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Label-Free Detection of Antibody−Antigen Interactions on (*R*)-Lipo-diaza-18-crown-6 Self-Assembled Monolayer Modified Gold Electrodes

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Novel (*R*)-diaza-18-crown-6 has been prepared by a simple two-step synthetic method and characterized for its ability to form a uniform self-assembled monolayer (SAM) on gold as well as to immobilize proteins using atomic force microscopy, quartz crystal microbalance, and electrochemical impedance spectroscopy (EIS) experiments. The (*R*)-lipo-diaza-18-crown-6 was shown to form a well-defined SAM on gold, which subsequently captures the antibody (Ab) molecules that in turn capture the antigen (Ag) molecules. The Ab molecules studied include antibody C-reactive protein (Ab-CRP) and antibody ferritin (Ab-ferritin) along with their Ag's, i.e., CRP and ferritin. Quantitative detection of the Ab−Ag interactions was accomplished by EIS experiments with a $Fe(CN)_6^{3-/4-}$ redox probe present. The ratios of the charge-transfer resistances for the redox probe on the SAM−antibody-covered electrode to those with the antigen molecules attached show an excellent linearity for log[Ag] with lower detection limits than those of other SAMs for the electrochemical sensing of proteins.

Immunosensors that transform antibody−antigen (Ab−Ag) interactions to measurable signals such as fluoresence,[1,2] chemiluminescence,[3] electrical current,[4] and surface plasmon resonance[5] have been studied extensively for clinical diagnosis,[6] detection of environmental pollutants,[7,8] and food analysis.[9] A core technique for immunoassay is the development of advanced methods of immobilizing the proteins and controlling their orientation. Thus far, a number of immobilization methods on solid substrates, surfaces of which are chemically activated or functionalized by aldehydes,[10] conducting polymers,[11] biopolymers,[12,13] and streptavidin−biotin,[14,15] have been developed for constructing biological assemblies on solid matrixes. However, these methods have a few limitations such as minimally optimized orientations of proteins, weaker interactions, poor adsorption, loss of protein functions, and need for labeling agents. Self-assembled monolayers (SAMs) using thiol derivatives can be a useful tool as a capture probe for proteins providing high density and appropriate protein orientations while maintaining their activity.[16–18] For instance, bifunctionalized calix[4]crown-5 has been utilized for assembling highly sensitive protein microarrays for fluorophore labeled target proteins.[2a,b] The ammonium ions on the outer sphere of protein molecules were captured by the crown ether moiety of the calixcrown through the host−guest interactions. It was demonstrated by quartz crystal microbalance (QCM) and fluorescence scanning experiments that the Fc domain of an Ab molecule is preferentially immobilized on the calixcrown through the host−guest interaction between the ammonium ions on the Ab molecule and the crown having electron-donating ligand atoms (oxygen and nitrogen), with the Fv domains exposed to the solution to capture Ag's.[2a]

We report here that the (*R*)-lipo-diaza-18-crown-6 (hereafter called "lipo-diaza crown") molecule shown in Figure 1 can more effectively be used as a protein sensing probe. Although the

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(1) Sanchez-Martinez, M. L.; Aguilar-Caballos, M. P.; Gomez-Hens, A. *Anal. Chem.* **2007**, *79*, 7424.

(2) (a) Lee, Y.; Lee, E. K.; Cho, Y. W.; Matsui, T.; Kang, I. C.; Kim, T. S.; Han, M. H. *Proteomics* **2003**, *3*, 2289. (b) Oh, S. W.; Moon, J. D.; Lim, H. J.; Park, S. Y.; Kim, T.; Park, J. B.; Han, M. H.; Snyder, M.; Choi, E. Y. *FASEB J.* **2005**, *19*, 1335. (c) Lee, J.-Y.; Park, S.-M. *J. Phys. Chem. B* **1998**, *102*, 9940. (d) Choi, S.-J.; Choi, B. G.; Park, S.-M. *Anal. Chem.* **2002**, *74*, 1998.

(3) Fu, Z.; Hao, C.; Fei, X.; Ju, H. *J. Immunol. Methods* **2006**, *312*, 61.

(4) Fu, X. H. *Electroanalysis* **2007**, *19*, 1831.

(5) Kurita, R.; Yokota, Y.; Sato, Y.; Mizutani, F.; Niwa, O. *Anal. Chem.* **2006**, *78*, 5525.

(6) Watterson, J. M.; Stallcup, P.; Escamilla, D.; Chernay, P.; Reyes, A.; Trevino, S. C. *J. Clin. Lab. Anal.* **2007**, *21*, 162.

(7) Gonzalez-Martinez, M. A.; Puchades, R.; Maquieira, A. *Trends Anal. Chem.* **1999**, *18*, 204.

(8) VanEmon, J. M.; Lopez-Avila, V. *Anal. Chem.* **1992**, *64*, 79A

(9) Zacco, E.; Adrian, J.; Galve, R.; Marco, M. P.; Alegret, S.; Pividori, M. I. *Biosens. Bioelectron.* **2007**, *22*, 2184.

(10) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760.

(11) Cao, T. B.; Wei, F.; Jiao, X. M.; Chen, J. Y.; Liao, W.; Zhao, X.; Cao, W. X. *Langmuir* **2003**, *19*, 8127.

(12) Delgado, A. D.; Leonard, M.; Dellacherie, E. *Langmuir* **2000**, *17*, 4386.

(13) Muchova, L.; Jirsa, M.; Kuroki, M.; Dudkova, L.; Benes, M. J.; Marecek, Z.; Smid, F. *Biomed. Chromatogr.* **2001**, *15*, 418.

(14) (a) Nakanishi, K. H.; Muguruma, H.; Karube, I. *Anal. Chem.* **1996**, *68*, 1695. (b) Pei, R.; Cheng, Z.; Wang, E.; Yang, X. *Biosens. Bioelectron.* **2001**, *16*, 355.

(15) Mendoza, L. G.; McQuary, P.; Mongan, A.; Gangadharan, R. *BioTechniques* **1999**, *27*, 778.

(16) Kevin, L. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 10714.

(17) Huang, L.; Reekmans, G.; Saerens, D.; Friedt, J. M.; Frederix, F.; Francis, L.; Muyldermans, S.; Campitelli, A.; Van Hoof, C. *Biosens. Bioelectron.* **2005**, *21*, 483.

(18) Teramura, Y.; Iwata, H. *Anal. Biochem.* **2007**, *365*, 201.

