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Antibody regeneration on degenerate Si electrodes for calibration and reuse of impedance biosensors

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ABSTRACT

Mild denaturing agents were studied for antibody regeneration atop degenerate (highly doped) Si and Au electrodes using comparable 11-carbon chain linker reagents onto which the mouse monoclonal antibody to peanut protein Ara h 1 is covalently immobilized. Of the reagents studied, only 200 mM KSCN is effective for antibody regeneration and detection of Ara h 1 atop Au electrodes, allowing 15 days of sensor usage after daily antibody unfolding and refolding, while 200 mM KSCN + 10 mM HF is effective atop degenerate Si electrodes, allowing 30 days of sensor usage. The addition of HF is required for antibody regeneration atop Si electrodes, as demonstrated by cyclic voltammetry in the presence of 5.0 mM K3Fe(CN)$_6$/K4Fe(CN)$_6$ at pH 7.3, where the oxidation/reduction peaks can be observed only in the presence of HF. The impedance spectrum for detection of Ara h 1 gradually degrades during these multi-day regeneration trials, but calibration experiments performed within one day illustrate that sensor electrodes can be calibrated on the day of use to within about 2%.

1. Introduction

Biosensors that employ electrochemical impedance spectroscopy for direct and often label-free analyte detection are attractive due to their rapid performance and modest instrumentation requirements [1–3]. Their all-electrical nature, with no optical or acoustic components, offers significant advantages for portable and implantable applications, including simplicity, lower cost, and ease of miniaturization and integration with ultra large scale integration (ULSI) technology [1–3]. 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. One limitation of impedance biosensors has been the stability and reproducibility of biomolecule immobilization onto a conductive electrode material [4,5], usually accomplished by Au-thiol chemistry. A related concern is that biosensor calibration is normally required due to variations between subsequent electrodes, as is well known for electrochemical glucose biosensors [6].

For antibody-based biosensors, calibration is complicated by the strength and irreversibility of antibody-antigen binding. One cycle of exposure to a chaotropic agent and antibody refolding allows calibration of impedance biosensors, whereas multiple cycles allow regeneration of impedance biosensors. Antibody-based ELISA tests typically allow 50–60 cycles of regeneration, most commonly using strong acids or bases as chaotropic agents [7]. For impedance biosensors, high or low pH solutions may not be compatible with the chemistries employed for protein immobilization, and are particularly problematic for integration into ULSI or MEMS devices.

Here several mild chaotropic agents are tested for antibody regeneration of impedance biosensors with the antibody to peanut protein Ara h 1 covalently immobilized onto Au and degenerate Si electrodes. Biosensors for detection of peanut proteins, and other food allergens, have recently attracted significant interest [8–11]. Peanuts are a particularly problematic food allergen due to the prevalence of peanuts in a wide variety of food products, the high sensitivity of some individuals, and the stability of some allergenic peanut proteins during food manufacturing and human digestion [8–11]. Au-thiol self-assembly chemistry has been most commonly employed for antibody immobilization onto a conductive electrode [12]. However, depending on storage conditions, the shelf life of such sensors has been reported to be limited to days to weeks [13]. For these reasons, other substrate materials such as C, Si, Pt, Ti, and ITO have also been tested for impedance biosensors [14–23]. We recently reported degenerate (highly doped) Si as an alternative electrode material to Au, and demonstrated impedance detection of an allergenic food protein, peanut protein Ara h 1 [24,25]. Advantages of degenerate Si as a sensor electrode include the greater strength of Si–C covalent bonds relative to Au–S, simpler equivalent circuit relative to n-type or p-type Si, easier surface preparation relative to C, widespread

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availability of Si wafers, and ease of incorporation into ULSI devices [24]. Here antibody regeneration atop degenerate Si is successfully reported for a 30-day trial using 0.2 M KSCN and 10 mM HF as the chaotropic reagent, and compared to antibody regeneration atop Au electrodes.

