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Direct immobilization of sugar probes on bovine serum albumin-coated gold substrate for the development of glycan biosensors

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Glycan biosensors based on surface plasmon resonance (SPR) spectroscopy have attracted a great deal of interest due to their potential applications in numerous biological and biomedical fields. Controlled immobilization of sugar probes on a gold substrate is believed to be critical for the performance of these SPR biosensors. In this regard, herein the authors report a direct coupling of mannose probes with bovine serum albumin (BSA) layer on the gold substrate via a squaric acid-mediated reaction under mild conditions, in which the BSA layer provides not only reactive amine groups but also a nonfouling surface property. SPR measurements show that the resultant biosensor with an appropriate amount of mannose probes exhibits high affinity to its corresponding lectin (i.e., concanavalin A) and at the same time could resist nonspecific adsorption of other lectins. The limit of detection of the current SPR biosensor is 1.9 nM. Thus, the squaric acid-mediated immobilization strategy appears to be effective and useful for the fabrication of bioanalytical devices. *Published by the AVS.* https://doi.org/10.1116/1.5082005

I. INTRODUCTION

Over the past two decades, glycan biosensors have emerged as vital tools for a variety of biological and biomedical studies, such as cell behavior, pathogen detection, drug screening, and glycobiology research.[1–3] Since surface plasmon resonance (SPR) spectroscopy is widely accepted as a highly sensitive and label-free technique for real-time monitoring of carbohydrate-lectin interactions, the development of an SPR-based glycan biosensor has attracted considerable interest.[4–6] Considering the multivalent nature of the carbohydrate-lectin interaction, the hydrophilic glycan probes attached on the SPR substrate are required to have an appropriate lateral distribution and conformation.[7–9] Moreover, in order to avoid the interference of other lectins, it is highly desirable for an SPR-based glycan biosensor to resist the nonspecific adsorption of proteins.

Traditional immobilization method of small sugar probes on the SPR gold substrate involves both the derivation of sugar molecules and substrate modification with functional groups.[10–12] In order to obtain an optimum density of sugar probes, some researchers proposed the synthesis of glycoconjugates with various scaffolds, such as dendritic polymers, DNA, lipid, and proteins.[13–15] While the resultant glycoconjugates could adsorb easily on the gold substrate via hydrophobic interactions, both the separation and the purification of such glycoconjugates are tedious and time-consuming. Recently, several groups have demonstrated direct coupling of unmodified sugar probes on some specific substrates, such as hydrazide, graphene, and photochemically-active surfaces.[16–21] These strategies appeared to be simple and effective. However, these surfaces are prone to nonspecific adsorption of other proteins. Therefore, despite extensive research, it is still a big challenge to control the lateral distribution of sugar probes and at the same time to provide a nonfouling biosensor surface.

Bovine serum albumin (BSA) is the most widely used blocking agent for biosensor surfaces (e.g., ELISA chips) because it can be adsorbed easily on various materials and the resultant BSA-coated surface can resist nonspecific adsorption of proteins. On the other hand, it should be noted that BSA molecule has ca. 20 lysine residues at its outer surface and the distance between two lysines is between 2.0 and 2.4 nm,[22] which makes BSA a suitable scaffold to control the lateral distribution of small sugar probes.[13,23] For instance, Gildersleeve *et al.* prepared glycan microarrays by taking advantage of the direct spontaneous adsorption of BSA on the modified polystyrene substrate, in which BSA was used as linker molecules.[24,25] However, the reductive amination reaction employed in their work was slow and inefficient.

