

Recognition of Glycoprotein Peroxidase via Con A-Carrying Self-Assembly Layer on Gold

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We have successfully fabricated a self-assembled layer of concanavalin A (Con A) on a gold surface for recognition of glycoproteins. The type IV Con A is covalently bound to 11-mercaptopundecanoic acid (MUA) on gold with a 2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate (TNTU) linkage. The binding interaction between glycoproteins and self-assembled Con A is studied using horseradish peroxidase (HRP) as a model glycoprotein. Voltammetric, electrochemical impedance studies, and photometric activity measurements show the presence of both specific and nonspecific bindings of HRP to the Con A interface. The specific binding is attributed to the Con A–sugar interaction where Con A selectively recognizes the glycosylation sites of HRP. The catalytic current of the HRP-loaded electrode, because of catalytic oxidation of thionine in the presence of hydrogen peroxide (H_2O_2), is found to be proportional to the HRP concentrations in the incubation solution. A linear correlation coefficient of 0.993 was obtained over a wide HRP concentration range of 12.5 $\mu\text{g/mL}$ to 1 mg/mL . The approach described in this study provides a simple yet selective means to immobilize glycoproteins on a solid support. The specific binding achieved is desirable in biosensor fabrication, glycoprotein separation, recognition, and purification as well as in drug-releasing systems.

Introduction

The sugar chains located on the surface of the cell membrane or a cell wall play an important role in cell adhesion, a common phenomenon present in many important cellular processes such as cell activation, signal transduction, differentiation, and anticancer function.^{1–4} Cell malignancy, differentiation, and development usually cause structural changes in the sugar chains on cell surfaces. Therefore, identification of the properties and structures of these sugar chains is an effective means for disease diagnosis.^{5–9} Lectin, among many glycoproteins, shows high affinity toward specific sugar residues and thus has been widely used in various biological assays, including but not limited to (1) study recognition between glycoproteins and sugar residues, (2) purify glycoproteins, or (3) characterize properties of malignant cells.^{10–13}

Concanavalin A (Con A) is one of the lectin proteins found in Jack bean and exists as a tetramer with a molecular mass of 104 000 at a neutral pH.¹⁴ It possesses three unique binding sites: one for carbohydrates α -D-mannose and α -D-glucose; one for divalent cations (Ca^{2+} and Mn^{2+}), which activate the binding site of protein for carbohydrate interaction; and the third one for hydrophobic recognition.^{15–18} These properties allow Con A to be used as a versatile platform to immobilize a variety of glycoproteins/enzymes on a solid support, and thus Con A has been involved in several important applications, such as those in detection or purification of glycoproteins and sugars.^{6,19–24}

Con A has also been used to immobilize enzymes by reticulating Con A with glutaraldehyde^{28,29} or by forming enzyme–lectin–enzyme–lectin–enzyme multilayers on a Sepharose column.³⁰ Furthermore, with a careful selection of the enzyme to be immobilized, Con A-based solid electrodes have been demonstrated in biosensing. For example, taking advantage of high affinity between glycoprotein enzymes and Con A, Bucura et al. have immobilized acetylcholine esterase (AChE) on screen-printed electrodes in a sandwich format. The resulting sensor has displayed good operational stability and high sensitivity in detection of acetylthiocholine.^{33,34} Through biospecific complexation of Con A and sugar residues in glycoenzymes, multilayers of Con A and glycoproteins, such as glucose oxidase and horseradish peroxidase (HRP), have been successfully constructed on the surfaces of quartz slides and platinum electrodes. Electrochemical measurements have revealed that the enzymes on the Con A–enzyme multilayer-modified electrodes were catalytically active to phenolic compounds and were relatively stable against low concentrations of mannose and urea.^{35–41}

In those reported sensing approaches, the ability to immobilize Con A on a solid surface in a controllable fashion is found to be critical to ensure sensitive and reproducible sensing results. Therefore, numerous research efforts have been reported on the development and optimization of Con A attachment chemistry.^{28,29,33,34} Among these, covalent interaction between the surface-bound active groups and the lysine side chain amino groups in the protein has been demonstrated as a promising method for Con A immobilization. Since this binding is taking place in the lysine side chain, it does not block the carbohydrate binding site of Con A and is readily available to recognize glycoproteins and sugars through lectin–sugar interaction. To achieve this covalent reaction, the surface needs to be first