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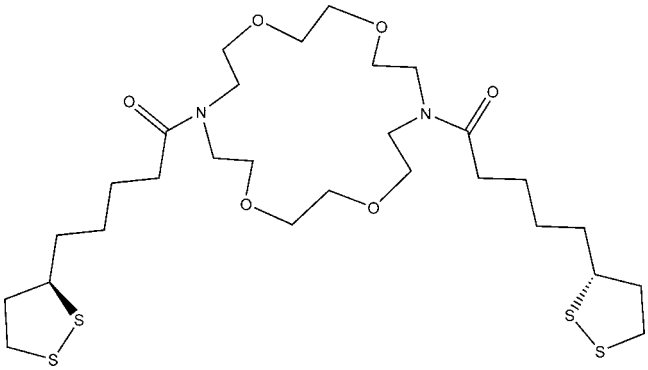

Figure 1. Structure of (*R*)-lipo-diaza-18-crown-6.

molecule has the same capability of capturing proteins through host−guest interactions as for thiolated calix[4]crown-5,[2a,b] it offers an improved conformation in that the crown ether ring is horizontally outstretched toward the protein surface, whereas the crown ether moiety of calix[4]crown-5 is attached vertically to the calixarene molecule, thus to the gold surface.[19] This difference in host conformations should affect its capability of capturing the guest molecule and thus the sensitivity of the sensor. Further, the lipo-diaza crown can be more tightly bound to the gold surface due to its two disulfide groups, which provide four thiol−gold bonds upon their cleavage.[20,21] Further, it is rather difficult to synthesize molecules containing more than two thiol groups due to their instability and the tendency to easily polymerize to form disulfides.

The blocking effect caused by the Ab−Ag adducts formed on the lipo-diaza crown SAM results in an increased charge-transfer resistance (R_{ct}) of the electrode for a redox probe. The ratio of R_{ct}'s before and after Ag's form adducts with the Ab molecules provides a basis for quantification of Ag's in solution. The increase in ratios was measured by simple, label-free electrochemical impedance spectroscopy (EIS) in a solution containing the $Fe(CN)_6^{3-/4-}$ redox probe. Two sets of Ab−Ag pairs, Ab-CRP and CRP (C-reactive protein) as well as Ab-ferritin and ferritin, were used for the study. The CRP is an inflammation marker for observing the progress of diseases and the efficacy of their treatment with their normal range below 3 mg/L.[22–25] Ferritin is an iron storage protein in our body with its nontoxic form serving as an iron overload or deficiency marker.[26,27] The capability of lipo-diaza crown to capture these proteins has been demonstrated by atomic force microscopy (AFM), QCM, and EIS experiments.

EXPERIMENTAL SECTION

Synthesis of Lipo-diaza-18-crown-6. Diaza-18-crown-6, thionyl chloride ($SOCl_2$), and solvents were obtained from Aldrich. (*R*)-Lipoic acid was purchased from Waterstone (U.S.A). (*R*)-Lipo-diaza-18-crown-6 (Figure 1) was synthesized by first converting the (*R*)-lipoic acid to the acid chloride using $SOCl_2$ according to the reaction scheme shown in Figure 2. A 2.92 mL aliquot of $SOCl_2$ was slowly dropped to the 4 mmol (*R*)-lipoic acid in 20 mL of chloroform, and the mixture was stirred overnight. After evaporation of the reaction mixture and the solvent, the residue was dissolved in chloroform. Then, 0.5 mmol of diaza-18-crown-6 and 50 μL of diisopropylethylamine were added to the solution, and it was stirred overnight. Finally, (*R*)-lipo-diaza-18-crown-6 thus synthesized was separated from the mixture through a short column with an eluent consisting of hexane/ethyl acetate = 3:1. A 1.0 mM lipo-diaza-18-crown-6 solution in chloroform was prepared as a stock solution for use in preparation of the SAM. The following data were obtained for the reaction product: mass peak calculated for $C_{28}H_{50}O_6N_2S_4$ (M^+) 638.255, found 637.269 in the mass spectrum (MALDI); ^{1}H NMR (300 MHz, $CDCl_3$), δ 3.67 (s, 8H), 3.58 (m, 2H), 3.14 (m, 4H), 2.46 (m, 2H), 2.34 (m, 4H), 2.03 (m, 4H), 1.93 (m, 2H), 1.72−1.64 (m, 16H), 1.55−1.43 (m, 8H).

Electrochemical Impedance Spectroscopy and Quartz Crystal Microbalance Experiments. The interaction of the Ab molecule with the Ag was detected by EIS experiments on the lipo-diaza crown SAM formed on a gold electrode. A BAS MF-2014 gold disk electrode (0.020 cm^2) was first polished successively with the alumina powder of 14.5, 5, 1, 0.3, 0.05 μm. How well the electrode was cleaned was confirmed by examining its cyclic voltammograms in 1.0 M H_2SO_4. Then, the lipo-diaza crown SAM was prepared by dipping the cleaned gold electrode into its 1.0 mM solution in chloroform for 3 h. The lipo-diaza crown SAM-covered gold electrode was then washed thoroughly with chloroform and acetone to remove any nonspecifically adsorbed molecules, followed by drying with a N_2 flow. The electrode thus modified was used as a working electrode with a Ag/AgCl reference electrode (in saturated KCl) and a platinum counter electrode.

Impedance data were obtained at the SAM-covered electrode in a 10 mM phosphate-buffered saline (PBS: pH 7.4) solution containing a redox probe (2.5 mM each $Fe(CN)_6^{3-/4-}$) and 0.50 M KCl at the dc bias potential set equal to an open circuit potential (OCP). At the OCP established by an equimolar concentration of $Fe(CN)_6^{3-/4-}$ (0.24 V vs the Ag/AgCl electrode), the overpotential is 0.0 V and the charge-transfer resistance (R_{ct}) obtained represents the resistance for the electron transfer to the redox pair.[28,29] "Polarization" resistances are obtained at other potentials such as at the peak potential, which can be shifted depending on the reversibility of the reaction;[29a] the reversibility is determined by the state of the electrode/electrolyte interface. The lipo-diaza crown thus immobilized captures Ab molecules when dipped in a 100 μL Ab solution (26 μg/mL Ab-CRP in a pH 7.0 bis-Tris buffer or 47 μg/mL Ab-ferritin in the same buffer solution) for 2 h. Then, the Ab molecules immobilized on the gold electrode recognize

