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Impedance Biosensor for Peanut Protein Ara h 1

Yin Huang, Melissa C. Bell, and Ian I. Suni*

Department of Chemical and Biomolecular Engineering and Center for Advanced Materials Processing (CAMP), 8 Clarkson Avenue, Clarkson University, Potsdam, New York 13699-5705

A reagentless electrochemical impedance biosensor for detection of peanut protein Ara h 1, one of the allergenic proteins found in peanuts, has been demonstrated using an Au substrate onto which an antibody film has been immobilized. Following initial stabilization of the self-assembled monolayer (SAM) through which the antibody is immobilized, the biosensor substrate exhibits stable impedance spectra at different stages of substrate preparation. By fitting the impedance spectra to a Randles equivalent circuit, one can demonstrate that the charge-transfer resistance (R_{ct}) increases and the differential capacitance (C_d) decreases with increasing concentration of Ara h 1, although R_{ct} exhibits greater sensitivity. The detection limit of this reagentless biosensor is estimated to be less than 0.3 nM. Assuming a Langmuir adsorption isotherm, the dissociation constant of the peanut protein Ara h 1 and its antibody can be calculated as 0.52 nM from the variation in differential capacitance with Ara h 1 concentration.

Because allergy immunotherapy is currently unavailable, food allergens present a significant public health hazard. Food allergies are especially common among children, afflicting about 6−8% of children less than 3 years of age.[1] Most food allergies are caused by crustaceans, fish, eggs, peanuts, milk, tree nuts, soybeans, or gluten-containing cereals. Peanuts are considered one of the most dangerous food allergens, with severe anaphylactic reactions causing over 100 fatalities in the United States alone each year.[2] Exposure to peanut allergens is often inadvertent, arising from ingestion of foods where the presence of peanuts is unexpected.[3] Food allergens are typically proteins or glycoproteins. Nine potentially important allergens within peanuts have been identified, Ara h 1 to Ara h 8, and peanut oleosin.[4,5] Ara h 1 and Ara h 2 have been widely described as the most important allergens, although this has sometimes been disputed.[4]

Surprisingly, biosensors for food allergens have not attracted substantial research attention. Current methods for detecting peanut proteins are based on enzyme-linked immunosorbent assays (ELISA),[6−9] which are time-consuming, require trained personnel, are difficult to automate and miniaturize, and are not fully standardized.[10] For these reasons, ELISA is unlikely to prove useful for point-of-use applications, where portable and immediate detection is needed, so alternative immunosensors to ELISA are considered highly desirable.[11] Biosensors for food allergens have been reported using capillary electrophoresis/laser-induced fluorescence,[12] liquid chromatography/mass spectrometry,[13] and electrochemical impedance spectroscopy (EIS).[14]

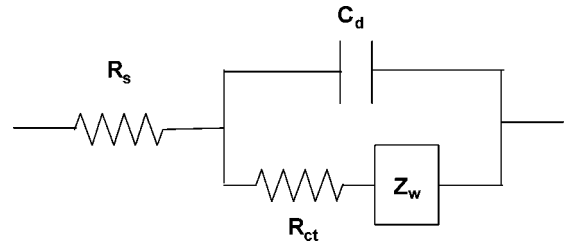

Figure 1. Randles equivalent circuit.

Impedance measurements involve application of a small sinusoidal ac voltage probe and determination of the current response.[15] Impedance measurements are often fit to the Randles equivalent circuit, which is shown in Figure 1, where R_{ct} is the charge-transfer resistance, C_d is the differential capacitance, and R_s is the solution-phase resistance. Impedance sensors detect a change in one of these equivalent circuit parameters upon analyte binding. For example, proteins can be most sensitively detected through the increase in the charge-transfer resistance (R_{ct}) with increasing protein concentration, since this causes an increase in the protein film thickness on the electrode, reducing the rate of electron transfer.[16,17] Alternatively, proteins can be detected through the decrease in the differential capacitance with increas-

* Corresponding author. Phone: 315-268-4471. E-mail: isuni@clarkson.edu.

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ing protein film thickness.[16,17] The use of electrochemical impedance spectroscopy as a transduction method for biological recognition has been recently reviewed,[16] and detection limits have been reported in the nanomolar to picomolar range for impedance biosensors.[18−23] The current report describes an impedance biosensor for detecting peanut protein Ara h 1.