2. Experimental

2.1. Materials and reagents

Glass slides with a 100-nm Au film atop a 5-nm Ti adhesion layer were purchased from Evaporated Metal Films (Ithaca, NY); and As-doped (n-type) degenerate Si (111) wafers with 500 μm thickness, 50 mm diameter, and <0.005 Ω-cm resistivity were purchased from University Wafers. Peanut protein Arah 1 and its monoclonal antibody were purchased from Indoor Biotechnologies. 11-mercaptoundecanoic acid (11-MUA) and 10 undecanoic acid were purchased from Aldrich; N-(3-dimethylaminopropyl)-N′-(ethylcarbodiimide hydrochloride) (EDC), potassium dihydrogen phosphate, di-potassium dihydrogen phosphate were purchased from Sigma; N-hydroxysulfosuccinimide sodium salt (NHSS) was purchased from Pierce biotechnology; and potassium ferri/ferrocyanide was purchased from Acros Organics. Potassium thiocyante was purchased from Fisher scientific. HF dip was purchased from J.T.Baker. Bovine serum albumin (BSA) was purchased from Jackson Immuno Research.

2.2. Biosensor fabrication

Parallel procedures were followed to create similar antibody films on both degenerate Si and Au electrodes. Bifunctional carboxylate-terminated linker reagents with an eleven-carbon backbone were used on both electrodes, 10-undecanoic acid for degenerate Si and 11-MUA for Au. The n-type degenerate Si(111) electrode was embedded within a virgin Teflon mount with an electrode area of 0.19 cm^2 and a cell volume of 1 ml, cleaned in ethanol and water, and etched in 10:1 HF dip to remove the native oxide. This was immediately immersed into 10% 10-undecanoic acid in deaerated toluene for 19 h and exposed to 352 nm ultraviolet light for photoactivated alkene insertion into Si–H bonds. The Au electrode was fixed by an O-ring onto an electrochemical cell constructed from virgin Teflon with an electrode area of 0.19 cm^2 and a cell volume of 6 ml. The Au electrode was cleaned with ethanol, dried, and immersed for 17 h into 1 mM 11-MUA and 50 mM phosphate buffer solution (pH = 10). After SAM formation, the surface was rinsed with 50 mM phosphate buffer solution 3 times.

Carboxylate-terminated Au and Si electrodes were then activated for 1 h in 75 mM EDC and 15 mM NHSS in 50 mM phosphate buffer solution (pH = 7.3). The antibody-coated electrodes were created by immersion for 1 h into a solution containing 50 μg/ml antibody and 50 mM PBS at pH 7.3, forming amide bonds to amine groups on the protein surface, and then immersed into 0.1% BSA for one hour to reduce the non-specific adsorption. This treatment with EDC and NHSS forms amide bonds between the carboxylate groups on the electrode surface and amine groups on the antibody surface [26].

2.3. Electrochemistry and spectroscopic ellipsometry measurements

Electrochemistry experiments were performed using a three-electrode configuration with a Pt spiral counter electrode and an Ag/AgCl reference electrode. Impedance measurements were performed using an EG&G PAR 263A potentiostat coupled to a Solartron 1250 frequency response analyzer (FRA) and Gamry instrument (Reference 600) over the frequency range from 0.01 Hz to 15 kHz with an AC probe amplitude of 5 mV. On Au electrodes, impedance measurements were taken at a DC potential of +200 mV vs. Ag/AgCl, close to the open circuit potential (OCP), whereas on degenerate Si electrodes, impedance measurements were taken at +50 mV vs. Ag/AgCl, This potential is chosen to suppress Si oxidation at more anodic potentials [24].

Spectroscopic ellipsometry measurements were performed in situ on the polymer-protein films using a J.A. Woollam M44 spectroscopic ellipsometer at wavelengths of 420–760 nm at a fixed angle of incidence of 70° from the surface normal. A refractive index of 1.45 was assumed for all polymer-protein film measurements [27]. The reproducibility of ellipsometric determination of film thickness was within ±0.2 nm.

