruses H1N1, H5N1 and H3N2 [28, 29]. The group was successful in detecting attomolar concentrations of lectins and influenza using their glycan-based EIS biosensor.

Identification and understanding of suitable multivalent carbohydrate scaffolds have become one of the crucial prerequisites in glycomics. Many researchers have systematically studied the structure–activity relationship of the multivalency of synthetic carbohydrate analogs with specific lectins, in attempts to mimic the complexity and heterogeneity of the structurally diverse carbohydrates presenting on cell surfaces, and their weak affinities of binding [30]. The extracellular membrane surface is known to contain a dense layer or network of polysaccharides, known as a glycocalyx, projecting from the cellular surface. Some of these saccharide residues, ranging from tri- to penta-saccharides, are found to be linked to the non-reducing ends of linear or branched oligosaccharides that interact prominently with the binding site of a carbohydrate recognizing domain of a lectin [31, 32].

The use of self-assembled monolayers (SAMs) on gold as scaffolds or mimetic systems, with variable and well-defined composition, has appeared to provide a good model to study carbohydrate–lectin multivalent interactions [14, 33]. The advantages of SAM systems include flexibility in preparing monolayers with functional saccharide moieties, the ease of controlling ligand density, and control over the ligand presentation pattern and orientation. These beneficial factors permit surface scientists to probe the interfacial behavior of polyvalent carbohydrate–lectin interactions. However, preparing a clean gold surface is crucial for the better reproducibility of the assay, and many current techniques have limitations in terms of simplicity, time of preparation and effectiveness in cleaning. Different types of mono- and oligo-saccharides and their interactions with lectins have been studied on gold surfaces before, but the comparison between the oligosaccharides by changing their glycosidic linkages and their effects on affinity have not been performed.

Herein, we report a simple method for effectively cleaning the gold surface and investigate the binding affinity of two linear (1-3) and (1-6) and two biantennary (1-3,1-6) and (1-4,1-6) α -D-mannopyranosides to lectin Con A on gold wire. SAMs containing these α -D-mannopyranoside terminal moieties on gold, before and after Con A exposure, were characterized using EIS. In addition, simple α -D-mannopyranosides were prepared to compare the binding preferences of Con A to this structurally different monosaccharide. Single-component and mixed SAMs of these derivatives prepared from 1:1, 1:2, 1:4, 1:9 and 1:19 solution molar ratios with 8-mercapto-3,6-dioxaoctanol (TEG-SH) onto gold surfaces, were examined by EIS and their binding affinity to Con A was studied. Our study confirms that Con A binding is mostly favored on SAMs of these thiolated mannosides when diluted with a spacer molecule. The influence of the linkage pattern for the dimannoside or trimannoside on the effective binding constant was also investigated.

2. Experimental Section

2.1 Cleaning of gold wires

The gold wires chosen for this study were previously used for immobilizing different types of biomolecules on the surface, so thorough cleaning of the wires is necessary for the reproducibility of the experiments. The gold wires (0.2 mm in diameter and 8.0 mm in length), slightly bent at one end, were dipped in nitric acid for 15 min to remove metallic and most of the organic impurities from the wires, followed by rinsing with copious amounts of milli-Q water (18.2 M Ω .cm at 25 °C). Freshly prepared piranha solution (3:1 mixture of concentrated H₂SO₄ and 30% H₂O₂) was used to remove any remaining organic residues from the surfaces (CAUTION: Freshly prepared piranha solution is a strong oxidizing agent and reacts violently with the organic solvents; it should be handled with extreme care). Piranha solution removes all the organic contaminants but forms oxide and hydroxide rich gold surfaces. Sodium borohydride (0.5 M in water) was used to remove oxides and hydroxides from the gold surface by dipping the wires in the borohydride solution for 17 h. Finally, the wires were rinsed with milli-Q water, left in ethanol solution for 1 h, dried in an oven at 110 °C for 10 min, and argon gas was passed over them for 2 min. The wires were then used either immediately or within the next day. For later or next day use, the vial containing gold wires was sealed after passing argon and stored at room temperature.

