Minimizing Nonspecific Adsorption in Protein Biosensors that Utilize Electrochemical Impedance Spectroscopy

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Peanut protein Ara h 1, a common food allergen, has been previously detected at antibody-coated Au electrodes using electrochemical impedance spectroscopy (EIS) in ideal solutions, free from interfering species. Here, peanut protein Ara h 1 is detected by EIS at antibody-coated Au electrodes in canned soup that is filtered, diluted, and spiked with peanut protein Ara h 1. In this system, the combined strategy of sample dilution and blocking of unreacted surface sites on the Au electrode with bovine serum albumin is sufficient to dramatically reduce nonspecific adsorption. This is demonstrated by a quantitative comparison of the impedance change at two Au electrodes, one coated with the antibody to peanut protein Ara h 1 and the other coated with the antibody to cockroach protein Bla g 1. These results suggest that nonspecific adsorption does not in general limit the utility of biosensors based on EIS.

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Electrochemical biosensors based on amperometry have been widely employed for glucose detection, including transdermal monitoring of blood glucose levels. This application to glucose sensing highlights some of the advantages of electrochemical biosensors, which can be made entirely “electrical,” making them simpler, easier to miniaturize, and more easily portable than biosensors that employ other detection schemes, such as optical and acoustic methods. In addition, electrochemical biosensors often have lower noise levels because high sensitivity optical biosensors require liquid nitrogen cooling, and acoustic biosensors are often sensitive to environmental noise.

Electrochemical glucose biosensors detect glucose by measuring the current associated with an electrochemical reaction. However, the development of electrochemical immunosensors suggests the use of alternative detection schemes based on impedance or capacitance because antibody–antigen binding does not involve electrochemical reaction. The use of electrochemical impedance spectroscopy (EIS) for biosensor applications has attracted substantial interest, as indicated by several recent reviews. Detection limits reported for impedance-based biosensors vary from the nanomolar to picomolar range, which are sufficient for many analytical applications. However, the application of impedance biosensors has been limited by their sensitivity to nonspecific adsorption, which often limits the selectivity of biosensors applied to complex matrices such as food, blood, and environmental water samples.

We have recently reported impedance detection of peanut protein Ara h 1, a common food allergen, at Au and degenerate Si electrodes. The present paper considers methods to reduce or compensate for nonspecific adsorption during detection of peanut protein Ara h 1 in a real world food matrix, canned soup. In this system, the combined strategy of sample dilution and blocking of unreacted surface sites on the Au electrode with bovine serum albumin (BSA) is sufficient to dramatically reduce nonspecific adsorption. This is demonstrated by the use of a reference electrode at which an antibody to a cockroach protein allergen is immobilized.

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), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and IgG-free BSA were purchased from Sigma-Aldrich; and N-hydroxysulfosuccinimide sodium salt (NHSS) was purchased from Pierce Biotechnology; and peanut protein Ara h 1, rabbit polyclonal antibody to Ara h 1, and rabbit polyclonal antibody to cockroach protein Bla g 1 were purchased from Indoor Biotechnologies. All reagents were used as received. The Ara h 1 protein was received in monomeric form.

Electrode preparation.—The Au electrode was fixed by an O-ring onto an electrochemical cell constructed from virgin Teflon. The electrochemical cell was then cleaned with isopropanol alcohol, water, and acetone and washed with Alconox solution in ultrasound for 45 min. After rinsing thoroughly with water, the cell was cleaned for 30 min in 25% HNO3 + 25% H2SO4 and for 10 min in a freshly prepared Piranha solution (70% H2SO4 + 30% H2O2), followed by thorough rinsing with water. The electrode was then 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. To immobilize the antibody to peanut protein Ara h 1 and the antibody to cockroach protein Bla g 1, the carboxylate groups were activated for 30 min in 25% HNO3 + 25% H2SO4 and for 10 min in a freshly prepared Piranha solution (70% H2SO4 + 30% H2O2), followed by thorough rinsing with water. The electrodes were then immersed for 17 h into a solution containing 1.0 M 11-MUA and 50 mM phosphate buffer solution (PBS) at pH 7.3. The electrodes were then immersed for 1 h into a solution containing 50 µg/mL antibody and 50 mM PBS at pH 7.3, which immobilizes antibodies onto the Au electrode by amide bond formation. This was then immersed into a solution containing 14.7 µg/mL BSA to block unreacted sites on the Au electrode. These antibody-coated Au electrodes were then exposed to peanut protein Ara h 1 in filtered and diluted soup.