EXPERIMENTAL SECTION

Materials. Glass slides with a 100 nm Au film atop a 5 nm Ti adhesion layer were purchased from Evaporated Metal Films (Ithaca, NY); 11-mercaptoundecanoic acid (11-MUA) was purchased from Aldrich; *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were purchased from Sigma; *N*-hydroxysulfosuccinimide sodium salt (NHSS) was purchased from Pierce Biotechnology; peanut protein Ara h 1 and its mouse monoclonal antibody were purchased from Indoor Biotechnologies. All reagents were used as received. The Ara h 1 protein was supplied in monomeric form.

Electrode Preparation. The Au electrode was fixed by an O-ring onto an electrochemical cell constructed from virgin Teflon. The conical electrochemical cell was designed with an electrode area of 0.32 cm^2 and a cell volume of 5 mL. The electrode was cleaned with ethanol and water, dried, and immersed for 17 h into a solution containing 1.0 M 11-MUA and 50 mM phosphate buffer solution (PBS) at pH 10 to form a carboxylate-terminated self-assembled monolayer (SAM). In order to immobilize peanut protein Ara h 1 antibody, the carboxylate groups were activated for 1 h in a solution containing 75 mM EDC, 15 mM NHSS, and 50 mM PBS at pH 7.3. The electrode was then immersed for 1 h into a solution containing 50 μg/mL peanut protein antibody and 50 mM PBS at pH 7.3. This procedure immobilizes the antibody to peanut protein Ara h 1 onto the Au electrode by amide bond formation to amine groups on the protein surface.[24] This electrode is then used to detect peanut protein Ara h 1 in solution.

Measurement Methods. All electrochemical measurements were performed with a three-electrode configuration using a Pt spiral counter electrode and a Ag/AgCl (1.0 M KCl) reference electrode. The background test solution contains 50 mM PBS and 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ at pH 7.3, with varying amounts of peanut protein Ara h 1. The addition of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox probe to the test solution allows observation of the *RC* impedance loop shown in Figure 1 rather than purely capacitive behavior. In other words, oxidation/reduction of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ occurs during the impedance measurements without altering the electrochemical interface. Impedance measurements were performed using an EG&G PAR 263A potentiostat coupled to a Solartron 1250 frequency response analyzer (FRA) over the frequency range from 0.05 Hz to 15 kHz with an ac probe amplitude of 5 mV. Each impedance spectrum takes about 2.5 min to acquire. The impedance results were obtained at a dc potential of +200 mV versus Ag/AgCl, which is slightly cathodic to the open circuit potential (OCP) in the electrolytes of interest.

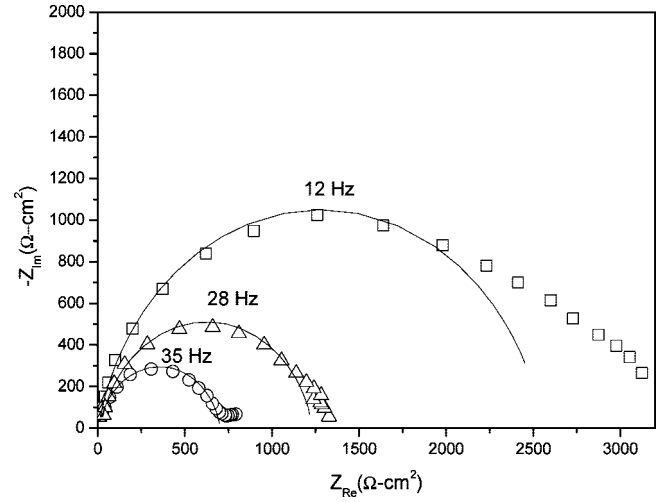

Figure 2. Impedance response of the Au electrode modified with 11-MUA (□), 11-MUA + NHSS (○), and 11-MUA + NHSS + anti-Ara h 1 (Δ) at +200 mV vs Ag/AgCl (1.0 M KCl). The test solution contains 50 mM PBS and 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ at pH 7.3. The solid lines correspond to Randles equivalent circuit fits.