2.2 Synthesis of thiolated mannosides

The five thiolated mannosides used for this study are 8-mercaptooctyl α -D-mannopyranoside, 8-mercaptooctyl 3-*O*-(α -D-mannopyranosyl)- α -D-mannopyranoside, 8-mercaptooctyl 6-*O*-(α -D-mannopyranosyl)- α -D-mannopyranoside, 8-mercaptooctyl 3,6-di-*O*-(α -D-mannopyranosyl)- α -D-mannopyranoside, and 8-mercaptooctyl 4,6-di-*O*-(α -D-mannopyranosyl)- α -D-mannopyranoside, see Chart 1. For simplicity, these mannosides in their thiolated forms will be abbreviated as Man-C₈-SH, Man(1-3)Man-C₈-SH, Man(1-6)Man-C₈-SH, Man(1-3,1-6)Man-C₈-SH, and Man(1-4,1-6)Man-C₈-SH, respectively. A detailed synthesis procedure of Man-C₈-SH was reported by our group previously [34] and that of the remaining mannosides are included in the supplementary information (S1).

2.3 Preparation of self-assembled monolayers (SAMs)

The SAMs were prepared using the established procedures [14, 34]. The cleaned gold wires were immersed either in a single-component solution of thiolated mannoside or mixed component solutions of thiolated mannoside and 8-mercapto-3,6-dioxaoctanol (TEG-SH) of total concentration 1.0 mM in methanol for 17 h at room temperature. The resulting SAMs on the gold surfaces were rinsed thoroughly with methanol and dried for EIS measurement or further rinsed with Tris buffer and incubated in lectin solution for the immobilization of lectin.

2.3 Lectin preparation and immobilization

The lyophilized powder of lectin Concanavalin A (Con A) from *Canavalia ensiformis* (Jack Bean) and peanut agglutinin (PNA) from *Arachis hypogaea* were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. The lectins solution was prepared fresh in Tris buffer (pH 7.4, 10 mM) supplemented with NaCl (0.1 M), CaCl₂ (1 mM), and MnCl₂ (1 mM). and diluted further to the desired concentrations. The SAM-modified gold wires were incubated in the desired concentration of lectins solution for 1 h at room temperature and thoroughly rinsed with Tris buffer and water before making the measurement. Our prior studies of Con A binding to SAMs containing Man-C₈-SH [18] and of binding of the lectin soybean agglutinin to SAMs of a globotriose derivative [14] indicate that one hour is sufficient for binding equilibrium to be reached. Lectin PNA, which binds to galactosyl (β -1,3) *N*-acetylgalactosamine structures, was used as a negative control in this study.

2.4 Assembly of gold wire electrode

Gold wire with or without SAM and protein immobilized was held by an alligator clip at the bent end and wrapped around by Teflon tape in such a way that only 5.0 mm of the wire would be exposed to the electrolyte solution.

2.5 Electrochemical characterization

All the electrochemical experiments were carried out using a PARSTAT 2273 potentiostat (EG & G Princeton Applied Research) operated by PowerSINE software and a three-electrode cell having Ag/AgCl (KCl sat.) as a reference electrode (CH Instruments Inc., TX) and platinum wire (99.997% purity, 0.5 mm dia.) as a counter-electrode (Alfa Aesar, MA).

2.5.1 Electrochemical impedance spectroscopy (EIS)—EIS was performed in a solution of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1 molar ratio) redox probe of total concentration 5 mM prepared in 10 mM Tris buffer pH 7.4, over a frequency range of 100 kHz to 1 Hz and at a bias potential of 0.22 V. ZSimpWin software was used for determining the charge-transfer resistance from Nyquist plots by fitting the data to the Randles equivalent circuit model.

2.5.2 Cyclic voltammetry (CV)—CV was used to determine the electrochemically accessible surface area using the same redox probe as in EIS by providing potential between -0.2 to 0.6 V at a scan rate of 100 mV/s. The surface area of the clean gold wire was determined previously by our group using CV and was found to be 0.035 ± 0.001 cm² [35].