(19) Park, J.-Y.; Kim, B.-C.; Park, S.-M. *Anal. Chem.* **2007**, *79*, 1890.
(20) Carot, M. L.; Esplandiu, M. J.; Cometto, F. P.; Patrito, E. M.; Macagno, V. A. *J. Electroanal. Chem.* **2005**, *579*, 13.
(21) Kitano, H.; Makino, Y.; Kawasaki, H.; Sumi, Y. *Anal. Chem.* **2005**, *77*, 1588.
(22) Nystrom, T. *Clin. Sci.* **2007**, *113*, 79.
(23) Koenig, W.; Sund, M.; Frohlich, M.; Fischer, H. G.; Lowel, H.; Doring, A.; Hutchinson, W. L.; Pepys, M. B. *Circulation* **1999**, *99*, 237.
(24) Blake, G. J.; Ridker, P. M. *Circ. Res.* **2001**, *89*, 763.
(25) (a) Ford, E. S.; Giles, W. H.; Myers, G. L.; Mannino, D. M. *Clin. Chem.* **2003**, *49*, 686. (b) Lin, S.; Lee, C. K.; Wang, Y. M.; Huang, L. S.; Lin, Y. H.; Lee, S. Y.; Sheu, B. C.; Hsu, S. M *Biosens. Bioelectron.* **2006**, *22*, 323.
(26) Andrew, S. C.; Harrison, P. M.; Yewdall, S. J.; Arosio, P.; Levi, S.; Bottke, W.; Darl, M. v.; Briat, J.-F.; Laulhere, J.-P.; Lobreaux, S. *J. Inorg. Biochem.* **1992**, *47*, 161.
(27) Theil, E. C. *Annu. Rev. Biochem.* **1987**, *56*, 289.
(28) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods*, 2nd ed.; Wiley: New York, 2001; Chapters 1 and 3.
(29) (a) Park, S.-M.; Yoo, J.-S. *Anal. Chem.* **2003**, *75*, 455A. (b) Chang, B.-Y. *Anal. Chem.* **2006**, *78*, 1052. (c) Chang, B.-Y. *Anal. Chem.* **2007**, *79*, 1890.

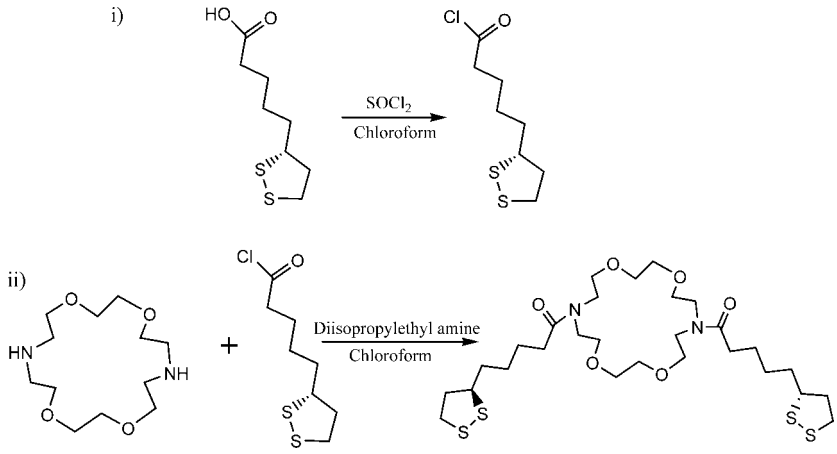

Figure 2. Synthetic scheme for (*R*)-lipo-diaza-18-crown-6.

varied concentrations of Ag molecules dissolved in a pH 7.0 bis-Tris buffer solution for 2 h. Impedance data were obtained before and after the Ab molecules bind with Ag's in the same solution. The dc bias potential of 0.24 V was applied with an ac voltage of ±5 mV (peak-to-peak) of a frequency range of 100 kHz to ~100 mHz overlaid, and the impedance measurements were made using a Solartron model SI 1255 HF frequency response analyzer connected to an EG&G 273 potentiostat/galvanostat. The measurements were stopped when the impedances began to show a Warburg component at low frequencies due to mass transfer effects as it has no significance in our measurements. Only the R_{ct} value at the OCP presents the degree of interactions between Ab and Ag molecules. The R_{ct} values were then obtained by fitting the impedance data with the ZsimpWin program (Princeton Applied Research) with an appropriate equivalent circuit.

A Biomechatron model 1000L EQCN (Jeonju, Korea) electrochemical quartz crystal analyzer (EQCA) was used to measure the amount of (*R*)-lipo-diaza-18-crown-6 molecules adsorbed on a gold-coated, AT-cut quartz crystal QCM electrode with a fundamental frequency of 9.0 MHz and an area of 0.196 cm^2. The decrease in frequency was monitored upon addition of a 100 μL aliquot of 1 mM lipo-diaza crown in chloroform to 2.0 mL of chloroform in an EQCA cell. The sensitivity of the machine, 1.41 (±0.02) ng/Hz/cm^2, was obtained by calibration with silver deposition. A 100 μL aliquot of the Ab solution (26 μg/mL Ab-CRP or 47 μg/mL Ab-ferritin) was injected to the 10 mM bis-Tris buffer (2 mL) solution in the EQCA cell, and then the change in frequency was monitored after the baseline was stabilized. After immobilization of the Ab, the EQCA electrode was washed with a 10 mM bis-Tris buffer (pH 7.0) without disassembling the cell, and 100 μL of the Ag solution (23 μg/mL CRP or 1.0 μg/mL ferritin) was then added to a 2 mL blank solution of 10 mM bis-Tris buffer after the EQCA cell was stabilized.

Atomic Force Microscopy Observations. Gold-on-silicon electrodes (Inostek, Ansan, Korea: 200 Å thick gold) were utilized as substrates for the AFM observation after they were cleaned in a piranha solution (70% H_2SO_4/30% H_2O_2) for 1 min and annealed with a H_2 flame for 3 min. The cleaned substrate was immersed in a 1 mM lipo-diaza crown solution for 3 h. After washing with chloroform and acetone, the SAM-covered electrode immobilized the Ab molecules in a bis-Tris buffer (pH 7.0) containing 26 μg/mL Ab-CRP or 47 μg/mL Ab-ferritin, which was then immersed in an Ag solution containing CRP (2.3 μg/mL) or ferritin (1.0 μg/mL). The gold-on-silicon electrode with Ab's or Ag's immobilized on its surface was then washed with a 10 mM PBS buffer, and their images were recorded using a Veeco Dimension V in the National Center for Nanomaterials Technology (NCNT), POSTECH, Korea. The AFM tips were etched silicon probes with their apex radius of less than 10 nm, and the images were recorded in a tapping mode.