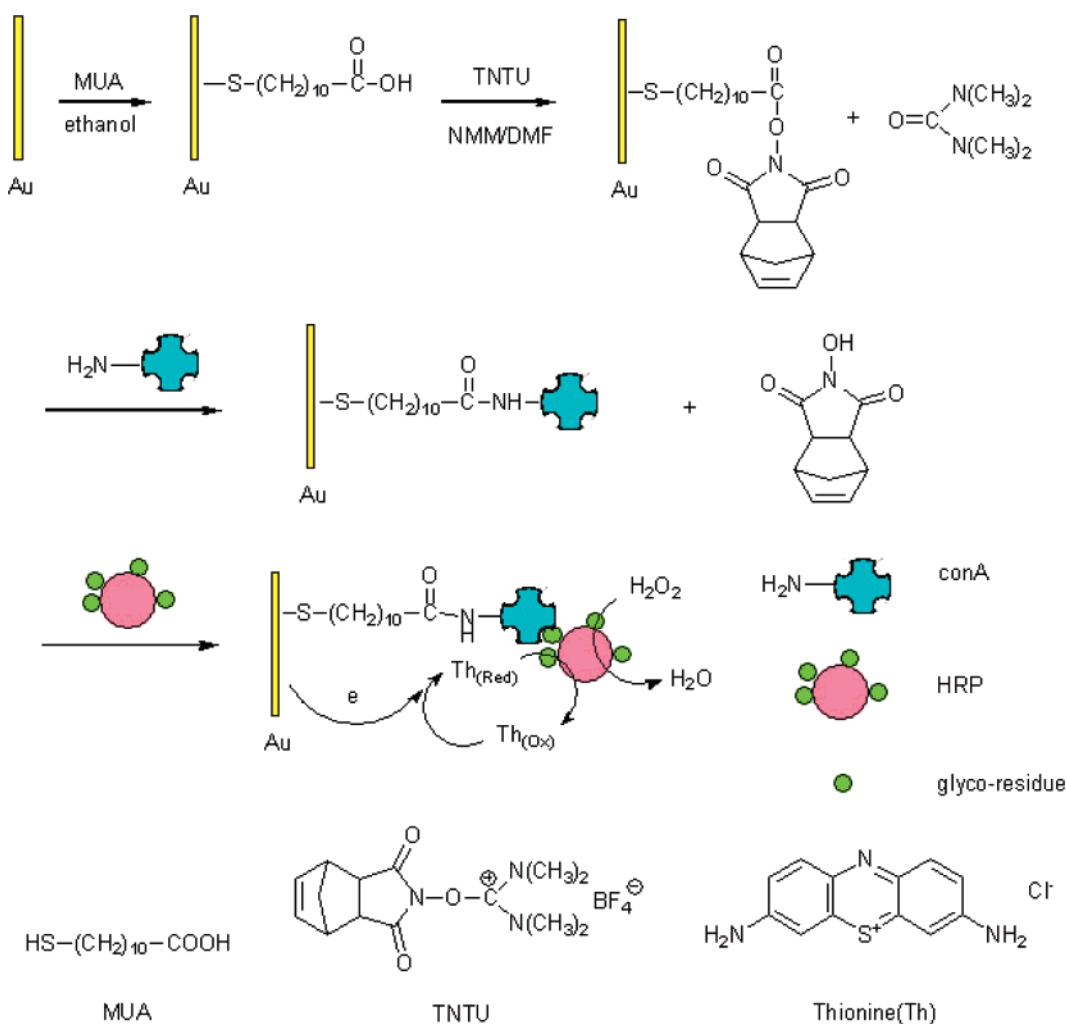
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Scheme 1



modified with function groups such as hydroxyl-, amino-, thiol-, aldehyde-, or carboxyl groups. Following well-documented activating steps, these function groups react with the amino groups on lysines in Con A molecules and subsequently affix Con A to the substrate surface. For examples, surface-anchored hydroxyl groups have been activated with cyanogen bromide,⁴² bisepoxirane,⁴³ organic sulfonyl chlorides,⁴⁴ or carbodiimidazole to interact with the primary amine in lysine;⁴⁵ amino groups have been activated with glutaraldehyde,^{28,46,47} while carboxyl groups have been activated with *N*-hydroxysuccinimide.⁴⁵ In the present work, we report a simple and robust chemical scheme to activate the carboxyl group of surface-attached 11-mercaptoundecanoic acid and then to immobilize Con A on the resulting surface in a controllable fashion to investigate the binding nature between glycoproteins and surface-attached Con A. The work is also motivated by the promising applications of specific Con A–sugar interaction in separation, purification, and detection of glycosylated proteins. In this study, Con A was formed on the gold surface by covalently bound Con A to surface-attached 11-mercaptoundecanoic acid with a 2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate linkage. Horseradish peroxidase (HRP) was chosen as the model enzyme for its high affinity to Con A and accessible sugar groups for binding,⁴⁸ and bovine serum albumin (BSA), a non-glycoprotein, was used as the control molecule in comparison. The study demonstrated that glycoprotein peroxidase can be effectively bound to a Con A interface through both specific and non-specific bindings. The applications of the resulting surface in

hydrogen peroxide sensing and HRP binding quantification were demonstrated as well.

Materials and Methods

Chemicals. Horseradish peroxidase (HRP, EC 1.11.1.7, RZ > 3.0, activity > 250 U/mg, from horseradish) was purchased from Huamei Biological Engineering Co. (China) and was used without further purification. Bovine serum albumin (BSA), 11-mercaptoundecanoic acid (MUA), concanavalin A (Con A, type IV, activity < 500 $\mu\text{g/mL}$), 3,3',5,5'-tetramethylbenzidine dihydrochloride, and thionine chloride were obtained from Sigma and were used as received. *N*-methylmorpholine (NMM) was from Fluka (Germany). 2-(5-Norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate (TNTU) was from Calbiochem (San Diego, CA). Dimethylformamide (DMF) was from Mallinckrodt Baker (Griesheim, Germany). All other chemicals were of analytical grade and were used as supplied. All solutions were prepared with double-distilled water. Measurements were performed in 0.1 M phosphate buffer solutions (PBS) of different pHs, as indicated in the text.

Preparation of Con A Self-Assembled Monolayer. The preparation of the Con A self-assembled layer on gold is illustrated in Scheme 1. TNTU (2.9 mg, 7.9 μmol) was dissolved in a mixture of 1 μL of NMM (9.6 μmol) and 100 μL of DMF to obtain solution 1. Solution 2 was prepared by adding 2 mg Con A to 1 mL PBS buffer solution (0.1 M, pH 6.8, containing 0.1 M MnCl_2 , 0.1 M CaCl_2 , and 0.1 M KCl).³⁵ Prior to use, solution 2 was diluted 10 times by 0.1 M, pH 6.8, PBS.