Recently, people found that squaric acid dialkyl esters could react selectively with amine groups at $p\mathrm{H} = 7$ and room temperature without any interference of transesterification or hydrolysis reactions.[26] Of particular interest, due to the lower reactivity of the reaction product as compared to the starting material, only squaric acid ester monoamides are formed, which can be isolated and characterized. Moreover, by adjusting the reaction medium's *p*H value to 9, the ester amide intermediates can be conjugated to another amine

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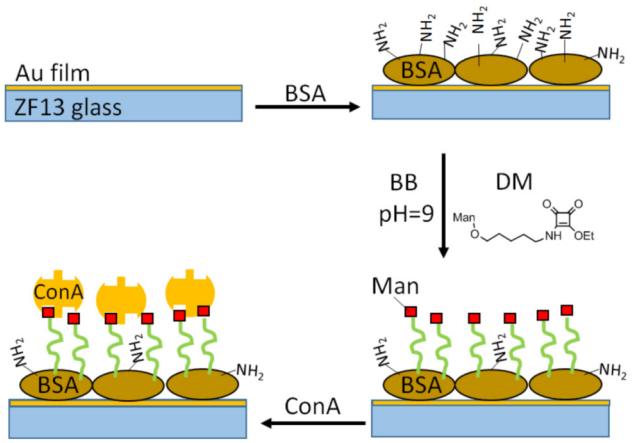

FIG. 1. Schematic representation of the immobilization of DM on BSA-coated gold substrates via the squaric acid-mediated coupling reaction, and the subsequent specific binding with ConA.

functional compound in a second amine-selective and hydroxy-tolerant reaction. Consequently, the squaric acid-mediated coupling reaction has been employed for amine-selective modification of proteins[27,28] and for the synthesis of neoglycoconjugates.[29]

Considering the merits associated with BSA molecule and squaric acid-mediated coupling reaction, herein we demonstrated a facile and effective strategy for the construction of an SPR-based glycan biosensor, as depicted in Fig. 1. First, BSA molecules were adsorbed spontaneously on the gold substrate without any surface modifications. Then, derived mannoses (DMs) were coupled with BSA via the squaric acid-mediated reaction in borate buffer (pH = 9).[30] Mannose (Man) was chosen as a model sugar probe in this work because it exhibited specific recognition with concanavalin A (ConA), which is the most extensively studied carbohydrate-lectin pair.[31] SPR measurements indicated that the resulting glycan biosensor could detect the ConA with a limit of detection of 1.9 nM and at the same time could resist peanut agglutinin (PNA) adsorption.

II. EXPERIMENT

A. Materials and substrates

BSA, ConA, PNA, and phosphate buffered saline (PBS) tablet were purchased from Sigma-Aldrich (Shanghai, China). Mannose, DES, palladium, $Na_2B_4O_7{\cdot}10H_2O$, and H_3BO_3 were obtained from Energy Chemical Co. (Shanghai, China). The synthesis of DM [6′-*N*-(2-Ethoxy-3,4-dioxocyclobut-1-en-1-yl)-aminohexyl-α-D-mannopyranoside] was performed according to the previously described method.[13] Borate buffer (pH = 9.0) of 0.2M was prepared by dissolving 12.39 g of H_3BO_3 and 19.07 g of $Na_2B_4O_7{\cdot}10H_2O$ in 1000 ml water. All chemicals and reagents were of analytical grade and were used as received unless indicated otherwise. Deionized water was used throughout the experiments. The protein solutions were prepared immediately prior to use by dissolving the proteins in 0.01M of PBS buffer (pH = 7.4). The substrates for SPR measurements were 25 × 25 mm of ZF13 glass slides ($n_{ZF13} = 1.8396$ at $\lambda = 632.8$ nm), which were cleaned using 2% Hellmanex solution, rinsed with copious water, and dried with pure nitrogen gas. These glass slides were coated with approximately 2 nm of Cr and 47 nm of Au using a thermal evaporator (ZHD-400, Technol, China).

B. BSA adsorption on the SPR gold substrate

Freshly deposited gold substrates were immersed in BSA solution of different concentrations and then placed on the orbital shaking incubator for 1 h at 70 rpm and T = 20 °C. Subsequently, the BSA-coated substrates were washed with copious deionized water and then dried with a stream of nitrogen gas.