Measurement methods.—All electrochemical measurements were performed with a three-electrode configuration using a Pt spiral counter electrode and a saturated calomel reference electrode. Figure 1 illustrates the electrochemical cell that was constructed with two working electrodes, with one working electrode serving as the measurement channel and the other serving as a reference channel. The counter electrode is −2 to 3 mm from one working electrode WE (measurement channel) and −4 to 6 mm from the other working electrode (reference channel). Impedance measurements were performed using an EG&G PAR 273A potentiostat coupled to a Solartron 1250 frequency response analyzer at a constant frequency of 5 Hz. Impedance measurements were multiplexed between the two working electrodes in Fig. 1 using the ZPlot software. Whenever the working electrode was switched, the system was allowed to stabilize before impedance measurements were recorded.

Test solution.—The test solution was obtained from a can of chicken soup purchased at the supermarket. The soup was filtered successively through a kitchen strainer, nylon mesh filter with a pore size of 20 µm, and a nanoporous alumina filter with a nominal pore size of 100 nm.
size of 100 nm. This solution was then diluted, and known concentrations of peanut protein Ara h 1 were added. The dilution levels studied were $8 \times 10^{-3}$, $8 \times 10^{-4}$, and $8 \times 10^{-6}$, and the peanut protein concentrations studied were 0.033, 0.066, 0.132, and 0.264 $\mu$g/mL. These peanut protein concentrations were sufficiently low to avoid saturating the antibody film, for which the dissociation constant was $\sim 0.52$ nM when immobilized onto a Au electrode. Sample dilution was employed in part to prevent saturation of the antibody film. In addition, sample dilution is often a highly effective method for reducing the extent of nonspecific adsorption.

**Results and Discussion**

*Biosensors that employ EIS.*— We previously reported the detection of peanut protein Ara h 1 using EIS at Au electrodes onto which the peanut antibody is immobilized by the methods described above. Impedance results as a function of peanut protein concentration are illustrated in Fig. 2 in ideal solutions. However, commercial biosensor applications would more likely involve monitoring only one or a few frequencies of the impedance spectrum. When fit to a Randles equivalent circuit, the impedance results in Fig. 2 show that frequencies from 1 to 10 Hz, which sample the charge-transfer resistance ($R_{ct}$), are most sensitive to the binding of peanut protein. For this reason, only single frequency measurements at 5 Hz are reported in the present study.

As discussed above, one might expect that the application of impedance-based biosensors to the detection of food allergens is limited by interference from nonspecific adsorption arising in complex food matrices. Such interference is not, however, unique to electrochemical detection methods but is observed in a wide range of sensor technologies. In immunosensors that employ surface plasmon resonance (SPR) spectroscopy, a reference channel has been successfully employed to correct for the contribution of nonspecific adsorption. This is accomplished by immobilizing a closely similar antibody in the reference channel whose antigen is absent from the samples of interest and subtracting the reference channel from the measurement channel. In addition to correcting for the effects of nonspecific adsorption, this method also compensates for variations in temperature and refractive index.

SPR spectroscopy can detect changes in the optical properties of an antibody film upon antigen binding, whereas EIS can detect changes in the electrical properties of an antibody film. This analogy motivated the use of the electrochemical cell shown in Fig. 1, where the antibody to peanut protein Ara h 1 is immobilized at one Au working electrode (measurement channel), and the antibody to cockroach protein Bla g 1 is immobilized at the other Au working electrode (reference channel). In addition to correcting for the effects of nonspecific adsorption, the reference channel in Fig. 1 may also help correct for variations in the sample viscosity and ionic strength.