3. Results and Discussion

3.1 Preparation and cleaning of gold wire electrodes

Clean, stable and reproducible gold surfaces are indispensable for different types of analytical techniques including surface plasmon resonance (SPR), localized surface plasmon resonance (LSPR), quartz crystal microbalance (QCM), voltammetry and electrochemical impedance spectroscopy (EIS) for the detection and sensing of biomolecules. There are different common pretreatment methods for producing a clean gold surface and most are performed just before the modification of the surface. Common pretreatment methods include thermal treatment, mechanically polishing with slurry of alumina, oxygen plasma cleaning, chemical oxidation with piranha solution or ethanol and electrochemical polishing using potential cycling [36]. Using a combination of the techniques is a better way to get a clean gold surface; however, high thermal treatment and mechanical polishing can introduce roughness on gold surfaces. On the other hand, cleaning with chemical oxidation methods such as piranha treatment removes all the organic contaminants but simultaneously forms the gold oxide layer on the surface [37]. It has been reported that some SAMs terminated with hydrophilic groups ($-OH$, $-COOH$ and $-PO_3H_2$) immobilized on gold oxide surface show packing disorder [38], so there is a necessity to remove the oxides from the gold surface for better packing and reproducibility of the electrode. The electrochemical techniques are useful to remove oxide layers when used after the piranha treatment, but they are time-consuming as cleaning a single gold electrode requires multiple scanning cycles. As an alternative, we have found that the piranha treated gold wire (GW) can be effectively freed from the oxides layer by simply reducing the oxides by treating the wires with sodium borohydride solution for 17 h. This method avoids surface roughness and can be used to clean a large number of gold wires at once.

Fig. 1A shows the Nyquist plot of GW before and after cleaning using different techniques. Borohydride cleaned wire (N+P+B) with the charge-transfer resistance of 52 ± 2 Ω represents the clean surface, for which R_{ct} is nearly 125 times smaller than that of the as-purchased gold wire (A) or piranha cleaned gold wires (P). The error bar for the piranha cleaned wires is nearly 600 times higher than that for the borohydride cleaned wires, as seen in Fig. 1B, which proves the better reproducibility of the borohydride cleaned wires at the beginning of the experiment. Fig. 1C is a modified Randle's equivalent circuit used for

fitting the EIS data, incorporating solution resistance (R_s), constant-phase element (Q), charge-transfer resistance (R_{ct}), and Warburg impedance (Z_w). The R_{ct} represents the kinetics of the redox reaction of the diffusing probe, whereas Q can be affected by the microscopic roughness of the surface as well as by chemical inhomogeneity and ion adsorption creating a heterogeneous electrode-electrolyte interface [39, 40]. Therefore, commonly R_{ct} is used as an indicator for the study of affinity binding in Faradaic-based biosensing, whereas Q, a non-ideal capacitor, is used for non-Faradaic-based biosensing [40]. On the other hand, Z_w is related to the diffusion properties of the redox probe in the solution near the surface of the electrode and does not represent the dielectric properties of the electrode-electrolyte interface [41].

Fig. 1D is the CV of bare GW before and after the borohydride-based cleaning. The potential difference between the peak anodic and cathodic currents (E_p) of the CV was used to determine the cleanliness of the GW surface. Theoretically, E_p for a single-electron transfer reaction like $[\text{Fe}(\text{CN})_6]^{3-/4-}$ on a perfect gold surface was found to be 58 mV [37]. The closer the value of E_p to 58 mV, the cleaner should be the surface. We found that $E_p = 74 \pm 4$ mV for the N+P+B cleaned GWs compared to $E_p = 268 \pm 10$ mV for the as-purchased GW.