C. Immobilization of derived mannose on the BSA-coated gold substrate

The DM solutions were obtained immediately prior to use by dissolving a certain amount of DM in 0.2M of borate buffer. The BSA-coated gold substrates were immersed in DM solutions and then placed on the orbital shaking incubator at 70 rpm. After 20 h of the coupling reaction, the resulting mannose-modified substrates were washed with deionized water and then dried with a stream of nitrogen gas prior to SPR analysis. The surface morphology of the mannose-modified substrate was observed using a JSPM-5200 atomic force microscope in the tapping mode. As shown in Fig. S1,[48] the resultant glycan biosensor exhibited a very flat surface with a roughness $R_q = 1.3$ nm, which was comparable to that of a bare gold substrate.

D. SPR analysis

SPR measurements were carried out in a custom-built SPR setup based on Kretschmann's configuration.[32,33] Briefly, an He-Ne laser ($\lambda = 632.8$ nm, laser power <5 mW) was passed through a chopper and two polarizers and was then reflected from the prism base, which was detected by a photodiode and a lock-in amplifier. A flow cell of about 0.5 ml was attached to the prism and then connected to a peristaltic pump for liquid exchange. The flow rate used in this study was 700 μl/min, unless otherwise indicated. Both the control of SPR setup and the data collection were achieved through a WASPLAS software. The SPR spectra (i.e., the reflectivity vs incidence angle curve) could be fitted by using a Winspall software, which was based on Fresnel's multireflection theory and developed by Professor Knoll's group in the Max-Planck-Institute for Polymer Research, Mainz, Germany. The SPR kinetic curves (i.e., the reflectivity vs time curve at a fixed angle) could also be measured. The simulation of SPR kinetic data was performed by using an OriginPro software.

III. RESULTS AND DISCUSSION

A. BSA adsorption on the bare gold substrate

It is well known that BSA molecule can be adsorbed spontaneously on the bare gold substrate via multiple interactions, including hydrophobic interactions, covalent bonds between

the thiol group and gold, etc.[34] The adsorbed BSA can form a monolayer on the gold substrate, and the adsorption rate is dependent mainly on the BSA's concentration, the *p*H value of the solution, and the temperature.[35–39] Considering the fact that a high density of BSA on the gold substrate can contribute to not only the formation of nonfouling surface but also the coupling of sugar probes; consequently, we first compared the density of the adsorbed BSA by using different concentrations of BSA solution at room temperature. The inset in Fig. 2 shows the SPR spectra before and after the BSA adsorption on the bare gold substrate. One can find that the SPR dips shifted to higher angles when using a higher concentration of BSA, which suggested that more BSA molecules adsorbed on the gold substrate. On the other hand, the adsorption process was monitored in real time using an SPR kinetic mode [see Fig. S2 (Ref. 48)]. The result clearly showed that when a BSA solution of 10 mg/ml was introduced into the flow cell, the protein adsorption would take place immediately, and reached equilibrium within ca. 20 min. Moreover, the adsorbed BSA layer remained relatively stable after PBS rinsing for ca. 20 min. This result suggests that BSA could form a compact monolayer on the gold substrate quickly and spontaneously. Therefore, the immersion time of 1 h was chosen in this work for BSA adsorption on the gold substrate.

By assuming that the refractive index of BSA is $n = 1.45$,[40] the thickness of the adsorbed BSA can be obtained by fitting SPR spectrum. The BSA density on the gold surface can then be calculated using de Feijter's equation:[41,42]

$$M = d_A \times \frac{n_A - n_S}{d_n/d_c}.$$

Here, d_A and n_A are the thickness and the refractive index of the adsorbed protein layer, respectively. n_s is the refractive index of the surrounding medium on the metal surface. d_n/d_c is the refractive index increment, which is equal to approximately 0.182 cm^3/g for BSA protein. Figure 2 shows the BSA density on SPR gold substrates as a function of BSA's concentration. One can find that, when the BSA concentration increased from 0.5 to 5 mg/ml, the BSA density increased from 89 ± 8 ng/cm^2 to 228 ± 11 ng/cm^2. But when the BSA concentration continued to increase to 10 mg/ml, the BSA density further increased to 250 ± 8 ng/cm^2. This result suggests that the adsorption of BSA was saturated when the concentration of BSA was 10 mg/ml. The result indicates that a monolayer of BSA adsorbed on the gold surface, which is in good agreement with the literature.[43]