**Impedance results at Au electrodes with immobilized antibodies.**— Upon addition of peanut protein Ara h 1 to the test solution, the real component of the impedance at 5 Hz increases both for the measurement channel, at which the antibody to peanut protein Ara h 1 is immobilized, and for the reference channel, at which the antibody to cockroach protein Bla g 1 is immobilized. The cumulative impedance increase at 5 Hz upon introduction of peanut protein Ara h 1 at the two different working electrodes is quantitatively compared in Tables I and II for the three different dilution levels. These results are obtained concurrently by multiplexing between the working electrodes in the measurement and reference channels.

For all dilution levels, the impedance change at the peanut protein antibody-coated electrode in Table I rises monotonically with increasing concentration of peanut protein. This increase is quite consistent between the two higher dilution levels but is somewhat larger for the lowest dilution level ($8 \times 10^{-6}$). Intuitively, one would like to ascribe this discrepancy to an increasing extent of nonspecific adsorption with a decreasing level of dilution. However, the results in Table II for the impedance change at 5 Hz for the cockroach antibody-coated electrode suggest that this is only a par-

<table>
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<tr>
<th>Ara h 1 concentration (µg/mL)</th>
<th>$\Delta Z_{real}$ (Ω cm$^2$)</th>
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<tr>
<td></td>
<td>Dilution by $8 \times 10^{-6}$</td>
</tr>
<tr>
<td>0.033</td>
<td>61.2</td>
</tr>
<tr>
<td>0.066</td>
<td>88.6</td>
</tr>
<tr>
<td>0.132</td>
<td>102.3</td>
</tr>
<tr>
<td>0.264</td>
<td>112.0</td>
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Thus, only a modest change in the effective thickness or coverage of the antibody film can result in a much larger change in the measured impedance.

The utility of the sample dilution methods discussed here depends critically on the relative values of the allergenic threshold for peanut protein Ara h 1 and the antibody–antigen dissociation constant. As discussed above, to avoid saturating the immobilized antibody film, the concentration of peanut protein Ara h 1 should be less than \( \sim 0.3 \, \mu g/mL \). A comparison to the allergenic threshold for the immobilization of peanut protein is complicated by the uncertainty in the exact threshold for an allergic reaction and variations in this threshold between allergic individuals.30 However, a general guideline for the detection of food allergens is 1–100 mg/kg of food.31 Using the density of water, this translates into a detection range of 1–100 \( \mu g/mL \). Peanut protein concentrations tested here are 0.033–0.264 \( \mu g/mL \), significantly lower than those needed to test food for the presence of peanut protein Ara h 1.

### Conclusions

Peanut protein Ara h 1, a common food allergen, has been previously detected at antibody-coated Au electrodes using EIS in ideal solutions, free from interfering species. Here, peanut protein Ara h 1 is detected by EIS at antibody-coated Au electrodes in canned soup that is filtered, diluted, and spiked with peanut protein Ara h 1. Sample dilution is employed both to prevent saturation of the immobilized antibody film and to limit the extent of nonspecific adsorption from the food matrix. Nonspecific adsorption can be approximately quantified by comparing the impedance change at two Au electrodes, one coated with the antibody to peanut protein Ara h 1 and the other coated with the antibody to cockroach protein Bla g 1. At the three levels of sample dilution studied, the impedance change at the antibody-coated electrode is far greater than that at the cockroach antibody-coated electrode. This suggests that nonspecific adsorption does not in general limit the utility of biosensors based on EIS.

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### References


### Table II. Impedance increase at 5 Hz upon successive introduction of peanut protein Ara h 1 to cockroach protein antibody-coated Au electrode.

<table>
<thead>
<tr>
<th>Ara h 1 concentration (( \mu g/mL ))</th>
<th>( \Delta Z_{\text{real}} ) (( \Omega \text{ cm}^2 ))</th>
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<tbody>
<tr>
<td></td>
<td>Dilution by 8 \times 10^{-6}</td>
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<tr>
<td>0.033</td>
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<td>0.132</td>
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