3. Results and discussion

3.1. Detection of peanut protein Ara h 1 by electrochemical impedance spectroscopy

Figs. 1A and B illustrate Nyquist plots of the impedance response to introduction of peanut protein Ara h 1 at a Si and Au electrode, respectively, onto which the corresponding mouse monoclonal antibody is immobilized. The impedance spectra in Figs. 1A and B approximate semicircular behavior at intermediate to high frequencies, and can be fit with the Randles equivalent circuit shown in Fig. 2, where the differential capacitance (C_d) is replaced with a constant phase element (CPE). Here R_s corresponds to the solution resistance, R_{ct} to the charge transfer resistance, and Z_w to the Warburg impedance, which is not fit. The impedance of the CPE is [28–30]:

$$Z(CPE) = \frac{1}{T(j\omega)^n} \quad (1)$$

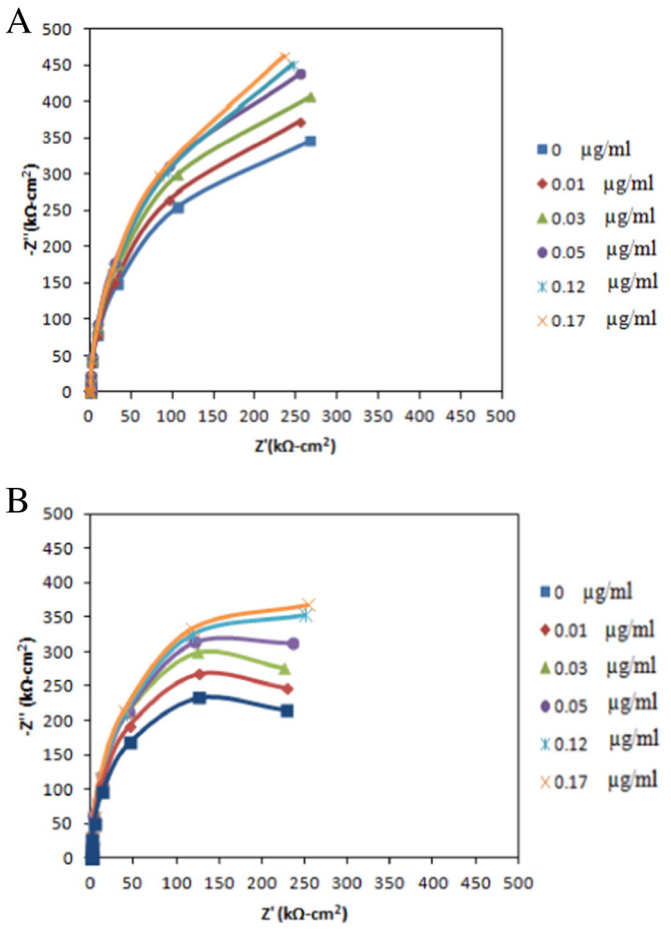

Fig. 1. A. Nyquist plots of the impedance response of antibody-coated degenerate Si electrode after exposure to increasing concentrations of peanut protein Ara h 1 in an electrolyte also containing 50 mM PBS buffer and 5.0 mM $K3Fe(CN)_6/K_4Fe(CN)_6$ at pH 7.4. B. Nyquist plots of the impedance response of antibody-coated Au electrode after exposure to increasing concentrations of peanut protein Ara h 1 in an electrolyte also containing 50 mM PBS and 5.0 mM $K3Fe(CN)_6/K_4Fe(CN)_6$ at pH 7.4.