B. Immobilization of derived mannose

Direct coupling of the derived mannose with the BSA-coated gold substrate was monitored in real time using an SPR kinetic mode, as shown in Fig. 3. Firstly, borate buffer was introduced into the flow cell. Then, the DM solution of 5 mg/ml was injected at a flow rate of 700 μl/min and kept contact with the BSA-coated gold substrate at room temperature. One can find that the SPR reflectivity first exhibited a sharp jump due to the change of solutions' refractive index. Then, the reflectivity changed slowly at the beginning 2 h. After that, the reflectivity increased linearly as a function of time and slowed down after 20 h, which appears to follow the classic sigmoidal shape. The result is in good agreement with the literature.[44] Noted that when the SPR reflectivity becomes higher than a certain value (depending on the shape of SPR spectrum), it would not be proportional to the shift of SPR spectrum. Thus, Fig. 3 gives qualitative rather than quantitative information. After 28 h of the coupling reaction, the SPR reflectivity increased from 24.1% to 49.8%, suggesting the successful immobilization of mannose probes. The resultant biosensor was then exposed to ConA solution of 1 μM in PBS buffer. Rinsing with PBS buffer could not remove all the captured ConA, indicating that the freshly-prepared glycan biosensor could bind specifically with ConA.

Since the concentration of the derived mannose is a critical parameter for the squaric acid-mediated coupling

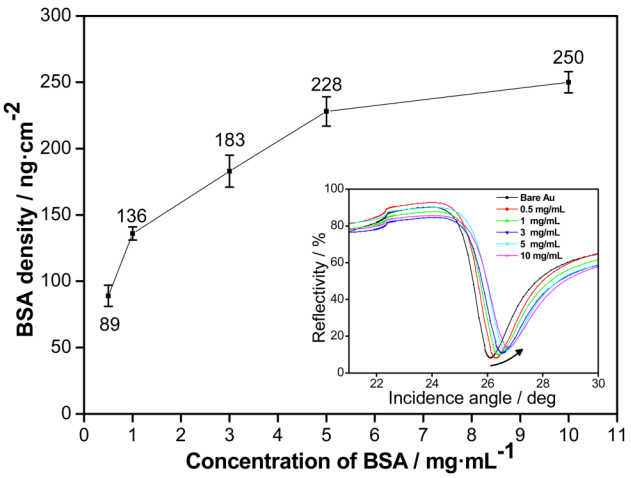

FIG. 2. Calculated surface density of the adsorbed BSA on the bare gold substrate for different concentrations of BSA solution. The corresponding SPR spectra are given in the inset.

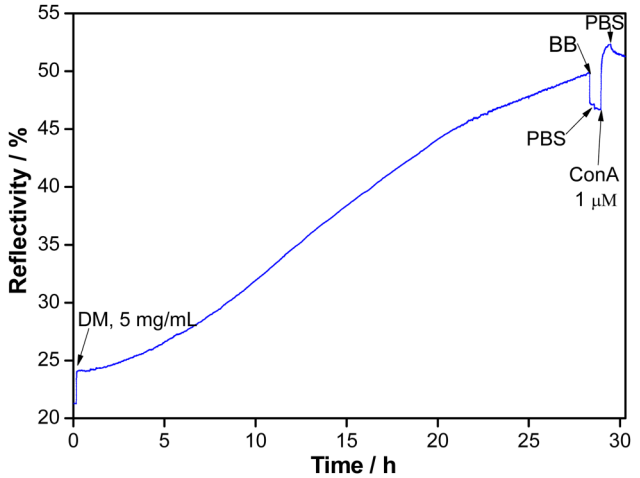

FIG. 3. SPR kinetic monitoring of the immobilization of the derived mannose on a BSA-coated gold substrate, and the subsequent binding of ConA.

reaction, we compared the performance of a series of glycan biosensor prepared with different DM's concentrations. To this end, BSA-coated gold substrates were immersed in various DM solutions ranging from 1.0 to 7.0 mg/ml and then placed on the orbital shaking incubator at T = 20 °C for 20 h. It should be noted that, since the DM probes are much smaller compared to BSA proteins, it is difficult to directly quantify the amount of the immobilized DM probes to the best of authors' knowledge. Therefore, after each coupling reaction, the resultant glycan biosensor was employed to monitor the capture of ConA lectin using SPR technique.