3.2 Single-component SAMs of thiolated mannosides and their interactions with Con A

Chart 1 shows the molecular structures of different types of thiolated mannosides used for this study. Besides the thiolated monomannoside, we have used two linear thiolated dimannosides and two biantennary thiolated trimannosides. We synthesized these di- and tri-mannosides because it has been reported that di- or tri-mannosides have higher affinity for Con A than methyl α -D-mannopyranoside in solution and also because their structures closely resemble the N-linked biantennary mannose structures of glycoproteins [42]. SAMs of these thiolated mannosides on a GW were formed simply by dipping a clean GW in methanolic solution of desired thiolated mannosides at room temperature for 17 h. Then the SAM formed on GW was incubated in 0.5 μM Con A solution for 1 h to study the interactions, see Fig. 2. Each step can be monitored using EIS and CV. Fig. 3 shows Nyquist plots on GW before and after the formation of SAMs of pure TEG-SH and thiolated mannosides. A specific trend of increasing or decreasing R_{ct} with the size of the molecules was not observed. The SAMs of larger molecules often show higher R_{ct} value as larger molecules more effectively hinder the transfer of charge between the ferro/ferri-cyanide redox probe and the gold surface. As expected, SAMs of Man-C₈-SH show a larger $R_{ct} = 169 \pm 18 \Omega$ compared to SAMs of the shorter TEG-SH for which $R_{ct} = 62 \pm 6 \Omega$. However, this trend does not continue to SAMs of di- and tri-mannosides, as it is obvious from the plots that R_{ct} of di- and tri-mannosides are smaller compared to Man-C₈-SH. There are also variations in R_{ct} within the di- or tri-mannosides when only the glycosidic linkages between mannose are different. SAMs of dimannoside Man(1-3)Man-C₈-SH have lower R_{ct} ($80 \pm 4 \Omega$) compared to R_{ct} ($144 \pm 14 \Omega$) of Man(1-6)Man-C₈-SH and trimannoside Man(1-4,1-6)Man-C₈-SH has lower R_{ct} ($84 \pm 5 \Omega$) compared to R_{ct} ($127 \pm 21 \Omega$) of Man(1-3,1-6)Man-C₈-SH. The variation in R_{ct} value of different SAMs infers that not all the SAMs are formed in the same manner. Though di- and tri-mannosides are larger structures they have a smaller R_{ct} value because of their greater difficulty alone in forming well-

ordered SAMs, allowing better electron transfer between electrode and solution across these SAMs as compared to that across the SAMs of Man-C₈-SH.

Fig. 4 represents some of the control experiments performed on GW before studying mannosides-Con A interactions. GWs were directly incubated in 0.5 μ M Con A solution for 1 h to study their physical interaction with Con A. The wires were cleaned with buffer followed by water before the data were obtained. We found that a significant amount of protein gets immobilized nonspecifically on the clean gold surface as evidenced by the larger semicircle and hence increase in R_{ct} value, resulting in a change in R_{ct} (R_{ct}) = (R_{ct} after 0.5 μ M Con A immobilization – R_{ct} before Con A immobilization) = $243 \pm 12 \Omega$, Fig. 4A. In the next step, GW was functionalized with SAMs of TEG-SH, and its interaction with 0.5 μ M Con A was studied. Binding of Con A to the electrode surface is significantly decreased with $R_{ct} = 63 \pm 13 \Omega$. A small increase in R_{ct} value due to immobilization of 0.5 μ M Con A on TEG-SH surface should be because of either nonspecific interactions of TEG-SH with Con A or interaction of some bare spots on the gold surface with Con A, as TEG-SH is a short molecule and might not be able to fully block the interaction of Con A with the gold surface. We used peanut agglutinin (PNA) as a negative control to see its interaction to the SAMs of thiolated-mannosides surface. Fig. 4B clearly demonstrates negligible increase in R_{ct} value when 0.5 μ M PNA was exposed to the surface compared to the 0.5 μ M Con A where $R_{ct} = 299 \pm 51 \Omega$.

3.3 Determination of optimal composition in mixed SAMs for Con A binding

A series of experiments were performed to determine the optimal ratio of thiolated mannosides and TEG-SH for capturing Con A. A well-defined semicircle in the Nyquist plot was seen for every ratio (1:1, 1:2, 1:4, 1:9 and 1:19) investigated, suggesting the formation of organized and resistive monolayers at all these compositions. On incubating these SAMs in Con A solution, an increase in the R_{ct} was observed, as indicated by the increase in diameter of the semicircle in the Nyquist plots. The result clearly shows that a layer of Con A is being added at the interface of these two component SAMs. This suggests that the bulky mannose termini may have either adopted a standing orientation, or the mannose moiety is being presented above the molecular termini and is available to interact with a saccharide binding site of Con A. In accordance with literature reports, a better response for carbohydrate–lectin binding is seen upon dilution of ligand density on the surface[34] [43] as it is likely that the mannose surface density on the gold surface for pure SAMs is too high, creating steric hindrance for proper binding of Con A. Fig. 5 shows the change in R_{ct} when Con A was immobilized in a single-component SAM of Man-C₈-SH and mixed SAMs of Man-C₈-SH/TEG-SH on gold wire at 1:1, 1:2, 1:4, 1:9 and 1:19 solution molar ratios. The greatest response to Con A binding was obtained for mixed SAMs formed at a 1:4 solution molar ratio. Interestingly, it has been found that at 1:1 molar ratio the response decreases, and it might be because of unfavorable orientation of mannose for capturing Con A. The response starts increasing with 1:2 molar ratio, reaches a maximum at 1:4 and decreases for SAMs formed at 1:9, 1:19, and for TEG-SH.