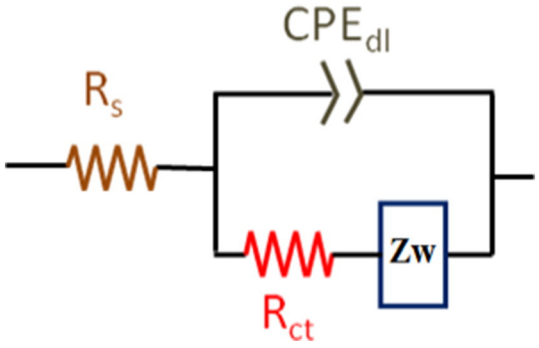

Fig. 2. Randles equivalent circuit for CNLS data fit.

The best fit equivalent parameters during impedance detection of peanut protein Ara h 1 are given in Tables 1A and 1B for the degenerate Si and Au sensor electrodes, respectively. Here the charge transfer resistance (R_{ct}) is the most sensitive equivalent circuit element to antigen binding. R_{ct} rises approximately linearly with the antigen concentration at low concentrations, and eventually approaches saturation at high concentrations, as is typical for the Langmuir adsorption isotherm [24,31].

The charge transfer resistance (R_{ct}) shown in Tables 1A and 1B is found to be consistently lower at Au relative to degenerate Si electrodes. Since the polymer-protein films at both electrodes should have similar thickness and continuity, this difference likely arises from the higher catalytic activity of Au electrodes relative to degenerate Si. At the equilibrium potential [32]:

$$\boldsymbol{R_{ct}} = \frac{\boldsymbol{RT}}{\boldsymbol{Fi_0}} \tag{2}$$

where R is the ideal gas constant, T is the absolute temperature, F is Faraday's constant, and i_0 is the exchange current density for oxidation/reduction of $Fe(CN)_6^{3-/4-}$. The exchange current density is the intrinsic electrochemical rate constant for surface reaction, so Eq. (2) illustrates that R_{ct} is inversely related to catalytic activity.

3.2. Detection of peanut protein Ara h 1 by spectroscopic ellipsometry

The results for impedance detection of peanut protein Ara h 1 at degenerate Si and Au electrodes use bifunctional linker reagents 10-undecanoic acid and 11-mercaptanoic acid, respectively, both of which contains an eleven-carbon backbone. To verify that these antibody surface immobilization methods are approximately comparable, they were also studied by spectroscopic ellipsometry. Table 2 summarizes the results of film thickness measurements during exposure of antibody films on both degenerate Si and Au electrodes to increasing concentrations of Ara h 1. In both cases, behavior typical of a Langmuir adsorption isotherm is observed [24,31], as described above. At low concentrations of Ara h 1, the film thickness rises linearly with concentration, but eventually approaches saturation at high concentration. In addition, the film thickness values reported in Table 2 on the degenerate Si and Au electrodes are equal to within the experimental uncertainty both for the bare antibody film, and for antigen binding, verifying that these two biosensor interfaces are approximately comparable.

3.3. Regeneration of antibody-coated Si and Au electrodes

The capability to repeatedly detect Ara h 1 at these same electrodes was tested in several different chaotropic agents that had been previously reported as mild antibody denaturants, including 200 mM tris–glycine-HCl, KSCN, and l-O-octyl-fi-D-glucopyranos(OG) [33]. For Au electrodes, only KSCN was found to be an effective chaotropic reagent. For degenerate Si electrodes, low HF concentrations were also added to this denaturing solution to remove Si oxide that forms during electrode storage. The following procedures were employed for daily impedance biosensor tests on Si:

1) The antibody-coated Si electrode was stored in 50 mM PBS buffer at pH 7.3.
2) The Si electrode was removed each day and exposed to 200 mM KSCN and 10 mM HF to unfold the antibody film and release the analyte.
3) The Si electrode was exposed to 100 mM BSA and 50 mM PBS buffer to refold the antibody film.

Table 1A
Best-fit equivalent circuit parameters (standard errors) during exposure of antibody-coated degenerate Si electrode to increasing concentrations of peanut protein Ara h 1.