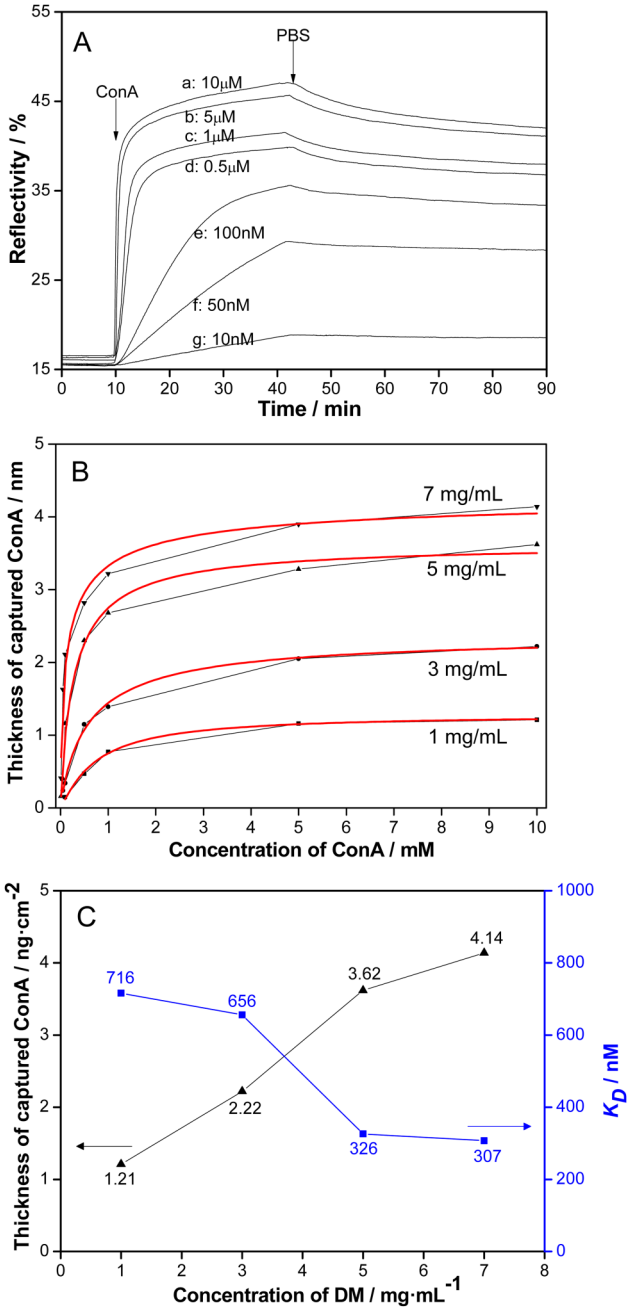

FIG. 4. (a) SPR kinetic monitoring of different concentrations of ConA on the glycan biosensor prepared with 5 mg/ml of DM at a reaction temperature of T = 50 °C. (b) Comparison of the thickness of the captured ConA on various glycan biosensors prepared with different DM concentrations ranging from 1, 3, 5 to 7 mg/ml. The red lines are the nonlinear fitting of the equilibrium isotherms based on the Hill equation. (c) Comparison of both the d_{max} and the K_D values for various glycan biosensors prepared with different DM concentrations ranging from 1, 3, 5 to 7 mg/ml. The reaction time is 20 h, and the reaction temperature is T = 20 °C.