Fig. 6 shows the representative Nyquist plots and corresponding CV of gold wires at the optimal ratio (1:4) of Man-C₈-SH/TEG-SH and after Con A immobilization. The small

semicircle domain of bare GW successively increases its diameter upon modification with SAMs and on the interaction of SAMs with 0.5 μM Con A. The lectin PNA (0.5 μM) was used as a control, which shows some nonspecific interactions with $R_{\text{ct}} = 80 \pm 10 \Omega$. However, this R_{ct} value is very small compared to the value obtained for Con A interactions where $R_{\text{ct}} = 495 \pm 15 \Omega$. The exponent for the constant phase element ($Q = Y_0 (j\Omega)^{-n}$) in the model circuit is interpreted as a measure of surface roughness, with deviation further below 1.0 taken as indicating a rougher surface. The exponent was found to be 0.79 for the SAM of Man-C₈-SH alone, and 0.72 for the Man-C₈-SH/TEG SAM of 1:4 solution molar ratio. After interaction with PNA, these values changed to 0.80 and 0.83, respectively. After interaction with Con A, a value of 0.92 was found in each case. This result may suggest that binding of Con A masks some of the underlying roughness of the gold surface. Similarly, CV of bare GW shows well-defined redox peaks whose peak currents decrease with the increase in thickness from SAMs and Con A due to the increase in barrier at electrode/electrolyte interface for the electron-transfer kinetics. Nyquist plots and CV of the mixed SAMs of thiolated di- and tri-mannosides/TEG-SH on a gold wire at their optimal molar ratios are shown in the supplementary information file (Fig. S1).

Fig. 7 shows the change in R_{ct} when 0.5 μM Con A was bound onto a gold wire by single-component SAMs of thiolated di- and tri-mannosides and mixed with TEG-SH at 1:1, 1:2, 1:4, 1:9 and 1:19 molar ratios. We found that the R_{ct} due to immobilization of Con A increases for the mixed SAMs, demonstrating the effectiveness of the mixed SAMs surface for capturing Con A. Thiolated dimannoside Man(1-3)Man-C₈-SH and trimannoside Man(1-3, 1-6)Man-C₈-SH show a sharp increase in R_{ct} with decreasing the molar ratio of mannoside to TEG-SH up to 1:4 that then sharply decreases on further dilution. However, thiolated dimannoside Man(1-6)Man-C₈-SH and trimannoside Man(1-4, 1-6)Man-C₈-SH show sharp increases in R_{ct} response at 1:1 molar ratio but this remains almost the same for the 1:9 molar ratio and then decreases sharply.

From the mole ratios study of mixed SAM, it can be inferred that the mole ratio should be chosen carefully for optimal response. Forming a mixed SAM might not always help for capturing a higher number of lectins, evident from 1:1 molar ratio SAMs of Man-C₈-SH/TEG-SH; choosing a random mole ratio without thorough investigation might yield negative results rather than improving the efficacy. In general, mixed SAMs of all the studied thiolated mannosides prepare in 1:4 molar ratio with TEG-SH was found to be the optimal condition.

3.4 Binding affinity of Con A to mixed SAM of mannosides at optimized molar ratio

Carbohydrate–lectins interactions can be further understood if we determine the equilibrium constants, expressed either as dissociation constant (K_d) or association constant (K_a). The smaller the value of K_d , the stronger will be the interactions. Data in Fig. 8 exhibits a change in R_{ct} with the varying concentration of Con A when captured by SAMs of the thiolated mannosides. Table 1 shows values of K_d obtained by fitting the curves using a nonlinear regression to the Hill equation (1) [44–46],

$$\Delta R_{ct} = \frac{\Delta R_{ct(max)} \cdot X^n}{K_d^n + X^n} \quad (1)$$

where $R_{ct(max)}$ is the change in charge transfer resistance for maximum specific binding, X is concentration of Con A, and n is the Hill coefficient, which represents the degree of cooperativity for the binding of Con A to carbohydrates SAMs.