Concentration of Ara h 1 (μg/mL)	0	0.01	0.03	0.05	0.12	0.17
R_s(Ω-cm^2)	34.62 (0.4)	35.12 (0.2)	33.27 (0.2)	34.50 (0.3)	33.28 (0.2)	33.15 (0.3)
CPE-T (μF cm^{-2} s^{n-1})	5.39 (0.01)	5.99 (0.01)	6.05 (0.01)	6.20 (0.01)	6.32 (0.01)	6.33 (0.01)
N	0.94 (0.001)	0.96 (0.001)	0.95 (0.001)	0.94 (0.001)	0.94 (0.001)	0.95 (0.001)
R_{ct} (kΩ-cm^2)	890.8 (5.8)	955.1 (6.8)	1005.6 (6.3)	1040.9 (6.2)	1090.8 (6.4)	1107.1 (6.2)

Table 1B
Best-fit equivalent circuit parameters (standard errors) during exposure of antibody-coated Au electrode to increasing concentrations of peanut protein Ara h 1.

Concentration of Ara h 1 (μg/mL)	0	0.01	0.03	0.05	0.12	0.17
R_s(Ω-cm^2)	26.43 (0.1)	26.72 (0.3)	23.11 (0.2)	24.90 (0.1)	25.19 (0.2)	26.51 (0.3)
CPE-T (μFcm^{-2} s^{n-1})	4.11 (0.01)	4.48 (0.01)	4.57 (0.01)	4.75 (0.01)	4.98 (0.01)	5.21 (0.01)
N	0.96 (0.01)	0.97 (0.01)	0.96 (0.01)	0.96 (0.01)	0.97 (0.01)	0.96 (0.01)
R_{ct} (kΩ-cm^2)	510.3 (6.3)	561.9 (6.3)	585.7 (6.9)	598.3 (6.9)	610.5 (6.7)	620.2 (6.4)

Table 2
Ellipsometric thickness measurements for polymer-protein films atop degenerate Si and Au electrodes.

Adsorbate	Measured thickness (nm)					
	Antibody layer	0.01 μg/mL	0.03 μg/mL	0.05 μg/mL	0.12 μg/mL	0.17 μg/mL
Degenerate Si electrode	1.62	2.87	3.20	3.59	3.68	3.76
Au electrode	1.53	2.58	2.99	3.41	3.58	3.71

4) The Si electrode was exposed to increasing concentrations (0.01, 0.03, 0.05, 0.12 and 0.17 μg/mL) of peanut protein Ara h 1.
5) Steps #2–4 were repeated once.

Identical protocols were used for Au, but without addition of HF. For both electrode materials, cycles were continued until the antibody film could not detect the peanut antigen. Repeated impedance detection of peanut antigen binding is illustrated in the Nyquist plots of Fig. 3A and B, where the degenerate Si and Au electrodes are capable of detecting Ara h 1 for 30 and 15 days, respectively. Both electrodes were exposed daily to the same Ara h 1 concentrations shown in Tables 1A and 1B. However, to avoid congestion, only the data taken every fifth day is illustrated in Fig. 3A, and every third day in Fig. 3B, and only two concentrations of Ara h 1 are shown (0 and 0.05 μg/ml). The results in Fig. 3A on degenerate Si electrodes were previously reported in a review article [25], but without comparison to results on Au electrodes, and without detailed explanation. Tables 3A and 3B illustrate the gradual changes in R_{ct} during daily testing.

The addition of 10 mM HF to the unfolding solution is necessary to remove Si oxide that forms on the surface during electrode storage. Without the addition of HF, the degenerate Si electrode only remain active for antigen detection for four days. This is consistent with recent studies of polymer film formation atop Si(111) that show gradual oxidation of the Si surface beneath a polymer film is enhanced by the larger areal footprint of ethylene groups relative to the Si-Si bond length [34]. Periodic removal of this Si oxide by HF, widely practiced during ULSI processing [35], is a straightforward method to extend the lifetime of degenerate Si electrodes for impedance biosensors. The effect of HF is illustrated by cyclic voltammetry of degenerate Si with and without HF in Fig. 4. This demonstrates that voltammetry peaks for $Fe(CN)_6^{3-/4-}$ oxidation/reduction are only observed when HF is present. Without the presence of HF, the oxidized surface is electrochemically inactive.