Gold wire electrodes were purchased from Nanjing Chemical Co. (99.95%, 0.5 mm diameter, geometrical area ca. 0.047 cm^2 , roughness

ca. 1.5, determined by the cyclic voltammogram in 1 M H_2SO_4 at the potential window from -0.2 to 1.6 V at 100 mV/s. The electrodes were pretreated by boiling in 3 M KOH solution for 2 h first, followed by soaking in a "piranha" solution (3:1 concentrated H_2SO_4 /30% H_2O_2 , hazard! Careful of handling!) for 30 min. After rinsing thoroughly with water, the clean Au-wire electrode was immersed in a 0.1 M MUA solution (2.2 mg MUA in 100 μL ethanol) for 12 h at 4°C to obtain the MUA-functionalized electrode. It was then rinsed with ethanol and subsequently with DMF and was incubated in solution 1 for 30 min to activate the carboxylic groups in the MUA monolayer. After rinsing thoroughly with DMF again, the TNTU-activated electrode was immersed in solution 2 for 2 h. This procedure allowed covalent bonding of Con A molecules to the Au surface (Con A-MUA-Au). Finally, the Con A-MUA-Au electrode was rinsed thoroughly with the PBS buffer solution and was incubated in the 2 mg/mL HRP solution (PBS, 0.1 M, pH 6.8) for 3 h. The HRP-Con A-MUA-Au electrode was rinsed with PBS three times and was stored in the same buffer. For the control experiments, HRP was covalently bound to TNTU-activated electrodes directly by incubating the TNTU-MUA electrode in 2 mg/mL HRP for 3 h to generate HRP-MUA-Au electrode. Alternatively, the Con A-MUA modified electrode was soaked in the solution containing 2 mg/mL BSA to generate BSA-Con A-MUA-Au electrode.

Electrochemical Measurements. Electrochemical measurements were performed with a CHI 750C electrochemical workstation (Shanghai, China). A conventional three-electrode electrochemical cell with a Pt-wire auxiliary electrode, a Ag/AgCl (saturated KCl) reference electrode, and the modified Au electrode as the working electrode was used for all electrochemical measurements. All experiments were performed at room temperature ($25 \pm 2^\circ\text{C}$) in 0.1 M PBS as the background electrolyte. The alternating current (AC) impedance experiments were carried out in 10 mM 1:1 $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ with frequencies ranging from 100 kHz to 0.1 Hz. The amperometric experiments were performed in a stirred cell with the successive addition of 5 μL of 10 mM H_2O_2 into 5.0 mL PBS solution (0.1 M, pH 7.0, containing 20 μM thionine) while the electrode potential was set at -0.2 V.

Photometric Activity Assay. Photometric measurements were performed with a Shimadzu UV-3150 spectrophotometer.⁴⁹ In the measurements of the HRP activity in a competitive binding assay, the rinsed peroxidase-modified electrode (HRP-Con A-MUA-Au) was incubated with 100 μL of 10 mM sorbitol solution in 0.1 M pH 7.0 PBS for 10 min. A mixture of 30 μL of 10 mM H_2O_2 and 870 μL of 0.2 mg/mL TMB in PBS was added to the above system, and solution absorption at 652 nm was measured for 10 min. The same electrode was dipped into 1 mL PBS containing 30 μL of 10 mM H_2O_2 , 870 μL 0.2 mg/mL TMB, and 100 μL pH 7.0 PBS for 10 min, and the absorbance at 652 nm was measured again to determine the binding activity of the electrode after sorbitol treatment. HRP-free solutions were used as reference.

Results and Discussion

Preparation of the Con A-Carrying Monolayer. The MUA-modified electrode was characterized by the reductive desorption peak of thiols and the degree of blocking of electrode reactions of redox active species. Figure 1 inset shows a cyclic voltammogram collected from a bare gold electrode in a solution containing 10 mM potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$). It displayed a pair of well-defined redox peaks of $\text{K}_3\text{Fe}(\text{CN})_6$. No apparent $\text{Fe}(\text{CN})_6^{3-/4-}$ peaks were observed from the MUA-modified electrode along with a reduced background current. These observations suggested that the formation of the MUA monolayer effectively blocked electron transfer between ferricyanide and the electrode, either by densely packed MUA molecules⁵⁰ or by the negatively charged surface that electrostatically repelled $\text{Fe}(\text{CN})_6^{3-/4-}$ ions (Figure 1b). The cyclic voltammogram of an MUA-modified gold electrode also

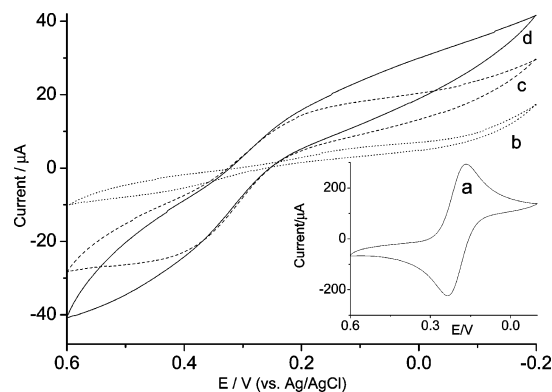


Figure 1. Cyclic voltammograms of (a) a bare Au electrode, (b) an MUA-Au, (c) a Con A-MUA-Au, and (d) an HRP-Con A-MUA-Au electrode in 0.1 M, pH 7.0, PBS containing 10 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 10 mM $\text{K}_4\text{Fe}(\text{CN})_6$. The scan rate was 100 mV/s.

displayed a reduction peak at -1.11 V in 0.5 M KOH at 100 mV/s (not shown). This peak is attributed to reductive desorption of thiolated compounds, which further confirms the attachment of MUA molecules on the gold surface.^{51–53} By integrating the reductive desorption peak area, the surface coverage, Γ ($\Gamma = Q/nFA$), is calculated to be 1.6×10^{-9} mol/cm² or 10^{15} molecules/cm². This surface coverage correlates well with the literature result of densely packed self-assembled monolayer (SAM) molecules on a Au surface.⁵⁴

Scheme 1 illustrates the major steps in Con A immobilization. Specifically, the carboxylic groups in the MUA monolayer were first activated using TNTU in the presence of NMM. The formed activated ester groups then interacted with the primary amines in Con A for permanent attachment. The cyclic voltammogram of the Con A-MUA-Au electrode displayed an increased redox current of a sigmoid shape, as shown in Figure 1c. This was probably due to the reduced surface negative charge density that allowed $\text{Fe}(\text{CN})_6^{3-/4-}$ ions to reach closer to the electrode surface.