RESULTS AND DISCUSSION

Quartz Crystal Microbalance Results on the Formation of the (*R*)-Lipo-diaza-18-crown-6 SAM. We performed QCM experiments to confirm quantitative formation of SAMs as well as interactions between Ab and Ag molecules. The binding reaction of disulfide bonds with the gold surface was confirmed through the QCM experiments. As shown in Figure 3a, the decrease in frequency of −21 Hz was recorded for the immobilization of lipo-diaza crown, which corresponds to about 1.4×10^{14} molecules/cm^2. This is between adsorbed molecular densities of 0.83×10^{14} molecules/cm^2 for thiolated β-cyclodextrin (β-CD) and 1.9×10^{14} for α-CD.[2c,d] The area occupied by a lipo-diaza-18-crown-6 is calculated to be about 71 $Å^2$, which is about 1.7 times of that of thiolated calix[4]crown-5.[19] Thus, the lipo-diaza crown has a little larger available intercrown space than for calix[4]crown-5 to immobilize protein molecules for more effective Ab binding, which can be advantageous for more specific Ab−crown interactions. This is because diaza-18-crown-6 has a little larger crown size in comparison to calix[4]crown-5. After the SAM formation, the electrode was cleaned with chloroform and dried by purging with the N_2 gas. Then, 100 μL of the Ab ferritin solution (47 μg/mL) was added to the stabilized EQCA cell containing 2 mL of the bis-Tris buffer (pH 7.0) solution. Figure 3b shows the result of the immobilization of Ab ferritin on the lipo-diaza crown SAM, in which a few breaks are noted in the curve. The breaks probably indicate that Ab-ferritin molecules get adsorbed at a certain rate, followed by their stabilization through reorientation on the surface. This process is repeated a few times until there is no further decrease in frequency due to thermodynamic stability. The final frequency decrease was −141 Hz, which corresponds to approximately 4.1×10^{12} molecules/cm^2 of bound molecules. This surface density is in excellent agreement with a theoretical value of 4.06×10^{12} molecules/cm^2.[2a] After washing the nonspecifically

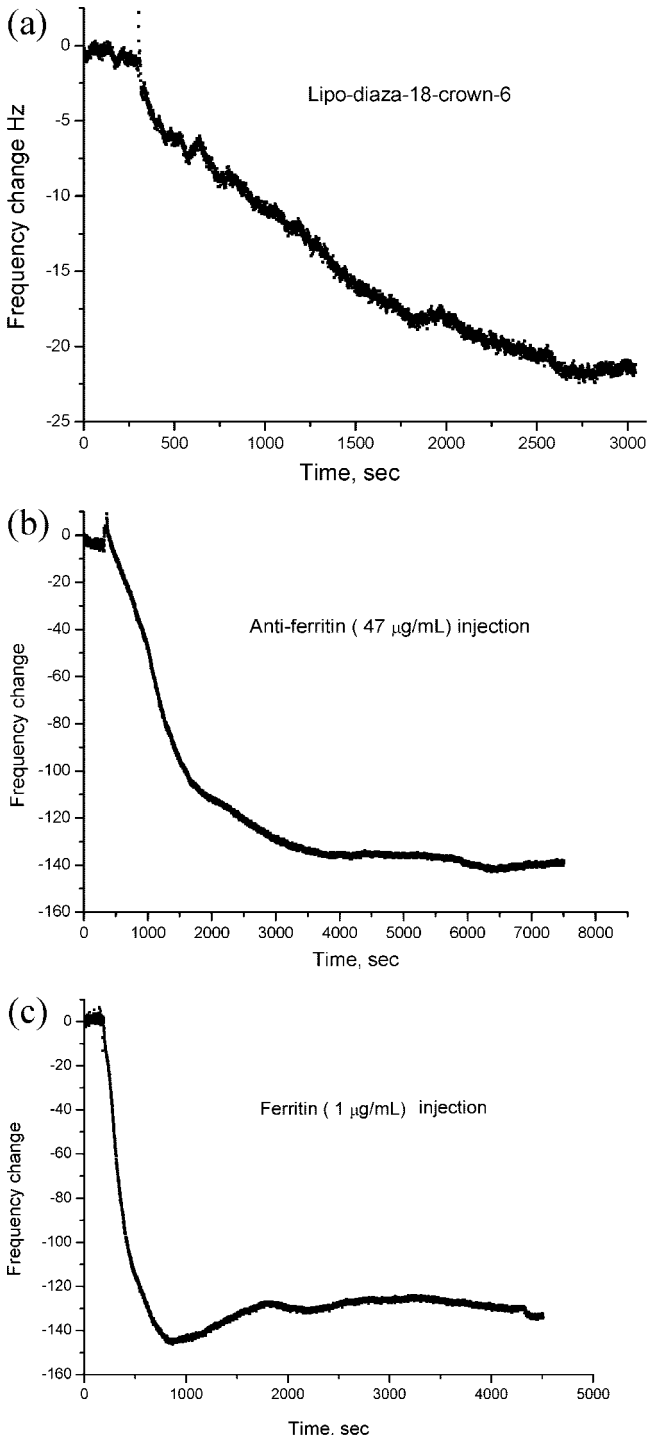

Figure 3. Results of QCM experiments: (a) decrease in frequency due to the formation of the lipo-diaza crown SAM, (b) further decrease observed upon injection of 100 μL of the Ab-ferritin solution (47 μg/mL) to 2 mL of pH 7.0 bis-Tris buffer solution, and (c) further decrease upon addition of 100 μL of the Ag, ferritin, solution (1 μg/mL).

binding Ab's with the bis-Tris buffer 7.0, the electrode was exposed to a 1 μg/mL ferritin Ag solution in the bis-Tris buffer solution. The decrease in frequency was −126 Hz, corresponding to 1.2×10^{12} molecules/cm^2.

Similar results were obtained for Ab-CRP and CRP. The decrease in frequency was −166 Hz for immobilization of Ab-CRP, which was followed by −152 Hz of the frequency decrease for subsequent binding of CRP. The adsorption densities of bound molecules are 4.8×10^{12} and 2.9×10^{13} molecules/cm^2, respectively, for Ab-CRP and CRP. Through the QCM experiments, we see that lipo-diaza crown molecules effectively capture the ammonium ions on the spherical surfaces of protein molecules through the host−guest interactions as the thiolated calix[4]crown-5 molecules do.

Atomic Force Microscopy Observations. The AFM images of adsorbed Ab and Ag molecules were obtained on lipo-diaza crown SAM-modified gold-on-silicon electrodes. As control experiments, bare and lipo-diaza crown SAM-covered gold electrodes were subjected to the AFM analysis. The result was that the image of the SAM-covered surface is hardly different from that of the bare gold as shown in Figure 4a; only large globular particles of gold are seen regardless of whether or not the lipo-diaza crown molecules are present on the surface. This is because each lipo-diaza crown molecule is too small to be resolved from each other by AFM imaging. After dipping the SAM-modified surface into the Ab solution (26 μg/mL in bis-Tris buffer pH 7.0) for 3 h, spherical Ab-CRP particles compactly spread on the gold surface modified with lipo-diaza crown were rather clearly seen as can be seen in Figure 4b. The change in morphology between bare gold and Ab-modified surfaces is easily recognized when the height profiles shown on the right of Figure 4, parts a and b, are compared; the annealed surface of the gold globule is smooth as seen in Figure 4a, whereas three Ab-CRP molecules with an average diameter of 14.4 nm and an approximate height of 1 nm are clearly seen on the top of a gold globule. After the Ab-CRP molecules bound to the lipo-diaza crown SAM are exposed to the 2.3 μg/mL CRP solution in bis-Tris buffer pH 7.0 for 2 h, smaller CRP particles of about 9.9 nm are observed (Figure 4c). This change in size of the particles when the surface layer was changed from Ab-CRP to CRP is reasonable because the CRP (23 kDa), whose molecular size has been reported to be about 10 nm,[25] should be smaller than that of Ab-CRP (150 kDa). Similar observations were made for Ab-ferritin and ferritin (not shown), except that the particle size became larger for ferritin than for Ab-ferritin. This is consistent with the molecular weight of ferritin (440 kDa), which is larger than that of Ab-ferritin (150 kDa). The Ab-ferritin is similar to Ab-CRP in size as it is the same type of Ab as immunoglobulin G.