Figure 4(a) shows the SPR kinetic monitoring of a typical glycan biosensor when exposed to different concentrations of ConA. After the association process reached equilibrium, SPR angular spectrum was collected. By assuming the refractive index of ConA is $n = 1.57$,[45] the thickness of the captured ConA can be obtained by fitting SPR spectrum. Figure 4(b) gives the thickness of the captured ConA for various glycan biosensors as a function of ConA concentration. The red line is the nonlinear fitting of data points based on the Hill equation:[8]

$$d_{eq} = d_{max}\frac{C^h}{K_D + C^h},$$

where K_D is the equilibrium dissociation constant, h is the Hill coefficient, d_{eq} is the thickness of the captured ConA at a certain ConA concentration of C, and d_{max} is the maximal thickness of the captured ConA using the saturation concentration of ConA.

Figure 4(c) shows the comparison of the d_{max} and the K_D values for various glycan biosensors prepared with different DM concentrations. It is apparent that the d_{max} of the captured ConA increased from 1.21 nm for 1 mg/ml of DM to 4.14 nm for 7.0 mg/ml of DM, while the K_D values decreased from 716 to 307 nM by increasing the concentration of DM. All K_D values are consistent with the literature,[31] indicating that the multivalent interactions take place for all glycan biosensors regardless of the DM concentration used. Considering the lowest K_D value and the highest d_{max}, one can find that the DM concentration of 7 mg/ml could be employed for the construction of an SPR glycan biosensor.

Reaction temperature is another important parameter for the squaric acid-mediated coupling reaction. Considering that BSA can be denatured at a temperature higher than 55 °C,[46] we compared the performance of three glycan biosensors prepared at temperatures 20, 37, and 50 °C (noted that the DM concentration was 5 mg/ml here). The thickness of the captured ConA and corresponding simulation based on the Hill equation is given in Fig. 5(a). The d_{max} and the K_D values for those biosensors are shown in Fig. 5(b). One can find that by increasing the reaction temperature, the d_{max} of the captured ConA would increase from 3.62, 4.17 to 5.35 nm, while the K_D value would decrease from 326, 153 to 95 nM.

On the other hand, for the highest thickness of the captured ConA (i.e., 5.35 nm for the biosensor prepared at T = 50 °C), the corresponding surface density was calculated to be 697 ng/cm^2 by using de Feijter's equation. ConA has an MW of ca. 102 kDa with dimensions of 6.3 × 8.7 × 9.0 nm,[45,47] and thus the surface density of a densely packed monolayer of ConA will vary from 216 to 309 ng/cm^2. Therefore, it appears that the current biosensor can capture more than one monolayer of ConA. A possible reason to