Con A as a lectin protein displays high affinity toward sugar moieties in glycoproteins, such as acetylcholine-esterase.^{33,34} Thus, the Con A-functionalized self-assembled monolayer provides a generic scaffold to immobilize glycoproteic enzymes on a gold electrode in an oriented and reversible fashion. The presence of Con A also provides a cushion layer that improves bioactivity of the attached enzyme molecules. HRP with a glycosylation degree of approximately 16.8–21% was used as the model enzyme in this study.⁴⁸ The cyclic voltammogram of the HRP-modified Au-electrode (HRP-Con A-MUA-Au) shows a low background current in 0.1 M pH 7.0 PBS (Figure 2a). When the electrode was immersed in a solution containing $\text{Fe}(\text{CN})_6^{3-/4-}$, a pronounced increase in its CV current was observed in comparison to the signal from the Con A-MUA-Au electrode (Figure 1d). This increased current was a combined result of electron exchange between $\text{Fe}(\text{CN})_6^{3-/4-}$ and the electroactive heme groups of HRP and electron tunneling through HRP.⁵⁵ In a separate experiment, thionine, a positively charged probe, was used as an electron mediator to facilitate electron transfer between the electrode surface and the proteins. The cyclic voltammogram of HRP-Con A-MUA-Au in the solution containing thionine displayed a pair of well-defined peaks at -128 and -147 mV because of the electrochemical response of thionine (Figure 2b). Further addition of hydrogen peroxide to the buffer solution led to the observation of the increased reduction current with the shape of a catalytic wave (Figure 2c). The catalytic current for an HRP-Con A-MUA-Au in the presence of hydrogen peroxide and thionine is 21 times

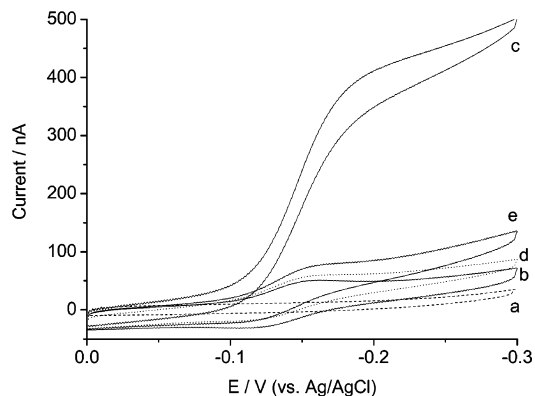


Figure 2. CVs of an HRP-Con A-MUA-modified gold electrode (a) in 0.1 M pH 7.0 PBS, (b) in 0.1 M pH 7.0 PBS containing 20 μ M thionine, (c) in 0.1 M pH 7.0 PBS containing 20 μ M thionine and 2 mM H_2O_2 , and CVs of a Con A-MUA-modified electrode without HRP (d) and HRP-MUA modified electrode without Con A (e) in the same buffer as c at 20 mV/s.

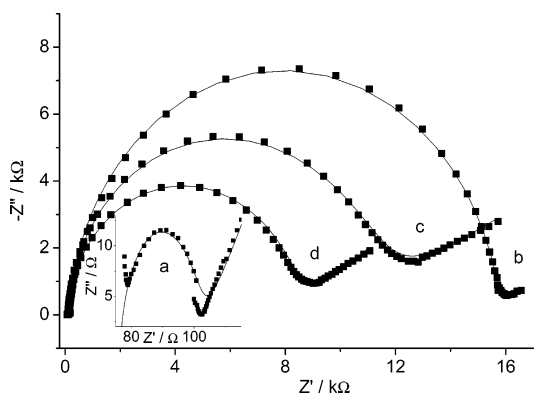


Figure 3. Electrochemical impedance spectra of (a) a bare Au, (b) an MUA-Au, (c) a Con A-MUA-Au, and (d) an HRP-Con A-MUA-Au electrode recorded at the open-circuit potential in 0.1 M pH 7.0 PBS containing 10 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 10 mM $\text{K}_4\text{Fe}(\text{CN})_6$.

larger than that of a Con A-MUA-Au-electrode without HRP (Figure 2d). On the other hand, when replacing HRP with BSA during the preparation of enzyme-modified electrodes, no catalytic wave was observed (not shown). These results collectively confirm the presence of HRP on the Con A-coated electrodes.

When HRP reacted directly with the TNTU-MUA modified electrode without Con A as the buffer layer, the resulting surface also showed a catalytic behavior of H_2O_2 reduction in the presence of thionine (Figure 2e). However, the catalytic current was 5.2 times smaller than that from the HRP-Con A-MUA-coated Au electrode. This was either due to ineffective binding of HRP to the TNTU-MUA interface directly or low electron-transfer efficiency in the absence of Con A. Regardless of the cause, our results suggested the critical roles played by Con A to sustain HRP's binding conformation and electroactivity.

The presence of HRP on the Con A-MUA-coated electrodes was further verified by the AC impedance measurements. Figure 3 shows the AC impedance spectra for (a) a bare Au electrode, (b) MUA-Au, (c) Con A-MUA-Au, and (d) HRP-Con A-MUA-Au recorded at the open-circuit potential in 0.1 M PBS (pH 7.0) containing 10 mM 1:1 $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$. An equivalent circuit $R_s(R_{ct}\text{CPE})$ was used to model the collected impedance data to extract electrical parameters, such as resistance and so forth.⁵⁶ In this circuit, R_s represents the uncompensated solution resistance, while the parallel combination of the charge-transfer resistance (R_{ct}) and the constant phase element (CPE)