In conclusion, biomolecules such as Ab-CRP and CRP as well as Ab-ferritin and ferritin are strongly and uniformly immobilized on the lipo-diaza crown SAM by the host−guest binding between the ammonium ions derived primarily from lysine molecules of proteins and the cavities of lipo-diaza crown molecules, as was shown by the results of QCM and AFM experiments. An immunosensor can thus be constructed using the newly synthesized lipo-diaza crown.

Electrochemical Impedance Spectroscopy Studies of Antibody−Antigen Binding. The detection technique for the Ab−Ag interactions using EIS does not need any labeling such as for fluorophores,[30–33] magnetic beads,[34,35] or enzymes,[36,37] which produce the measurable signals. It is based on the change in the electrochemical property of the electrode/solution interface due to the interaction of the probe proteins with target proteins. We thus used the EIS in this study to quantify the bound targets in this work. Under the experimental conditions used in this study, the electron transfer to/from the

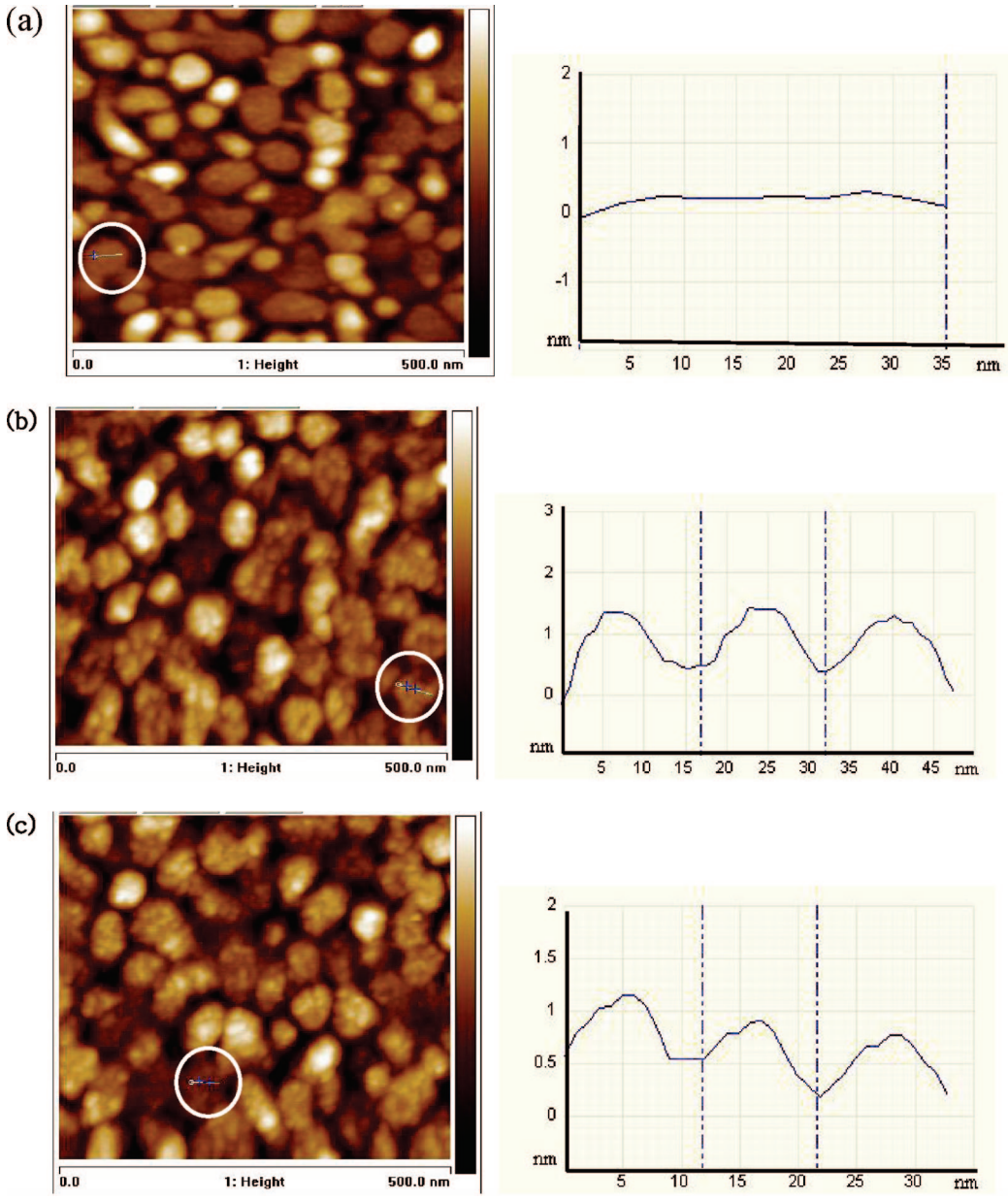

Figure 4. AFM images: (a) bare gold electrode after it is cleaned with the piranha solution and annealed with a H_2 flame, (b) lipo-diaza crown SAM-covered electrode with Ab-CRPs captured, and (c) the electrode with a topmost CRP layer. See the Ab-CRP particles with a diameter of about 14.4 nm in (b) and CRP globules with a diameter of about 9.9 nm (c) on the large gold globules. Graphs on the right show the expanded height profiles of the marked images shown on the left. The dotted lines indicate where the dips in heights are observed.

electrode surface from/to the redox probe can be simplified by the two paths shown in Figure 5a and the measured impedance data can be simulated using a circuit shown in Figure 5b. The first path (R_{ct1}), which is more probable than the second (R_{ct2}), should be through the vacant space between the SAM molecules. On the other hands, the second route, R_{ct2}, represents the electron transfer via lipo-diaza crown molecules. When the rate of electron transfer becomes faster than the frequency of the ac wave overlaid on the dc bias, the process would be limited by the Warburg impedance (W), i.e., mass transport of the redox probe ions to the reaction site on the electrode surface.[29,38] Because the SAM-modified surface provides nanoporous or microporous environments, well-defined capacitors are usually not formed, resulting in capacitive dispersions.[38] This is described as the constant phase element (CPE), Q. Finally, we should point out that R_{ct2}, which represents the electron transfer via protein molecules adsorbed on the lipo-diaza crown ring, is so large and is thus bypassed in the calculation of the total charge-transfer resistance. Thus, the binding of Ab to Ag affects more R_{ct1} by slowing down the electron transfer through blocking the intercrown space on the surface than R_{ct2} through the molecules on the crown.