We have found that K_d for the mannosides and Con A interactions are in the nanomolar range and are slightly smaller than previously reported values for mannose–Con A interaction using SPR (K_d = 135–400 nM) [47] and QCM (K_d = 250–400 nM) techniques [48, 49]. It has also been found that binding affinity of multivalent ligands to lectins are stronger compared to monovalent ligands [50, 51]. An isothermal titration calorimetric study has shown that K_d for the interaction of monomannoside with Con A in solution is 122 nM and that for dimannose to Con A ranges from 35–175 nM depending on ligand density on nanoparticles surface [52]. The K_d for the interaction of Con A with the surface immobilized mannosides and free mannosides has been compared previously [53]. The surface immobilized mannoside have been found to show stronger binding (K_d in nM range) compared to free mannosides in solution (K_d in μ M range). The strong binding between the surface bound mannosides and Con A is suggested as due to the multivalent interaction, and is possible whenever there is an optimal distance (neither at very high surface densities nor separated far apart) between the immobilized mannosides. SPR was used to determine K_d for Con A interacting to the mannose functionalized on a gold surface and was found to be 78 nM due to multivalent interactions [54], which is close to 71 nM obtained for 1:2 binding of Con A to mannose obtained using a quartz crystal microbalance [49]. However, binding affinities of Con A to the surface immobilized mannosides having different numbers of terminal mannose units Man1, Man4, Man8, and Man9 were found nearly the same with K_d of 73, 76, 80, and 90 nM, respectively [53]. We have found that Man-C₈-SH, Man(1-3)Man-C₈-SH, and Man(1-4, 1-6)Man-C₈-SH show the comparable K_d of 70 ± 19 , 75 ± 20 and 81 ± 31 nM, respectively with the reported value of K_d and are also similar in value to each other. However, we have found that even if the numbers of the terminal mannosides are same, changing their glycosidic linkage position changes their affinity. We have found that unlike their counterparts, Man(1-6) Man-C₈-SH and Man(1-3, 1-6)Man-C₈-SH show stronger interaction with Con A with K_d of 14 ± 4 and 30 ± 5 nM, respectively. It is possible that for the derivatives with the lower K_d values, the sugar units are oriented relative to the surface in a manner that is more accessible to a Con A binding site. The values of the Hill coefficient are all found to be less than 1.0, indicating negative cooperativity. The binding of a mannoside into a Con A binding site makes binding of a second mannoside slightly less favorable. The values of the Hill coefficient are found to be $n = 0.59 \pm 0.07$, 0.71 ± 0.10 , 0.73 ± 0.17 , 0.55 ± 0.05 , and 0.50 ± 0.06 for Man-C₈-SH, Man(1-3)Man-C₈-SH, Man(1-6)Man-C₈-SH, Man(1-3,1-6)Man-C₈-SH, and Man(1-4,1-6)Man-C₈-SH, respectively. Values of the Hill coefficient less than one have been recently reported for interaction of Con A with surface grafted glycopolymers on gold using surface plasmon resonance [55].

It has been found that the value of R_{ct} increases linearly with the increase in the concentration of Con A near the detection limit region. This information can be used to find the limit of detection (LOD) of the prepared mannosides-modified electrodes by plotting a linear regression line. The LOD in term of the standard error of the response (σ) and the slope (S) was expressed as $3\sigma/S$ [19, 56], where σ was obtained by the standard error of y-intercept of the regression lines. The detection limits of thiolated mannosides modified GW electrodes for Con A were found ranging from 4 to 13 nM. Thiolated monomannoside shows a better LOD of 4 nM compared to trimannosides Man(1-3,1-6)Man-C₈-SH and Man(1-4,1-6)Man-C₈-SH whose LOD are 13 and 11 nM, respectively whereas LOD for dimannosides Man(1-3)Man-C₈-SH and Man(1-4)Man-C₈-SH are 12 and 6 nM, respectively. These LOD values are comparable to the detection limit of 5 nM for the detection of Con A on mannose modified gold surface obtained using voltammetry [57] and the localized surface plasmon resonance technique [58]. These LOD values are also comparable to the mannose-modified boron-doped diamond surface used for the detection of lectin *Lens culinaris* agglutinin [59]. The LOD has been improved compared to several reported methods for Con A detection. A colorimetric sensing method using mannose stabilized gold or silver nanoparticles was used to detect Con A with LOD of 40 and 100 nM, respectively [60]. In another study, it has been shown that using extinction spectroscopy LOD down to 78 nM can be obtained [61]. Using complex labeling techniques, designing nanostructures based support, and by using sophisticated instrumentation LOD down to picomolar concentration has been reported for similar interactions [43, 61, 62]. However, for a simple, cheap and sensitive alternative GW is a suitable substrate for the study of different types of biological interactions.