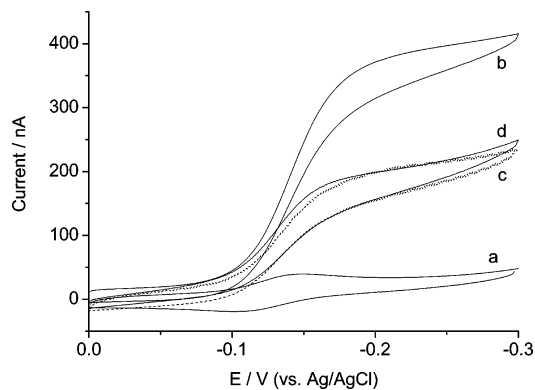


Figure 4. CVs of an HRP-Con A-MUA-modified gold electrode (a) in 0.1 M pH 7.0 PBS containing 20 μ M thionine and (b) in 0.1 M pH 7.0 PBS containing 20 μ M thionine and 2 mM H_2O_2 at 20 mV/s. (c) Electrochemical response of an electrode after an incubation for 30 min in 0.1 M pH 7.0 PBS containing 10 mM sorbitol and (d) after an additional incubation in 2 mg/mL HRP solution in 0.1 M pH 7.0 PBS for 3 h. Both electrodes were in 0.1 M pH 7.0 PBS containing 20 μ M thionine and 2 mM H_2O_2 and were scanned at 20 mV/s.

leads to a depressed semicircle in the corresponding Nyquist impedance plot. CPE is defined as CPE-T and CPE-P. If CPE-P equals 1, then CPE is considered as a capacitor C_{dl} . The charge-transfer resistance (R_{ct}) of each electrode was estimated and ranked in the following order:

$$\text{Au (124 } \Omega) \ll \text{HRP-Con A-MUA-Au (8032 } \Omega) < \text{Con A-MUA-Au (10 129 } \Omega) < \text{MUA-Au (15 550 } \Omega)$$

These results suggest that (1) the presence of organic materials atop Au electrodes, including MUA, Con A, and HRP, behaved as physical barriers and resulted in significance increases in charge-transfer resistance; (2) the binding of Con A reduced the charge-transfer resistance of the MUA SAMs because of the decrease of the surface charge density of the MUA interface, which allows the $\text{Fe}(\text{CN})_6^{3-/4-}$ ions to reach closer to the electrode surface and transfer the electrons through protein tunnels; and (3) the binding of HRP to Con A-MUA resulted in further decrease of this charge-transfer resistance as a result of HRP-facilitated redoxation of $\text{Fe}(\text{CN})_6^{3-/4-}$ ions. All findings are in agreement with the previous conclusions from the CV measurements.

Investigation of the Binding Nature of HRP on Con A-Coated Electrodes. The formation of Con A-HRP complexes is a reversible process in the presence of competing carbohydrate moieties. To determine the relative binding affinity of HRP to the Con A monolayer, sorbitol was used in a displacement assay to compete for the same sugar binding sites on Con A. After immersing the HRP-Con A-MUA-modified electrode in a buffer solution (pH 7.0) containing 10 mM sorbitol in excess, the catalytic current of H_2O_2 decreased to approximately 49.3% of its initial value in 30 min (Figure 4c). Considering that sorbitol is not large enough to remove nonspecifically bound HRP molecules, this decrease in the catalytic response could be quantitatively attributed to the loss of HRP that was specifically bound to Con A through carbohydrate binding sites. When the same electrode was subsequently incubated in a solution containing 2 mg/mL HRP for 3 h, no increase in the catalytic current of H_2O_2 was observed (Figure 4d), suggesting minimal displacement of sorbitol by HRP and thus a significantly higher binding affinity of sorbitol to the Con A-coated electrodes. The amount of HRP released was also verified in a photometric activity assay using tetramethyl benzidine (TMB) as the chromatic substrate.⁴⁹ The

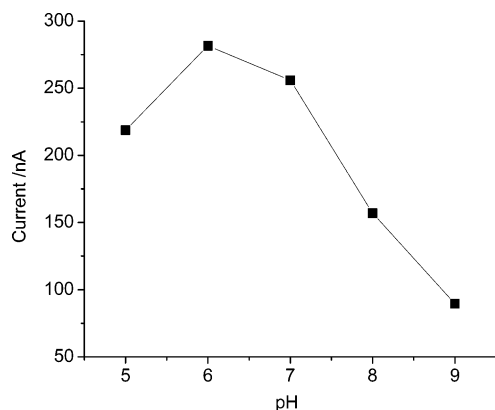


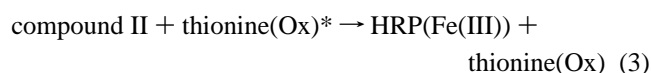
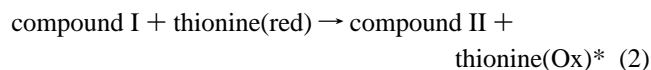
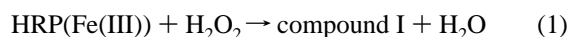
Figure 5. Effect of the pH value on the electrocatalytic activity of the HRP-Con A-MUA-Au.

measurements revealed that 0.1196 nU of bound HRP was lost during sorbitol incubation (55.2%) and that 0.0972 nU of HRP was left behind on the electrode (44.8%). It is in good agreement with the results from the amperometric measurements.

The same phenomena were observed when other monosaccharides, such as glucose, fructose, galactose, and rhamnose, were used in place of sorbitol. The catalytic current of HRP-Con A-MUA-Au decreased to approximately 75.6, 74.8, 68.0, and 36.7% of its initial value after contact with 10 mM monosaccharides solution in PBS for 30 min, respectively (see Supporting Information). These results show that (1) most monosaccharides have higher binding affinities to Con A than HRP does, (2) HRP bound both specifically and nonspecifically on the electrode surface, and (3) the strength of the interaction of sugars with the Con A layer is found having the follow sequence: D-glucose > D-fructose > galactose > sorbitol > rhamnose, which is in agreement with the literature observation reported by Al-Arbabi et al. using CZE.⁵⁷ Extending the electrode immersion time did not further decrease the catalytic reduction current, suggesting that the ligand exchange equilibrium was reached.

Together, our results show that binding of HRP to the Con A interface includes both specific and nonspecific bindings. The specific binding is from affinity interaction between HRP and Con A where Con A specifically recognizes the glycosylation sites on HRP. This binding interaction is reversible in a competitive assay using various monosaccharides. As will be shown later, this is the basis of a method for the determination of the glycosylated protein and its substrates.