(30) Hatch, A.; Kamholz1, A. E.; Hawkins, K. R.; Munson, M. S.; Schilling, E. A.; Weigl, B. H.; Yager, P. *Nat. Biotechnol.* **2001**, *19*, 461.
(31) Horie, M.; Yanagisawa, H.; Sugawara, M. *Anal. Biochem.* **2007**, *369*, 192.
(32) Wang, Z. H.; Zhu, Y.; Ding, S. Y.; He, F. Y.; Beier, R. C.; Li, J. C.; Jiang, H. Y.; Feng, C. W.; Wan, Y. P.; Zhang, S. X.; Kai, Z. P.; Yang, X. L.; Shen, J. Z. *Anal. Chem.* **2007**, *79*, 4471.
(33) Su, F. Y.; Endo, Y.; Saiki, H.; Xing, X. H.; Ohmura, N. *Biosens. Bioelectron.* **2007**, *22*, 2500.

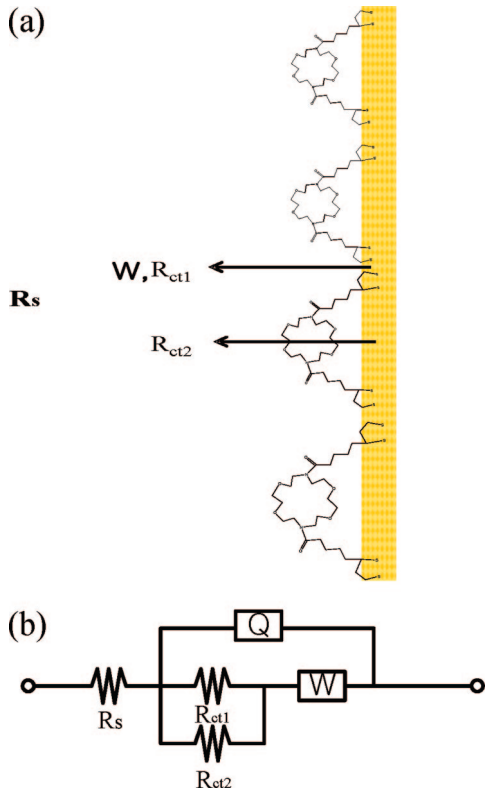

Figure 5. (a) Model for electron transfer to/from the redox probe at the SAM-covered electrode and (b) an equivalent circuit for the interface shown in (a). Here R_s is the solution bulk resistance, R_{ct1} is the resistance for electron transfer through the space between the lipo-diaza crown molecules of the SAM, and R_{ct2} is through the SAM layer, W is the Warburg impedance, and Q (constant phase element) represents the dispersion in local capacitances at the surface.

The simulation results of impedance responses shown in Figure 6 are listed in Table 1. Note that R_{ct2}values are significantly larger than R_{ct1} values as expected from the model shown in Figure 5a and discussed above. When the cavities of the lipo-diaza crown SAM-covered electrode immobilize Ab-CRP, the total resistance of charge transfer to the $Fe(CN)_6^{3-/4-}$ redox pair is increased as shown in Figure 6 due to the blocking effect of the electrode. The charge-transfer resistances increase progressively upon formation of the lipo-diaza crown SAM, SAM's immobilizing Ab-CRP molecules, Ab-CRPs binding with CRPs, and increasing the CRP concentration. The ratios of the total charge-transfer resistances before and after the Ag binding, obtained by the parallel connection of the two parallel charge-transfer resistances ($R_{ct1}//R_{ct2}$, i.e., $1/R_{total} = 1/R_{ct1} + 1/R_{ct2}$) are shown to be linearly dependent on log[CRP] as shown in Figure 7. An excellent linear relation is seen between the ratio of impedances before and after the Ag binding and log[CRP], when the impedance data were obtained in an aqueous solution containing 10 mM PBS (pH 7.4), a redox probe (2.5 mM $Fe(CN)_6^{3-/4-}$ each), and 0.50 M KCl. In the case of ferritin also, a similar plot showing the linear relation between the charge-transfer resistances and log(ferritin) is obtained under the same experimental conditions as for CRP except that the solution has 0.25 M $LiClO_4$ supporting electrolyte and 10 mM bis-Tris buffer (pH 8.5) (Figure 7b). Again, similar results were obtained for the Ab-CRP/CRP pair at the calix[4]crown-5 SAM as shown in Figure 6b and summarized in Table 1. However, the R_{ct} values are generally larger at the calix[4]crown-5 SAM than those at the lipo-diaza crown SAM with smaller R_{ct} ratios when the experiments are run under identical conditions. This is probably because the crown ring hanging vertically to the calix[4]crown-5 ring, and thus to the surface also, plugs up the calixarene ring and prevents the redox probe from approaching, as already pointed out.

In order to check how nonspecific adsorption of proteins affects the results, we ran EIS experiments on a bare gold electrode with and without Ag's present in the solution. A typical result is shown in Figure 6c, in which the EIS data obtained at a bare gold electrode with and without ferritin present are shown. As can be seen, no significant increase in the R_{ct} value was observed due to the presence of ferritin. We also ran experiments on the other Ab and Ag molecules, but the increases in R_{ct} values were insignificant in comparison to those experiments run with the Ab's immobilized on the lipo-diaza crown SAM. We also found that an electrode, which displayed an increase in the R_{ct} value upon nonspecific adsorption of Ab's or Ag's, reverted almost to the bare electrode when it was washed as was the case for immobilized Ab's on the lipo-diaza crown SAM. Although a bare gold electrode exposed to solutions containing Ab or Ag showed small increases in R_{ct} values due to nonspecific adsorption as shown in Figure 6c, quantitative adsorption of Ag via interactions with Ab is shown to deter the nonspecific adsorption at the Ab-immobilized electrode as shown by the QCM experiments and the linearity in Figure 7. We should also point out that Ab's were found to bind the crown ring with their Fc domain[2a] because it has 38 amine (lysine) groups sticking out on its surface in immunoglobulin G, e.g., whereas only six lysine groups are on the Fv domain.[39] For this reason, the Fc domain binds strongly and stably to the crown ring on the electrode surface with the Fv domain facing up to capture Ag's.