RESULTS AND DISCUSSION

Impedance Studies of Electrode Preparation. Figure 2 shows Nyquist plots of the impedance spectrum at different stages of Au electrode preparation. As has been reported in the literature, the 11-MUA SAM on a Au electrode is highly passivated and stable in a laboratory environment. However, during impedance measurements, this interface did not reach steady state until about 30 min after immersion into 50 mM PBS and 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$, at which point successive impedance scans were identical. Another research group recently reported the same observation from their impedance analyses of SAMs formed from 11-MUA on Au.[25] Therefore, during all impedance studies, the 11-MUA-covered electrode was allowed to stabilize for 30 min. During this time, the OCP remains constant in the range from +215 to +230 mV, as determined by the standard reduction potential of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$.

After the NHSS layer is immobilized, the charge-transfer resistance (R_{ct}) decreases significantly, as illustrated in Figure 2 by the decrease in the semicircle diameter on the Nyquist plot. This is expected given the electroactive terminal groups of the NHSS-coated surface. Subsequent immobilization of peanut protein Ara h 1 antibody through amide bond formation increases R_{ct} as the Au electrode is obscured, slowing charge transfer. The OCP remains constant as subsequent layers are immobilized, indicating that the OCP is determined by the solution composition rather than the electrode composition.

Impedance Studies of Ara h 1 Binding. Figure 3 illustrates the impedance spectra that were obtained upon gradual addition of peanut protein Ara h 1 to the test solution. After each protein addition, successive impedance scans were obtained until the system reached steady state. With proper mixing, this occurred

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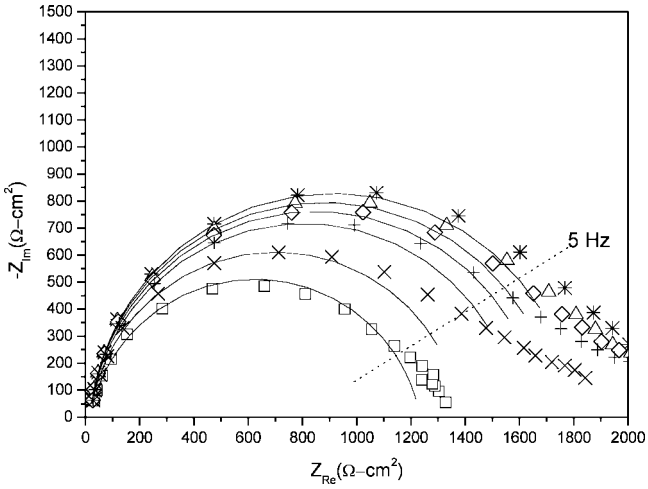

Figure 3. Impedance response of the Au electrode modified with 11-MUA + NHSS + anti-Ara h 1 after adding varying concentrations of peanut protein Ara h 1. The test solution also contains 50 mM PBS and 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ at pH 7.3. The concentrations of Ara h 1 protein from the innermost to the outermost semicircular arcs are 0, 0.02, 0.04, 0.08, 0.16, 0.24 μg/mL, respectively. The solid lines correspond to Randles equivalent circuit fits.

after the second impedance scan, about 5 min after protein addition. As the protein concentration is increased, the semicircle diameters in the Nyquist plots shown in Figure 3 also increase. These semicircle diameters correspond to the charge-transfer resistance (R_{ct}), as will be discussed below when complex nonlinear least-squares (CNLS) impedance fitting is considered. As the protein coverage at the Au electrode surface increases, the rate of electron transfer is reduced, causing the observed increase in R_{ct}. However, as the concentration of peanut protein increases, the surface coverage must eventually saturate, since all available antibody sites will have already bound peanut protein Ara h 1. This can be seen in Figure 3, where the impedance spectrum changes only modestly as the protein concentration is increased from 0.24 to 0.36 μg/mL.

The impedance results given in Figures 2 and 3 have been fit by CNLS analysis[26,27] using ZView 3.0a (Scribner Associates) to the Randles equivalent circuit[28] shown in Figure 1. During CNLS fitting, the sum of the squares of the difference between the experimental measurements and model predictions for the real and imaginary components of the impedance are minimized, most often with a Marquard−Levenberg algorithm.[15] Here the data are weighted by the calculated modulus. CNLS fitting routines have been criticized because multiple equivalent circuits can sometimes fit the same data set equally well.[15] However, this limitation typically does not impact the ability to understand simple electrochemical systems that can be described by the Randles equivalent circuit employed here.