HRP-Con A-MUA-Au Electrodes in Biosensing. The catalytic reduction current of the HRP modified electrode may be described by the following scheme:⁵⁸



Equation 4 suggests that the pH of the buffer solution greatly affects the reaction equilibrium and subsequently the activity of HRP. Figure 5 shows the catalytic currents as a function of solution pH values. In this experiment, the Con A-MUA-Au electrode was first incubated in 2 mg/mL HRP solution in 0.1

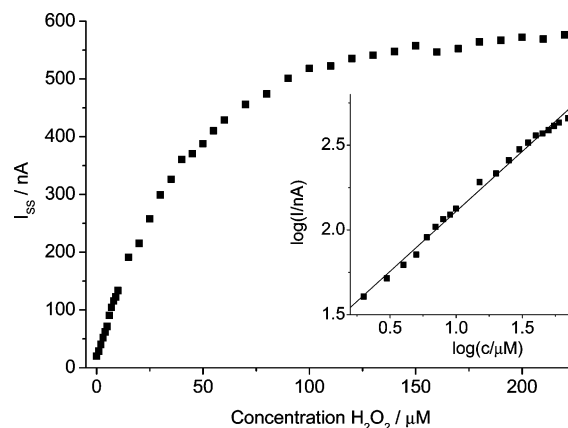


Figure 6. Plot of the reduction current of HRP-Con A-MUA-Au vs H₂O₂ concentration. The electrocatalytic current was measured at -0.2 V of the CV of the enzyme-loaded electrode in 0.1 M pH 7.0 PBS containing 20 μM thionine and 2 mM H₂O₂. Inset: Linear fitting of the calibration curve at concentrations ranging from 1 to 70 μM .

M pH 7.0 PBS for 3 h. The catalytic current was then measured in 0.1 M PBS of different pH values but containing the same amounts of 2 mM peroxide and 20 μM thionine in solutions. The results were normalized on the basis of the reduction currents from the Con A-MUA-Au electrode under different pH's. Accordingly to eq 4, an acidic environment, that is, a higher concentration of $[\text{H}^+]$, would result in the right shift of the reaction equilibrium and a higher redox current readout. As expected, higher electrocatalytic responses were observed when the solution pH decreased initially. However, continuous decrease in the solution pH denatured HRP and subsequently resulted in a decreased redox current. The maximal catalytic current was reached at pH 6.0.

Figure 6 shows that the HRP-modified electrode exhibited an increasing amperometric response to H₂O₂ from 1 μM to 0.22 mM, a basis for quantification of hydrogen peroxide in solution. At the low concentration range, a linear response was observed from 1 to 70 μM , as seen in the inset of Figure 6. Linear regression fitting yielded an equation of $\log I (\text{nA}) = 1.4 + 0.71 \log C (\mu\text{M})$, with a correlation coefficient of 0.997. The limit of detection was estimated to be 0.11 μM at a signal-to-noise ratio of 3σ . A detection plateau was observed when the concentration of H₂O₂ was increased beyond 70 μM , characteristic of Michaelis–Menten kinetics. The apparent Michaelis–Menten constant (K_M^{app}) for HRP-Con A-MUA-Au was found to be 38.4 μM , obtained from the electrochemical version of the Lineweaver–Burk equation.⁵⁹ This value is much lower than those reported in the literature: 2.28 mM for cyt c/Au-CPE,⁶⁰ 3.69 mM for HRP-Au-CPE,⁶¹ 5.5 mM for membrane-entrapped HRP,⁶² and 2.3 mM for HRP/Au colloid self-assembled monolayer electrode.⁶³

Given the fact that the net redox reaction in solution follows the equation of $\text{HRP(Fe(III))} + \text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow \text{HRP(Fe(II))} + 2\text{H}_2\text{O}$, the catalytic current measured is expected to be a function of the amount of HRP bound on the surface as well. Therefore, by keeping the redox solution constant, the same sensing concept could also be used in reverse to quantitate HRP. Specifically, a stock HRP solution (2 mg/mL solution in 0.1 M pH 7.0 PBS) was diluted to a series of different concentrations using 0.1 M pH 7.0 PBS. The Con A-MUA modified electrodes were incubated separately in the synthesized HRP solutions at room temperature for 3 h. Figure 7 shows a plot of the catalytic current at -0.2 V as a function of the HRP solution. The measured current was found to be proportional to the HRP concentration in the range from 12.5 $\mu\text{g/mL}$ to 1 mg/mL, as

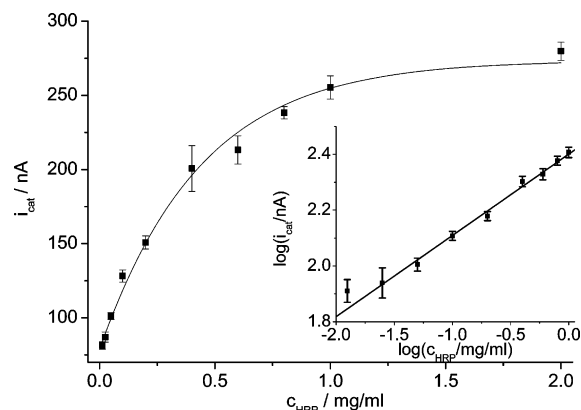


Figure 7. Plot of the electrocatalytic current at -0.2 V vs HRP concentration of the incubation solution. Inset: linear fitting of the calibration curve at concentrations ranging from $12.5 \mu\text{g/mL}$ to 1 mg/mL .

shown in the inset of Figure 7. The linear regression equation was fitted as $\log I (\text{nA}) = 2.4 + 0.292 \log c (\text{mg/mL})$, with a correlation coefficient of 0.993. Above the solution concentration of 1 mg/mL , the surface-immobilized HRP approached a full surface coverage, which gave a steady oxidative current value of $\sim 295 \text{ nA}$.