4. Conclusions

The structure of the glycan at the end of the alkyl chains of a thiolated derivative from which a SAM or mixed SAM can be formed clearly has a profound effect on organization and lectin binding response. The terminal glycan is a sterically demanding group that affects the composition of the mixed SAM at which the optimal lectin binding response was observed, the lateral distribution of the molecules in a mixed SAM, and the overall binding affinity. The single mannoside derivative Man-C₈-SH is the least sterically demanding, and hence forms a relatively ordered structure giving higher R_{ct} value. However, maximum Con A binding can be achieved when diluted with TEG-SH to 1:4 molar ratio. This suggests that at the higher density of mannosides on surface, the proximity of the mannose units inhibits binding of Con A. Similarly, for thiolated dimannosides and trimannoside Man(1-3,1-6)Man-C₈-SH, molar ratio of 1:4, and for trimannoside Man(1-4,1-6)Man-C₈-SH, molar ratio of 1:9 with TEG-SH were found to be the optimal conditions. The branched di- and tri-mannosides assemble into less ordered arrangements at the gold surface with many glycans possibly oriented in a manner not as well suited for binding such as lying flat, bent and oriented away from the interface, or entangled in small clusters. Dilution with TEG-SH increases or reduces the overall value of R_{ct} for the SAMs depending on types of SAMs used, but mostly yields more accessible glycans and an improved response in R_{ct} upon Con A binding. K_d values of Con A binding to all the mannosides were determined

and were found in tens of nanomolar region. LOD of thiolated mannosides immobilized electrodes were also determined and are found ranging from 4 to 13 nM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A simple novel method was developed for cleaning gold wire electrodes
- Effect of change in glycosidic linkage position of mannosides on Con A binding was studied
- Optimal ratios of thiolated mannosides to prepare the mixed self-assembled monolayers was examined
- Binding affinities between thiolated mannosides and Con A were found in nanomolar range

Fig. 1.

Comparison of bare GW before and after pretreatment A) Nyquist plots of the bare GW as-purchased (A) and after piranha cleaning (P), piranha + one-step chronoamperometric cleaning in 0.5 M H_2SO_4 providing potential of -1.0 V for 1 min, 5 times (P+E), nitric acid + piranha + ethanol cleaning (N+P+Et), nitric acid + piranha + sodium borohydride + ethanol cleaning (N+P+B). B) Bar graph showing error bars in R_{ct} of different pretreatment methods, where error bars indicate the SD of four measurements. C) The modified Randles equivalent circuit used for fitting all the EIS data. The solid lines in Figure A represent a fit to this circuit. D) CV of the bare GW before (A) and after borohydride-based cleaning (N+P+B). Both CV and EIS spectra were recorded in 10 mM Tris buffer (pH 7.4) containing 100 mM NaCl, 2.5 mM $K_3Fe(CN)_6$ and 2.5 mM $K_4Fe(CN)_6$. EIS frequency range was from 100 kHz to 0.1 Hz at DC potential of 0.22 V, and scan rate for CV was 100 mV/s vs. Ag/AgCl.

Fig. 2.

A) Photographic image of gold wire electrode showing 5 mm long exposed gold surface and B) Schematic diagram showing the formation of mixed SAMs of Man-C₈-SH and TEG-SH on gold wire and the interaction of mannose with Con A. Mixed SAMs were prepared in different mole ratios (1:1, 1:2, 1:4, 1:9, and 1:19 of Man-C₈-SH/TEG) to find the optimal ratio for capturing the lectin Con A. Similar strategies were employed for forming mixed SAMs with thiolated di- and tri-mannosides on gold surfaces and studying their interactions with Con A.