Figure 7 shows that the ratios of the charge-transfer resistances before and after adding Ag's are linear with respect to log[Ag], not [Ag] itself. This is explained by the Butler−Volmer relation for the electrochemical current, i, with the overpotential, η, which has the form

$$i = i^0\left[e^{-\frac{\alpha nF}{RT}\eta} - e^{\frac{(1-\varepsilon)nF}{RT}\eta}\right] \quad (1)$$

where i^0 is the exchange current, α is the transfer coefficient, and other symbols have their usual meanings. The charge-transfer resistance, R_{ct}, is defined by $d\eta/di$,[29a] and has thus a mixed exponential relation with i, which in turn is directly related to the concentration of the electroactive species. In our case, i decreases due to the blockage of the electrode surface depending on [Ag] in the sample fluid. Therefore, R_{ct} would increase with a mixed exponential relation with i and thus with [Ag]. When the R_{ct} ratios

(34) Nichkova, M.; Dosev, D.; Gee, S. J.; Hammock, B. D.; Kennedy, I. M. *Anal. Biochem.* **2007**, *369*, 34.
(35) Mavre, F.; Bontemps, M.; Ammar-Merah, S.; Marchal, D.; Limoes, B. *Anal. Chem.* **2007**, *79*, 187.
(36) Rowe, C. A.; Tender, L. M.; Feldstein, M. J.; Golden, J. P.; Scruggs, S. B.; MacCraith, B. D.; Cras, J. J.; Ligler, F. S. *Anal. Chem.* **1999**, *71*, 3846.
(37) Eteshola, E.; Leckband, D. *Sens. Actuators, B* **2001**, *72*, 129.
(38) Barsoukov, E.; Macdonald, J. R. *Impedance Spectroscopy: Theory, Experiment, and Application*; John Wiley & Sons, Inc.: NJ, 2005.
(39) National Center for Biotchnology Information (NCBI) website. www.ncbi.nih.gov.

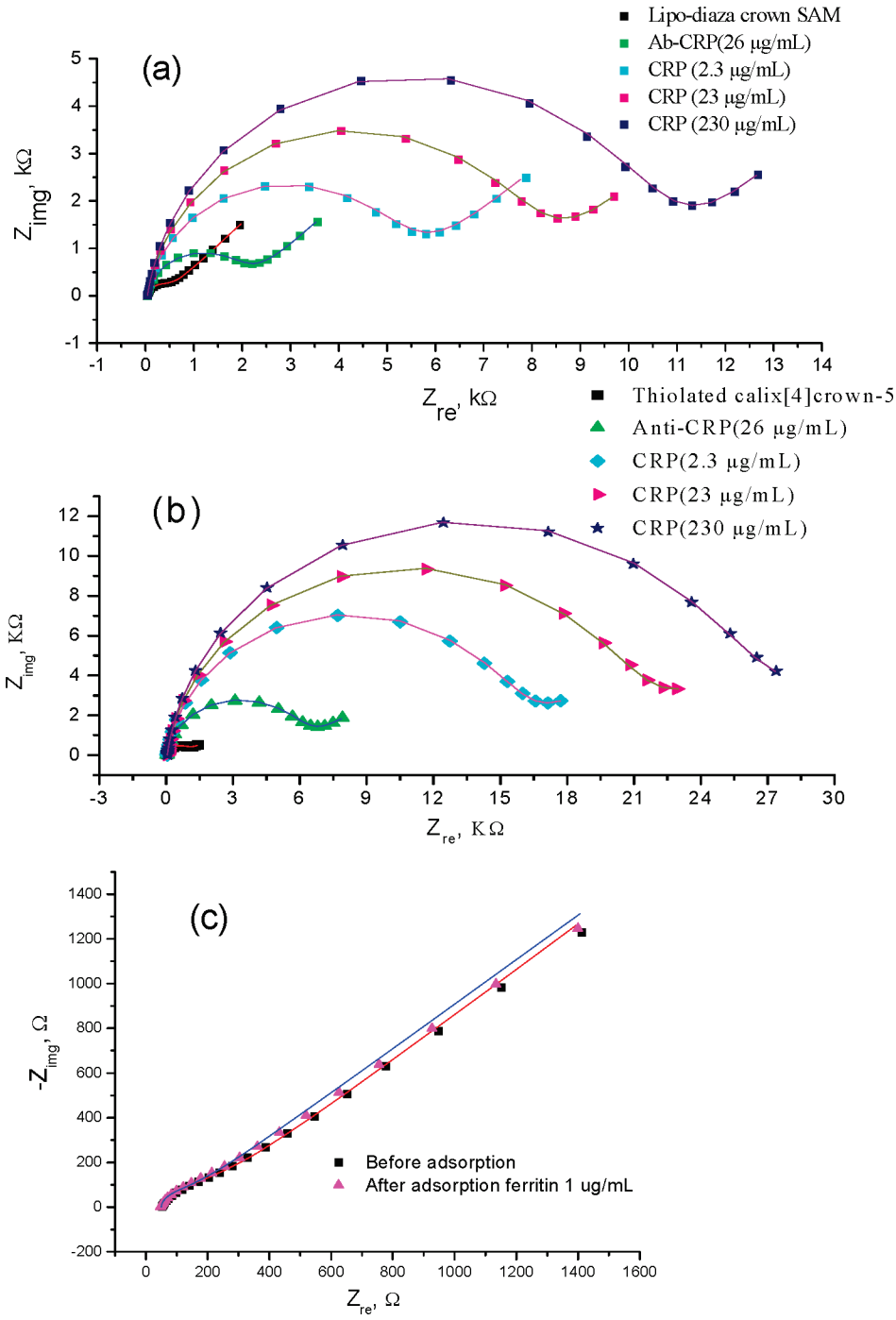

Figure 6. Comparison of the EIS results obtained from lipo-diaza crown (a) and thiolated calix[4]crown-5 (b) SAMs as well as interactions of Ab-CRP with different concentrations of CRP. The EIS measurements were made in the solution containing the redox probe (2.5 mM $Fe(CN)_6^{3-/4-}$ each) and 0.50 M KCl under identical experiment conditions.

are actually plotted against [Ag], it is linear in a small concentration range due to the linearity approximation[28] but becomes logarithmic in a large concentration range. In our case, the concentration spans over 2−3 orders of magnitude as seen in Figure 7, and thus, the relation is complex logarithmic.

Finally, we performed the CRP analysis on Olympus control blood serum 1 (Olympus Life and Material Science Europa GmbH, Hamburg, Germany), which contains 2.3 μg/mL of CRP along with a host of other components, to confirm that our method is applicable to real samples. For three analyses with the sample, we obtained an average R_{ct} ratio of 2.60 ± 0.01, which is translated into the CRP content of 2.4 ± 0.1 μg/mL. This is an excellent agreement; Olympus states that the concentration range of 1.7−2.9 μg/mL would be satisfactory for the CRP analysis kit they are marketing.