The best-fit impedance parameters are given in Table 1. The impedance results are well fit by the Randles circuit model at frequencies greater than 5.0 Hz. It must be acknowledged that the impedance behavior at frequencies less than 5.0 Hz is unexpected and is not currently understood. The increase in the charge-transfer resistance (R_{ct}) with increasing protein coverage has been reported in several electrochemical studies of protein binding at surfaces.[29−32] The other impedance parameter that is often sensitive to protein binding is the differential capacitance (C_d), which is expected to decline as the protein coverage increases.[17] The capacitance per unit area should vary with the thickness (t) of a polymer film according to

$$\frac{C}{A} = \frac{\varepsilon_d}{t} \tag{1}$$

where ε_d is the dielectric constant of the polymer film. Figures 4 and 5 illustrate the variations in R_{ct} and C_d with protein concentration in solution. As can be seen from Figures 4 and 5, the relative change in R_{ct} is much greater than the relative change in C_d following protein binding, as is often observed for impedance detection of proteins.[17]

Practical Considerations. Acquisition of the entire frequency spectrum in Figures 2 and 3 takes about 2 min. For commercial applications, measurements at only one or a few frequencies are more likely to be practical. As discussed above, R_{ct} is more sensitive to peanut protein binding than the other equivalent circuit elements in Figure 1, so one should monitor frequencies sensitive to this parameter. The high-frequency impedance in Figures 2 and 3 does not vary substantially with the protein concentration, being essentially equal to the solution-phase resistance (R_s). Therefore, the protein concentration can be monitored by choosing a frequency on the opposite side of the semicircular Nyquist plots. Thus, the largest change in the impedance signal upon protein binding occurs in the range of 1−10 Hz. However, since the noise level is often higher at low frequency, the optimal signal-to-noise ratio is sometimes observed to be closer to 100 Hz.[33] The optimal frequency varies between different sensor interfaces and different impedance measuring systems.[33]

Another practical consideration for impedance biosensors is the degree or reproducibility. The Au−thiol chemistry employed here has several shortcomings, including stability and reproducibility problems, which have limited its commercial utility. Au−thiol films have been shown to oxidize rapidly in ambient conditions.[34,35] In addition, reproducible preparation of Au−thiol SAMs depends on many factors, including the substrate crystallography, pretreatment, surface roughness, and degree of surface contamination, the nature of the thiol species, and the solvent and choice of SAM preparation conditions.[33,36]

In the current studies, impedance measurements following SAM formation from 11-MUA showed variations in the charge-transfer resistance (R_{ct}) on different Au electrodes of as much as 25%. However, despite this inconsistency in Au−thiol self-assembly

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Table 1. Impedance Parameters from the Randles Equivalent Circuit Fit to the Results in Figures 1 and 2

circuit element	11-MUA	11-MUA + NHSS	11-MUA + NHSS + anti-Ara h 1	Ara h1 protein concentration ($\mu g \cdot mL^{-1}$) 0.02	0.04	0.08	0.16	0.24
R_s ($\Omega \cdot cm^2$)	3.32(0.30)	7.74(0.60)	16.8(1.4)	15.0(1.3)	16.3(1.4)	14.9(1.3)	15.8(1.3)	17.2(1.4)
R_{ct} ($\Omega \cdot cm^2$)	2553(100)	695(31)	1217(54)	1393(61)	1598(70)	1693(74)	1742(77)	1822(80)
C_d ($\mu F \cdot cm^{-2}$)	5.21(0.09)	4.42(0.08)	4.34(0.08)	4.10(0.07)	3.97(0.07)	3.91(0.07)	3.85(0.07)	3.79(0.07)

chemistry, detection of peanut protein Ara h 1 is quite reproducible. From the clear separation between the impedance spectra in Figure 3 for the blank and for a protein concentration of 0.02 μg/mL (0.3 nM), the detection limit here appears to be less than 0.3 nM. This concentration could be detected in repeat experiments on different Au substrates, and in one case, a peanut protein concentration of 0.005 μg/mL could be detected. Although the detection limit was not rigorously determined, the detection limit for peanut protein can be conservatively estimated to be less than 0.3 nM.

The difficulties described above with Au−thiol self-assembly chemistry have led to the investigation of other chemistries to form polymer films on Au electrodes for sensing and biosensing applications.[37−40] The peanut sensor experiments reported here have also been performed on degenerate Si electrodes,[41] where the polymer film is attached to the surface by direct formation of Si−C bonds, which have a much higher bond energy than Au−S bonds. The variation in charge-transfer resistance (R_{ct}) between different experiments is reduced on degenerate Si relative to Au.