Fig. 3.

Nyquist plots of GW before and after the formation of SAMs of pure TEG or thiolated mannoses. EIS spectra were recorded in 10 mM Tris buffer (pH 7.4) containing 100 mM NaCl, 2.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 2.5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ in a frequency range from 100 kHz to 1 Hz at DC potential of 0.22 V. For the clarity of charge-transfer resistance region, the Warburg impedance region from 10 Hz to 1 Hz is not shown. The solid lines are the fit to the equivalent circuit of Figure 1C.

Fig. 4.

Nyquist plots of bare gold GWs before (black) and after A) physical immobilization of Con A on GW surface (blue), SAMs of TEG-SH was formed (red), and Con A was immobilized on TEG-SH immobilized GW surface (green) B) the formation of SAMs of Man-C₈-SH (red) and immobilization of Con A (green) and PNA (blue) on SAM formed surface. Lectins were immobilized by dipping the GW in 0.5 μM of their solution for 1 h. EIS spectra were recorded in the same conditions as in Fig. 3 and the solid lines are the fit to the equivalent circuit of Figure 1C.

Fig. 5. Change in R_{ct} when Con A ($0.5 \mu\text{M}$) was immobilized on GW modified with the mixture of Man-C₈-SH and TEG-SH in different molar ratios (total concentration = 1 mM). Error bars indicate the SD of three measurements.

Fig. 6.

Representative A) Nyquist plots showing an increase in R_{ct} and B) cyclic voltammograms showing a decrease in peak current at scan rate 100 mV/s, when 0.5 μM Con A was immobilized (green) on mixed SAMs formed by 1:4 mole ratio of Man-C₈-SH and TEG-SH (red) on GW (black). PNA (0.5 μM) was used as a control (blue). The experiment parameters used for CV and EIS measurement were same as in Fig. 1 and 3, respectively. The solid lines in A are the fit to the equivalent circuit of Figure 1C.

Fig. 7.

Change in R_{ct} with the immobilization of Con A ($0.5 \mu\text{M}$) on GW modified with the mixture of A) thiolated dimannosides and TEG-SH and B) thiolated trimannosides and TEG-SH, in different molar ratios (total concentration = 1 mM). Error bars indicate the SD of three measurements.

Fig. 8.

The binding affinity of Con A to mannosides at their optimal mixed SAMs ratios (A-E). Insets are the calibration curves obtained at the concentration range where there is a linear response. The error bars represent the SD of four measurements.

Chart 1.

Molecular structures of A) 8-mercaptooctyl α -D-mannopyranoside B) 8-Mercaptooctyl 3-*O*-(α -D-mannopyranosyl)- α -D-mannopyranoside (**11**), C) 8-Mercaptooctyl 6-*O*-(α -D-mannopyranosyl)- α -D-mannopyranoside (**8**), D) 8-Mercaptooctyl 3,6-di-*O*-(α -D-mannopyranosyl)- α -D-mannopyranoside (**13**), E) 8-Mercaptooctyl 4,6-di-*O*-(α -D-mannopyranosyl)- α -D-mannopyranoside (**14**), and F) 8-mercapto-3,6-dioxaoctanol

Table 1
Different parameters obtained for binding of Con A to SAM of mannosides at their optimal ratio with TEG-SH.

Man	Optimal ratio	K_d (nM)	Hill coefficient	Linear range (nM)	Regression equation (C = concentration, mM)	Standard error of y-intercept	Regression coefficient (R^2)	LOD (nM)
A	1:4	70 ± 19	0.59 ± 0.07	1–50	$R_{ct} = 4.20C + 89.50$	5.95	0.99	4
B	1:4	75 ± 20	0.71 ± 0.10	5–100	$R_{ct} = 2.68C + 79.89$	10.79	0.98	12
C	1:4	14 ± 4	0.73 ± 0.17	1–50	$R_{ct} = 4.56C + 90.74$	8.85	0.98	6
D	1:4	30 ± 5	0.55 ± 0.05	10–100	$R_{ct} = 2.67C + 246.42$	11.23	0.99	13
E	1:9	81 ± 31	0.50 ± 0.06	5–100	$R_{ct} = 1.57C + 122.78$	5.59	0.98	11