Last but not least, the measurement-to-measurement and electrode-to-electrode reproducibility was also studied. A relative standard deviation of 6.4% was found for six successive measurements at a H_2O_2 concentration of $50 \mu\text{M}$ using the same electrode. A relative standard deviation of 10.2% was found for six electrodes prepared independently. After being used for about six reversible binding (immersing of Con A-modified electrode in 2 mg/mL HRP for 2 h, followed by incubation in sorbitol for 30 min, is counted as one cycle; the cycle was repeated six times), the standard deviation of the decrease in the reduction current is smaller than 12.8%. The stability of HRP-Con A-MUA-Au electrodes was examined by storing the electrodes in PBS at 4°C for an extended period. It is found that the electrode retained 86% and 59% of its initial current response after a 3 day and 2 week storage, respectively.

Conclusions

In summary, we have successfully fabricated and studied HRP/Con A-modified gold wire electrodes. Con A molecules were covalently bound to the MUA monolayer on gold with a TNTU linkage, forming a self-assembled layer. The resulting interfaces display specific recognition to the glycosylation sites of HRP. Our electrochemical measurements and photometric results show that approximately 50% of the bound HRP was through specific binding. The catalytic current of H_2O_2 reduction in the presence of thionine on the HRP/Con A-modified Au electrode was proportional to the concentration of H_2O_2 and the initial HRP concentration during electrode preparation. This work offers a new way to build novel sensors of glycosylated proteins by specific binding of glycoproteins to a Con A self-assembled layer. The approach described in this study also provides a promising platform for further study of the separation and purification of glycoproteins in clinical applications.

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Supporting Information Available. Cyclic voltammograms of HRP/ConA/MUA/Au before and after incubation in 10 mM

rhamnose, glucose, galactose, or fructose. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

- George, N.; Reeke, J. R.; Joseph, W. B.; Gerald, M. E. *J. Biol. Chem.* **1975**, *250*, 1525–1547.
- Berlin, R. D. *Nat. New Biol.* **1972**, *235*, 44–45.
- Lee, M.; Park, S.; Shin, I. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5132–5135.
- Harris, T. J. C.; Siu, C.-H. *Bioessays* **2002**, *24*, 996–1003.
- Lebed, K.; Kulik, A. J.; Forró, L.; Lekka, M. *J. Colloid Interface Sci.* **2006**, *299*, 41–48.
- Sugawara, K.; Shirotori, T.; Hirabayashi, G.; Kuramitz, H.; Tanaka, S. *J. Electroanal. Chem.* **2004**, *568*, 7–12.
- Naumann, L.; Feist, E.; Straub, R. H.; Burmester, G.-R.; Buttgerit, F. *Biochem. Pharmacol.* **2006**, *71*, 530–539.
- Nishizaki, T. *Mol. Brain Res.* **2003**, *114*, 172–176.
- Veiga, V. F.; Holandino, C.; Rodrigues, M. L.; Capella, M. A. M.; Menezes, S.; Alviano, C. S. *Bioelectromagnetics* **2000**, *21*, 597–607.
- Stewart, D.; Forrest, J. M. S.; Muller, W. D. *Biotechnol. Adv.* **1996**, *14*, 553.
- Mizuno, M.; Noguchi, M.; Imai, M.; Motoyoshia, T.; Inazu, T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 485–490.
- Song, E. Y.; Kim, K. A.; Kim, Y. D.; Lee, E. Y.; Lee, H. S.; Kim, H. J.; Ahn, B. M.; Choe, Y. K.; Kim, C. H.; Chung, T. W. *Hepatol. Res.* **2003**, *26*, 311–317.
- Sauvion, N.; Nardonb, C.; Febvaya, G.; Gatehousec, A. M. R.; Rahbé, Y. *J. Insect Physiol.* **2004**, *50*, 1137–1150.
- Becker, J. W.; Reeke, G. N., Jr.; Cunningham, B. A.; Edelman, G. M. *Nature* **1976**, *259*, 406–409.
- Mandal, D. K.; Brewer, C. F. *Biochemistry* **1993**, *32*, 5116–5120.
- Loris, R.; Hamelryck, T.; Bouckaert, J.; Wyns, L. *Biochim. Biophys. Acta* **1998**, *1383*, 9–36.
- Lis, H.; Sharon, N. *Chem. Rev.* **1998**, *98*, 637–674.
- Kashimoto, E.; Sazaki, G.; Hasegawa, K.; Nakada, T.; Miyashita, S.; Komatsu, H.; Sato, K.; Matsuura, Y.; Tanaka, H. *J. Cryst. Growth* **1998**, *186*, 461–470.
- Miranda, M. V.; Magri, M. L.; del Canizo, A. A. N.; Cascone, O. *Process Biochem.* **2002**, *38*, 537–543.
- Cummings, R. D. Affinity chromatography of oligosaccharides and glycopeptides. In *Affinity Separations: A Practical Approach*; Matejtschuk, P., Ed.; Oxford University Press: New York, 1997; Chapter 5, pp 123–140.
- Sugawara, K.; Hirabayashi, G.; Kamiya, N.; Kuramitz, H. *Bioelectrochemistry* **2006**, *71*, 60–67.
- Ballerstadt, R.; Schultz, J. S. *Anal. Chim. Acta* **1997**, *345*, 203–212.
- Quinn, J. G.; O’Kennedy, R.; Smyth, M.; Moulds, J.; Frame, T. *J. Immunol. Methods* **1997**, *206*, 87–96.
- Barnes, C.; D’Silva, C.; Jones, J. P.; Lewis, T. J. *Sens. Actuators, B* **1991**, *3*, 295–304.
- Hatakeyama, T.; Murakami, K.; Miyamoto, Y.; Yamasaki, N. *Anal. Biochem.* **1996**, *237*, 188–192.
- Diniz, F. B.; Ueta, R. R. *Electrochim. Acta* **2004**, *49*, 4281–4286.
- Mislovicova, D.; Masarova, J.; Svitel, J.; Gemeiner, P. *Int. J. Biol. Macromol.* **2002**, *30*, 251–258.
- Koneke, R.; Menzel, C.; Ulber, R.; Schugerl, K.; Scheper, T.; Saleemuddin, M. *Biosens. Bioelectron.* **1996**, *11*, 1229–1236.
- Lebed, K.; Pyka-Fościk, G.; Raczowska, J.; Lekka, M.; Styczeń, J. *J. Phys.: Condens. Matter* **2005**, *17*, S1447–S1458.
- Faroqi, M.; Saleemuddin, M.; Ulber, R.; Sosnitzer, P.; Scheper, T. *J. Biotechnol.* **1997**, *55*, 171–179.
- Willner, I.; Rubin, S.; Wonner, J.; Effenberger, F.; Bäuerle, P. *J. Am. Chem. Soc.* **1992**, *114*, 3150–3151.
- Willner, I.; Rubin, S.; Cohen, Y. *J. Am. Chem. Soc.* **1993**, *115*, 4937–4938.
- Bucura, B.; Danetb, A. F.; Martya, J.-L. *Anal. Chim. Acta* **2005**, *530*, 1–6.
- Bucura, B.; Danetb, A. F.; Martya, J.-L. *Biosens. Bioelectron.* **2004**, *20*, 217–225.
- Anzai, J.; Kobayashi, Y. *Langmuir* **2000**, *16*, 2851–2856.
- Kobayashi, Y.; Anzai, J. *J. Electroanal. Chem.* **2001**, *507*, 250–255.
- Sato, K.; Imoto, Y.; Sugama, J.; Seki, S.; Inoue, H.; Odagiri, T.; Hoshi, T.; Anzai, J. *Langmuir* **2005**, *21*, 797–799.
- Yang, S.; Chen, Z.; Jin, X.; Lin, X. *Electrochim. Acta* **2006**, *52*, 200–205.