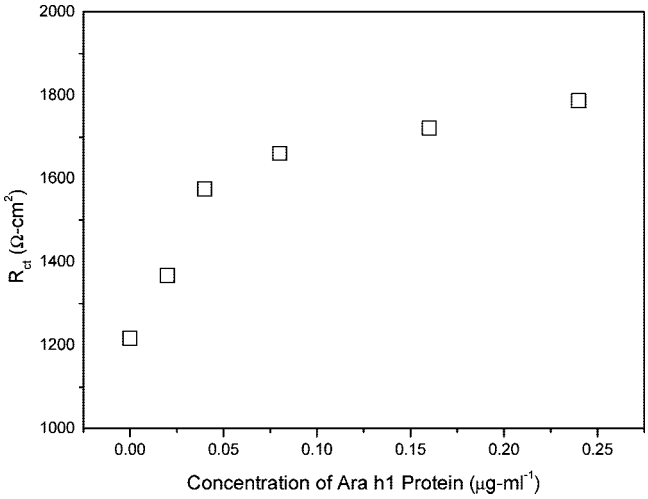

Figure 4. Charge-transfer resistance (R_{ct}) as a function of the concentration of Ara h 1 protein.

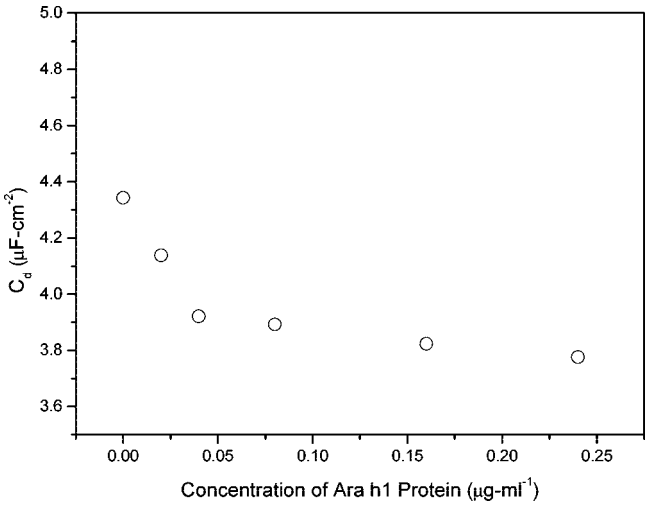

Figure 5. Differential capacitance (C_d) as a function of the concentration of the Ara h 1 protein.

In all of the results reported here, the test solution contains a relatively low concentration (5 mM) of the redox couple, $K_3Fe(CN)_6$/$K_4Fe(CN)_6$. The presence of this species allows for detection of a much higher current response than that obtained from an electrochemically inert test solution. On the other hand, if the concentration of this redox couple is too high, this may be harmful to biomolecules. This may have occurred in previously reported impedance studies, where gradual drift in the impedance signal was observed only in the presence of $K_3Fe(CN)_6$/$K_4Fe(CN)_6$.[42]

Upon repeated potential scanning over even a fairly narrow potential range (±200 mV vs OCP), the protein/polymer surface film becomes disrupted. This is supported by observation of a gradual increase in magnitude of the current peaks arising from oxidation/reduction of $K_3Fe(CN)_6$/$K_4Fe(CN)_6$. Therefore, a dc measurement potential of +200 mV was chosen for impedance studies, slightly cathodic to the OCP, to minimize unwanted electrochemistry, particularly unwanted oxidation reactions. Correct choice of this dc potential was critical to obtaining stable and reproducible impedance results.

Dissociation Constant. Antibody−antigen interactions are considered to be quite strong, with dissociation constants reported in the range from nanomolar to micromolar,[43] so one might expect the surface antibody layer to become saturated with peanut protein Ara h 1 even at quite low solution-phase concentrations. This can be verified by the gradual plateau seen in Figures 4 and 5 for the change in charge-transfer resistance (R_{ct}) and differential capacitance (C_d) with increasing peanut protein concentration.

The dissociation constant for peanut protein Ara h 1 and its surface-immobilized antibody can be determined from the differential capacitance (C_d) data in Figure 5 by assuming a Langmuir isotherm. Although the charge-transfer resistance (R_{ct}) is more sensitive to protein binding than the capacitance data, the adsorption isotherm should ideally be determined from the capacitance data. Two types of surface site exist, peanut protein-occupied and free sites, and these sites correspond to parallel

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electrical circuit elements. Since parallel capacitive elements combine in an additive manner, one expects a linear relationship between the capacitance and the surface coverage of peanut protein Ara h 1.