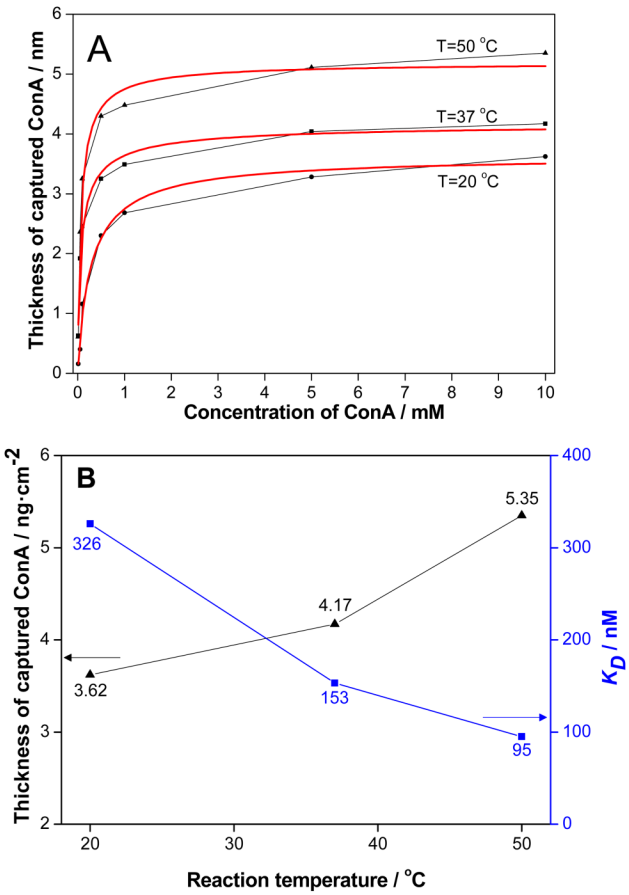

FIG. 5. (a) Comparison of the thickness of the captured ConA on various glycan biosensors prepared at different temperatures (i.e., 20, 37, and 50 °C). The red lines are the nonlinear fitting of the equilibrium isotherms based on the Hill equation. (b) Comparison of both the d_{max} and the K_D values for various glycan biosensors prepared at different reaction temperatures from 20, 37 to 50 °C. The reaction time is 20 h, and the DM concentration is 5 mg/ml.

explain this phenomenon is that BSA protein provides a 3D scaffold for DM immobilization and subsequent capture of ConA. Considering that the glycan biosensor prepared at T = 50 °C also has the lowest K_D value of 95 nM, it is apparent that a higher reaction temperature would contribute to the squaric acid-mediated coupling of sugar probes on the BSA-coated gold substrate.

C. Specificity and regeneration of the glycan biosensor

Nonspecific protein adsorption is known to reduce the specificity of various SPR-based biosensors. In order to investigate the specificity of the current glycan biosensor only to ConA rather than other lectins, another lectin PNA (i.e., peanut agglutinin, which binds specifically with galactose and lactose) was tested. As shown in Fig. 6, the binding of PNA with the current biosensor was negligible when compared with ConA of the same concentration. Moreover, the adsorption of PNA on the bare gold substrate was also measured [see Fig. S3 (Ref. 48)]. The result clearly indicates that the current glycan biosensor has very good specificity and could resist nonspecific adsorption of proteins. Regeneration of the current glycan biosensor was also investigated. As shown in Fig. 7, an NaOH solution of 10 mM was sufficient to remove all captured ConA from the surface, and the resulting biosensor could be reused for the detection of ConA for five times without a significant signal decrease. The results in Fig. 7 also suggest that the adsorbed BSA layer is very stable against the regeneration solution.

D. Limit of detection

In order to identify the limit of detection (LOD) of the current glycan biosensor, a series of ConA solutions ranging from 1 to 100 nM were tested sequentially, as shown in Fig. 8. One can find that 1 nM of ConA is sufficient to generate a clear increase in the SPR reflectivity. That means, the

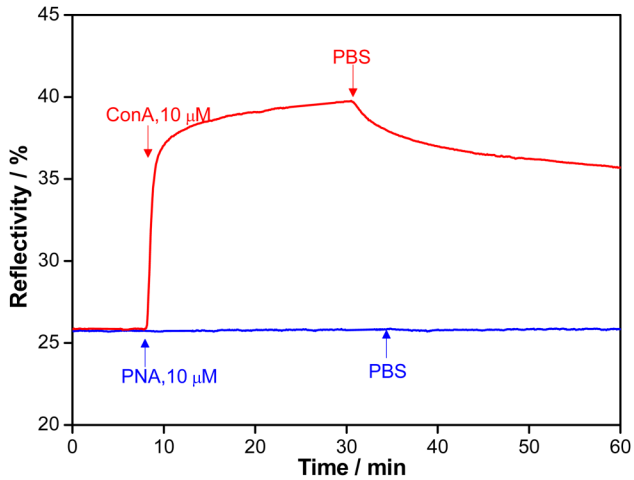

FIG. 6. Comparison of the binding of two lectins (i.e., PNA and ConA) with the glycan biosensor prepared with 5 mg/ml of DM at a reaction temperature of T = 50 °C.