The results in Fig. 3A show a continuous, gradual decline in the sensitivity of degenerate Si sensor electrodes. However, experiments within one day demonstrate that these sensors can be fairly rapidly calibrated, as illustrated in Fig. 5. Here successive measurements on the eleventh day demonstrate that the charge transfer resistance is consistent to within ~2%. Such a procedure can be employed for daily calibration of a sensor for peanut protein Ara h 1 during long-term storage.

Table 3A
Charge transfer resistance (R_{ct}) for peanut antibody-coated degenerate Si electrode during 30-day regeneration trial using 200 mM KSCN and 10 mM HF.

Day	R_{ct} (kΩ-cm^2)	
	Bare antibody	0.05 μg/mL Arah1
0	890 (5.8)	1040 (6.2)
1	880 (5.4)	988 (6.1)
2	750 (5.7)	924 (5.7)
5	695 (5.9)	867 (6.1)
10	643 (5.8)	819 (5.9)
15	566 (5.9)	697 (5.7)
20	514 (6.1)	618 (6.0)
25	493 (5.4)	541(6.1)
30	485 (5.6)	499 (5.9)

Table 3B
Charge transfer resistance (R_{ct}) for peanut antibody-coated Au electrode during 15-day regeneration trial using 200 mM KSCN.

Days	11-MUA-coated electrode R_{ct} (kΩ-cm^2)	
	Bare antibody	0.05 μg/mL Arah1
0	510 (6.3)	598 (6.9)
1	460 (6.7)	527 (7.1)
3	429 (6.5)	483 (7.1)
6	394 (6.2)	437 (6.9)
9	356 (6.4)	398 (6.2)
12	331 (6.1)	350 (6.3)
15	310 (6.5)	322 (6.6)