- (39) Langer, R.; Peppas, N. A. *Bioeng. Food Nat. Prod.* **2003**, *49*, 2990–3006.
- (40) Lvov, Y.; Ariga, K.; Ichinose, I.; Kunitake, T. *J. Chem. Soc., Chem. Commun.* **1995**, 2313–2314.
- (41) Lvov, Y.; Ariga, K.; Ichinose, I.; Kunitake, T. *Thin Solid Films* **1996**, *284/285*, 797–801.
- (42) Gupta, P.; Saleemuddin, M. *Biotechnol. Lett.* **2006**, *28*, 917–922.
- (43) Sundberg, L.; Porath, J. *J. Chromatogr.* **1974**, *90*, 87–98.
- (44) Nilsson, K.; Mosbach, K. *Methods Enzymol.* **1987**, *135*, 65–78.
- (45) West, I.; Goldring, O. Lectin affinity chromatography. In *Protein Purification Protocols*, 2nd ed.; Cutler, P., Ed.; Methods in Molecular Biology 244; Humana Press: Totowa, NJ, 2004; pp 159–166.
- (46) Ayhan, F. *J. Bioact. Compat. Pol.* **2003**, *18*, 297–310.
- (47) Anilyte, J.; Liesiene, J.; Niemeyer, B. *J. Chromatogr., B: Biomed. Sci. Appl.* **2006**, *831*, 24–30.
- (48) Aibara, S.; Yamashita, H.; Mori, E.; Kato, M.; Morita, Y. *J. Biochem.* **1982**, *92*, 531–539.
- (49) Stöllner, D.; Scheller, F. W.; Warsinke, A. *Anal. Biochem.* **2002**, *304*, 157–165.
- (50) Cannes, C.; Kanoufi, F.; Bard, A. J. *Langmuir* **2002**, *18*, 8134–8141.
- (51) Zhong, C. J.; Porter, M. D. *J. Am. Chem. Soc.* **1994**, *116*, 11616–11617.
- (52) Walczak, M. M.; Popenoe, D. D.; Deinhammer, R. S.; Lamp, B. D.; Chung, C.; Porter, M. D. *Langmuir* **1991**, *7*, 2687–2693.
- (53) Cecchet, F.; Marcaccio, M.; Margotti, M.; Paolucci, F.; Rapino, S.; Rudolf, P. *J. Phys. Chem. B* **2006**, *110*, 2241–2248.
- (54) Delamarche, E.; Juncker, D.; Schmid, H. *Adv. Mater.* **2005**, *17*, 2911–2933.
- (55) Shumyantseva, V. V.; Ivanov, Y. D.; Bistolas, N.; Scheller, F. W.; Archakov, A. I.; Wollenberger, U. *Anal. Chem.* **2004**, *76*, 6046–6052.
- (56) Chen, A.; La Russa, D. J.; Miller, B. *Langmuir* **2004**, *20*, 9695–9702.
- (57) Al-Arhabi, M.; Mrestani, Y.; Richter, H.; Neubert, R. H. H. *J. Pharmaceut. Biomed. Anal.* **2002**, *29*, 555–560.
- (58) Wollenberger, U.; Drungiliene, A.; Stöcklein, W.; Kulys, J. J.; Scheller, F. W. *Anal. Chim. Acta* **1996**, *329*, 231–237.
- (59) Kamin, R. A.; Willson, G. S. *Anal. Chem.* **1980**, *52*, 1198–1205.
- (60) Ju, H. X.; Liu, S. Q.; Ge, B.; Lisdat, F.; Scheller, F. W. *Electroanalysis* **2002**, *14*, 141–147.
- (61) Liu, S. Q.; Ju, H. X. *Anal. Biochem.* **2002**, *307*, 110–116.
- (62) Ferri, T.; Poscia, A.; Santucci, R. *Bioelectrochem. Bioenerg.* **1998**, *45*, 221–226.
- (63) Xiao, Y.; Ju, H. X.; Chen, H. Y. *Anal. Biochem.* **2000**, *278*, 22–28.

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