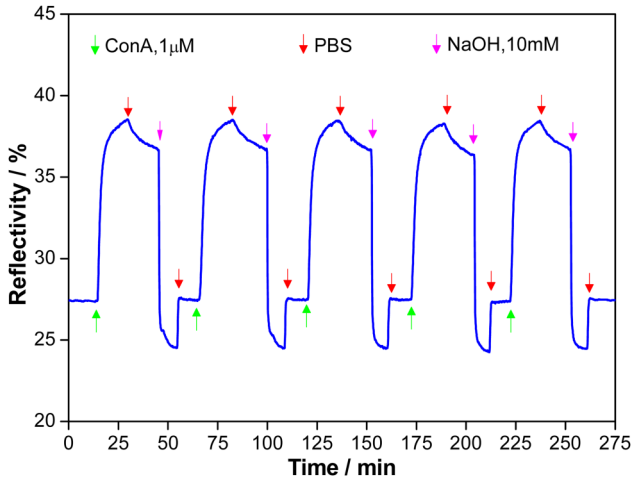

FIG. 7. SPR kinetic monitoring of the regeneration of a typical glycan biosensor (prepared with 5 mg/ml of DM at a reaction temperature of T = 50 °C) by using an NaOH solution of 10 mM.

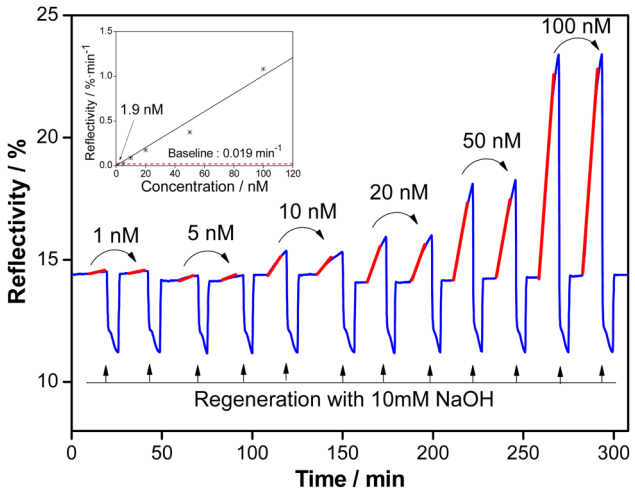

FIG. 8. SPR kinetic measurements for the binding of ConA with a concentration ranging from 1 to 100 nM on the glycan biosensor prepared with 5 mg/ml of DM at a reaction temperature of T = 50 °C.

lowest concentration that can be monitored is about 1 nM. At such a low concentration, the initial binding process is controlled by the diffusion of ConA from the bulk solution to the sensor surface, showing a linear response with time. The slope of the SPR reflectivity increase is proportional to the ConA concentration. A calibration curve is thus obtained by plotting the measured slopes vs the ConA concentration (see the inset of Fig. 8). The LOD is reached if the calibration curve intersects with the stability baseline. The baseline is defined as three times the standard deviation of the measurements of the time-dependent reflectivity without any ConA, which is about 0.019 min^{-1}. From the inset of Fig. 8, it can be seen that the LOD of the current SPR biosensor is 1.9 nM.

IV. CONCLUSIONS

SPR-based glycan biosensors with good selectivity and stability were simply obtained via a two-step process: (1) BSA adsorption on the bare gold substrate and (2) squaric acid-mediated coupling of the derived mannose probes with the lysine groups of BSA. SPR measurements of ConA binding indicated that both the DM concentration and the reaction temperature were critical for the immobilization of sugar probes, as well as the performance of SPR glycan biosensors. The glycan biosensor prepared either with 7 mg/ml of DM or at the reaction temperature of T = 50 °C gave rise to a higher thickness of the captured ConA and a lower K_D value. Moreover, the resultant biosensor exhibited a nonfouling surface property due to the use of BSA layer. The biosensor regeneration could also be achieved with an NaOH solution of 10 mM, which suggested that both the adsorbed BSA and the immobilized mannose probes were relatively stable. Therefore, the use of both BSA linker layer and squaric acid-mediated coupling reaction provides a facile and effective approach for the construction of SPR-based glycan biosensors. We believe that this strategy is also promising for the fabrication of other biomedical devices.

ACKNOWLEDGMENT

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