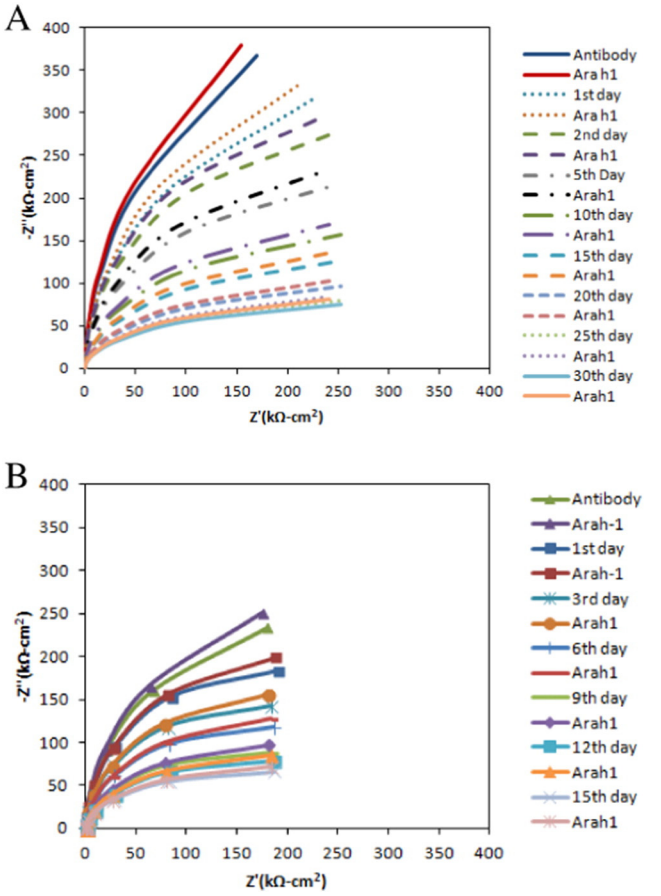

Fig. 3. A. Nyquist plots of impedance spectra during daily regeneration of peanut antibody-coated degenerate Si electrode in 200 mM KSCN and 10 mM HF. On each day, only two concentrations of Ara h 1 are shown (0 and 0.05 μg/ml). (reproduced with permission from reference #25). **B**. Nyquist plots of impedance spectra during daily regeneration of peanut antibody-coated Au electrode in 200 mM KSCN. On each day, only two concentrations of Ara h 1 are shown (0 and 0.05 μg/ml).

4. Conclusions

Antibody regeneration was tested using several reagents for the mouse monoclonal antibody to peanut protein Ara h 1 covalently

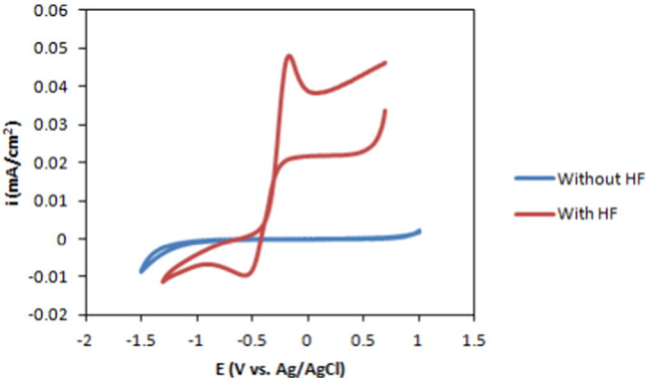

Fig. 4. Cyclic voltammogram of bare degenerate Si in an electrolyte containing 200 mM KSCN, 50 mM PBS buffer, and 5.0 mM $K3Fe(CN)_6/K4Fe(CN)_6$ at pH 7.3, with and without 10 mM HF.

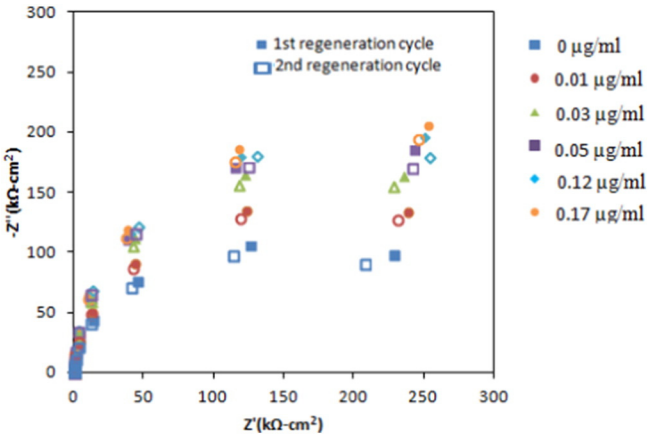

Fig. 5. Nyquist plots of impedance spectra during two consecutive trials for peanut antibody regeneration with 200 mM KSCN and 10 mM HF on degenerate Si for the eleventh day.

immobilized atop by degenerate (highly doped) Si and Au electrodes. Only 200 mM KSCN is effective for antibody regeneration and detection of Ara h 1 atop Au electrodes, allowing 15 days of sensor usage after daily antibody unfolding and refolding. On the other hand, 30 days of sensor usage were possible for degenerate Si using the same denaturing agent, but with addition of 10 mM HF. For degenerate Si, cyclic voltammetry in the presence of 5.0 mM $K3Fe(CN)_6/K4Fe(CN)_6$ at pH 7.3 showed that oxidation/reduction peaks are only observed in the presence of 10 mM HF, demonstrating that this reagent is necessary to prevent Si oxidation. Although the impedance spectrum for detection of Ara h 1 gradually degrades during these multi-day regeneration trials, experiments performed within one day illustrate that sensor electrodes can be calibrated on the day of use to within about 2%.

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