When an Ag, CRP or ferritin, was adsorbed on the lipo-diaza crown SAM *without* its Ab preimmobilized, the total resistance of charge transfer was slightly increased or even decreased, particularly when ferritin was immobilized. A ferritin molecule contains thousands of Fe^{3+} ions, possibly facilitating the electron transfer directly through the bound ferritin, i.e., the R_{ct2} route. Thus, the increase in resistance observed when the Ag's bind to

Table 1. Parameters Obtained from Simulation of Impedance Data[a]

lipo-diaza crown receptor	SAM lipo-diaza crown	Ab-CRP (26 μg/mL)	CRP (2.3 μg/mL)	CRP (23 μg/mL)	CRP (230 μg/mL)
R_s	42.6	44.1	41.2	41.6	42.0
Q	2.14×10^{-6}	1.66×10^{-6}	1.50×10^{-6}	1.33×10^{-6}	1.17×10^{-6}
n	0.892	0.888	0.891	0.897	0.904
R_{ct1}	455.1	2036	5705	7941	10 460
W	1.80×10^{-4}	2.19×10^{-4}	1.93×10^{-4}	2.45×10^{-4}	2.46×10^{-4}
R_{ct2}	110 500	100 500	75 770	3.31×10^{11}	2.29×10^{10}
R_{total} (= R_{ct1}/R_{ct2})	453.2	1995.6	5305.5	7941.0	10 460
ratio (= $R_{Ab\text{-}CRP}/R_{Ab}$)			2.66	3.98	5.24
calix[4]crown-5 receptor	SAM calix[4]crown-5	Ab-CRP (26 μg/mL)	CRP (2.3 μg/mL)	CRP (23 μg/mL)	CRP (230 μg/mL)
R_{total} ($R_{Ab\text{-}CRP}/R_{Ab}$) ratio	925.7	6042	15 553 2.57	20 782 3.44	25 851 4.28

[a] The parameters were obtained by simulating the impedance data as shown in Figure 6 employing the equivalent circuit shown in Figure 5b at both lipo-diaza crown and calix[4]crown-5 SAM-covered electrodes.

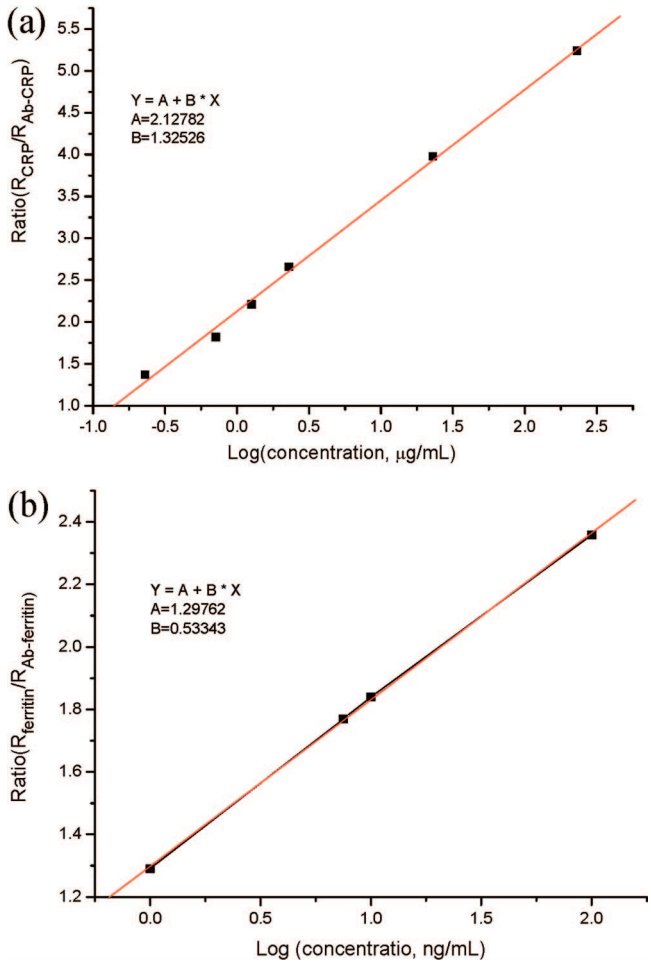

Figure 7. Ratios of charge-transfer resistances ($R_{CRP}/R_{Ab\text{-}CRP}$, $R_{ferritin}/R_{Ab\text{-}ferritin}$) plotted vs log[CRP] (a) and log[ferritin] (b). The detection limit is 220 ng/mL for CRP and 0.317 ng/mL for ferritin.

the Ab's must have originated from the formation of Ab−Ag adducts, not from the nonspecific binding of the Ag. As can be seen from Table 1, the binding affinity of Ab-CRP to the lipo-diaza crown SAM is significantly enhanced when compared with that to the thiolated calix[4]crown-5 SAM; the slope of the $R_{ab\text{-}CRP}/R_{Ab}$ ratio versus log[CRP] in Table 1 is 1.29 and 0.86, respectively, at the lipo-diaza crown and at the calix[4]crown-5 SAM-modified electrodes. The difference between the two crown ethers must have arisen from the orientation of the crown ether for the host−guest interaction. Again, the crown ring is horizontally exposed to the surface of proteins on the lipo-diaza crown SAM-covered electrode, so the ammonium ions of amino acids can easily bind to it. Thus, the sensitivity of the lipo-diaza crown SAM is enhanced for the recognition of target proteins compared to that of calix[4]crown-5 and it offers a better platform for immunoassay of enzymes and amines minimizing the nonspecific binding.

CONCLUSIONS

As demonstrated in this work, efficient surface chemistry plays a critical role in determining orientations of proteins and protecting their activity when they are immobilized on a solid matrix. Noncovalent binding methods have some limitations such as little control of orientations and quantitative binding of proteins. The lipo-diaza crown synthesized in our study has been shown to densely immobilize proteins without loss of their activity and nonspecific binding. The molecule was designed such that its host moiety would be horizontally oriented toward the guest ions of protein surfaces by tightly linking the diaza crown to the gold surface with four sulfur atoms. Although it is difficult to synthesize the multiply thiolated compound due to the easy formation of disulfide bonds, the lipo-diaza crown can be easily prepared through a simple two-step reaction. Its formation of the SAM and binding affinity of the Ab−Ag pair were confirmed by a variety of experiments. The proteins were shown to compactly cover the gold surface from the closeness of QCM measurements of the adsorbed molecules to the theoretical value[1,2] and by examination of AFM images. In conclusions, our study shows how orientations of host molecules affect the sensitivity of biosensors and how they can be designed to effectively capture analyte molecules.

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