The dissociation reaction for the allergen (A) and its antibody (Y) is given below for the equilibrium constant K_d:

$$AY \rightarrow A + Y \tag{2}$$

$$K_d = \frac{[A][Y]}{[AY]} \tag{3}$$

Assuming the surface coverage of the antibody−antigen complex is θ, the surface coverage of unbound antibody will be $1 - \theta$, so

$$K_d = \left(\frac{1-\theta}{\theta}\right)[A] \tag{4}$$

Assuming a Langmuir adsorption isotherm, the change in C_d can then be directly related to the surface coverage of the complex:

$$\Delta C_d = \theta \Delta C_{d,max} \tag{5}$$

where

$$\Delta C_d = [C_{d(\theta=0)} - C_d]/C_{d(\theta=0)} \tag{6}$$

and

$$\Delta C_{d,max} = [C_{d(\theta=0)} - C_{d(\theta=1)}]/C_{d(\theta=0)} \tag{7}$$

The use of unitless capacitance change (ΔC_d) data allows transformation of the adsorption isotherm into the Hanes−Woolf form, where overweighting of the low-concentration results is avoided:[44]

$$\frac{[A]}{\Delta C_d} = \frac{[A]}{\Delta C_{d,max}} + \frac{K_d}{\Delta C_{d,max}} \tag{8}$$

A plot of [A] versus $[A]/\Delta C_d$ is given in Figure 6. Linear regression yielded a slope of 6.76(0.35) and a *y*-intercept of 0.23(0.04) with a correlation coefficient of 0.996. The dissociation constant K_d can be obtained by dividing the *y*-intercept by the slope, yielding a value of 0.033(0.006) μg/mL, which corresponds to 0.52 nM. This method of finding the dissociation constant avoids the problem of not being able to accurately measure the maximum capacitance change, $\Delta C_{d,max}$.

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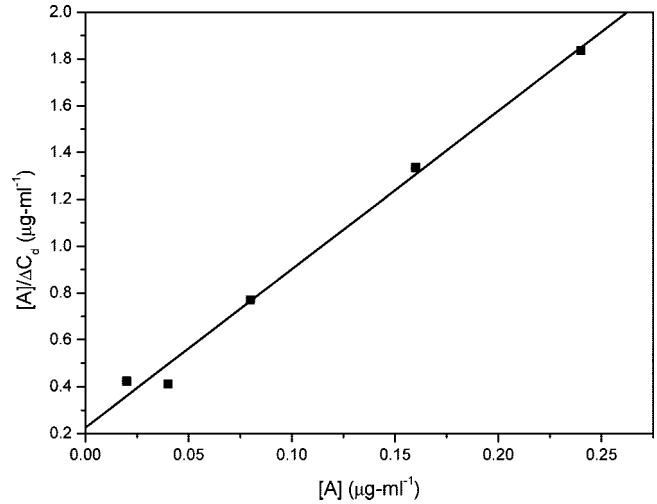

Figure 6. Hanes−Woolf plot for determining the dissociation constant, K_d.

CONCLUSIONS

Electrochemical impedance spectroscopy has been employed to demonstrate an impedance biosensor for peanut protein Ara h 1, one of the peanut protein allergens responsible for allergic reaction. This was accomplished by immobilizing a monoclonal antibody to Ara h 1 onto an Au electrode through amide bond formation to a carboxylate-terminated SAM. At each stage in the fabrication of the electrode, and after each protein addition, a steady-state impedance spectrum could be obtained. From the lowest protein concentration studied, a detection limit of better than 0.3 nM can be estimated.

From equivalent circuit CNLS fitting, the charge-transfer resistance (R_{ct}) increases and the differential capacitance (C_d) decreases with an increase in the concentration of peanut protein Ara h 1. The percentage change in R_{ct} is larger than the percentage change in C_d upon protein binding, suggesting that the peanut protein concentration can be more accurately determined through measurement of R_{ct}. On the other hand, the dissociation constant for the surface-immobilized antibody−antigen complex is best determined from the variation in C_d with peanut protein concentration, yielding a dissociation constant of 0.52 nM.

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