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# Faradaic Impedance Spectroscopy for Detection of Small Molecules Binding using the Avidin-Biotin Model

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ABSTRACT

The changes in the Faradaic impedance of gold/biomolecules system due to specific binding of small molecule to a significantly larger binding protein molecule were investigated. The biotin (244.31 Da) - avidin (66000 Da) couple was used as a model for small ligand - binding protein biorecognition. The study was carried out under open circuit potential in the presence of $[Fe(CN)_6]^{-3/-4}$ redox couple. An equivalent electrical circuit was proposed and used for the interpretation of the recorded impedance spectra.

Adsorption of thiolated avidin increased the electron transfer resistance, $R_{ct}$, by a factor of about 7.5 while subsequent addition of biotin within the concentration range of 4.1-40.9 nM reduced the value of $R_{ct}$ by amount proportional to the biotin concentration. The addition of biotin did not affect, however, the equivalent double layer capacitance or other equivalent circuit parameters.

A simple model based on effective surface coverage by the avidin molecules and the effect of the added biotin on electron transfer through the coated surface is proposed. A model for the minimum detection limit based on the random distribution of the binding protein and its dimensions is proposed.



## 1. Introduction

Label–free affinity biosensors are promising devices for point-of-care real time clinical diagnostics. Since the concept of immunoassay was first introduced by Yalow and Berson [1], a significant progress was made leading to the present generation of affinity biosensors. While affinity assays, especially enzyme-linked immunosorbent assay (ELISA), dominate a large fraction of clinical laboratory, a growing need for miniature, user-friendly, fast, sensitive and cost-effective analyzers calls for the development of new techniques for affinity biosensing. Such biosensors are expected to replace currently employed labor intensive affinity assays. The required new methods should combine the inherent specificity of biological recognition with the high sensitivity and convenient operation of modern electronic and electro-optic systems. Currently available detection methods for affinity biosensors which enable direct detection of biorecognition events include optical surface plasmon resonance (SPR), mechanical quartz crystal microbalance (QCM) and electrochemical impedance spectroscopy (EIS) [2]. EIS is an analytical method sensitive to both surface phenomena and bulk properties. The detection is based on molecular recognition events taking place at the electrode/electrolyte interface, affecting the real and imaginary parts of the system's impedance [3,4].

Avidin-biotin based system is one of the most investigated binding protein – ligand couple and used in many biotechnological applications, due to the high affinity of biotin binding ($K_D = 10^{15}$ $M^{-1}$) and readily implemented biotinylation labeling procedures, avoiding structural damage of the labeled molecule.

The majority of the protein-based conductive EIS biosensors incorporate a redox probe into the measurement solution which enables to detect changes in the charge transfer resistance ($R_{ct}$) of the redox reaction taking place at the electrode-solution interface. The changes in $R_{ct}$ are mostly attributed to two effects: (a) the physical blocking of the electrode's surface by the protein molecules, mostly resulting in increased $R_{ct}$ [5,6], and (b) the change in surface charge which affects the transport of the redox probe [7–9] due to the electrostatic interaction. Cyclic voltammetry (CV) measurements support these arguments as indicated by decrease in the current peak and shift in the redox potential due to protein adsorption. Given the above assumption it is expected that impedance based biosensors will be most sensitive to analytes, which are larger and/or have significant different charge state compared to the binding molecule. However, in this work and others, there are examples for sensing small molecules that do not fit these criteria [10,11]. Furthermore, Ding [10] reported that

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biotin detection by avidin-modified electrode is enabled by Faradaic EIS but not by CV which was not sensitive enough.

In this work, the binding of biotin to avidin molecules immobilized on the surface of gold working electrode was investigated. We demonstrate that small molecule, i.e. biotin, in very low concentrations can be detected by Faradic EIS using a probe molecule, which is two-order of magnitude larger in size, i.e. avidin. An equivalent electrical circuit for the data modeling is described, and possible explanations of the phenomena observed are proposed.

## 2. Experimental

### 2.1. Chemicals and reagents

Avidin, biotin, 2-imminothiolane hydrochloride (2-IT), $[Fe(CN)_6]^{-3/-4}$, PBS (pH 7.5, NaCl; $Na_2HPO_4$; $KH_2PO_4$; KCl), HEPES buffer (pH 7.5), eight-well strip of polystyrene tube, were all purchased from Sigma-Aldrich. $KNO_3$, 2-propanol, acetone, Amicon® centrifugal filters (10 kDa) were all purchased from Merck. Silicon substrates (p-Si <100> 1-10 Ω·cm, with 500 nm thick thermal oxide layer) were purchased from University Wafer Inc. All chemicals were ACS grade. All solutions were prepared with Nano-Pure® water (18.2 MΩ·cm).

### 2.2. Preparation of gold electrodes

Gold working electrodes were prepared by sputtering 20 nm thick titanium adhesion layer on the $SiO_2$/Si substrate followed by 300 nm sputtering of gold without breaking vacuum. The gold surface was cleaned by sonication for 10 min in acetone, washed with 2-propanol and dried under nitrogen flow. The Working Electrode's surface area was occluded by polystyrene tube (∅ = 6.8 mm, h = 10 mm) glued to the clean gold surface. Prior to use, the gold electrodes were washed with 2-propanol and dried under nitrogen flow. Voltammograms of the gold electrodes in sulfuric acid had shown a typical characteristic behavior of clean gold (supplementary Fig. S1).

### 2.3. Modification of working electrode

Avidin molecules were thiolated by 2-IT, which reacts with primary amines to introduce terminal sulfhydryl groups while maintaining charge properties similar to the original amino group. The procedure was performed as follows: 5 ml reaction mixture of 1.5 μM 2-IT and 150 nM avidin dissolved in PBS (molar ratio of 1:10 in favor of 2-IT), was incubated for 1 h at room temperature. Subsequently, free 2-IT was removed by ultrafiltration using centrifugal filters of 10 kDa MW cutoff. The retentate was resuspended in 0.1 M sodium phosphate buffer (PB) pH 5. Clean gold electrodes were incubated for 2 h with 100 μl of 150 nM thiolated avidin in PB (the electrode's surface coverage in terms of resistance, as will be describe later, was steady ± 10% for avidin concentration ≥ 75 nM, supplementary Fig. S2). Unbound protein was removed by washing three times (twice with the same PB buffer and once with 10 mM HEPES buffer). The avidin-modified electrodes were reacted for 30 min with different concentrations of biotin dissolved in 10 mM HEPES buffer or in the buffer solution itself (without biotin). All electrodes were washed three times and unbound biotin molecules were removed (twice with the same HEPES buffer and once with 100 mM $KNO_3$ and 5 mM HEPES buffer).

### 2.4. Electrochemical impedance spectroscopy (EIS)

Electrochemical measurements were carried out in a conventional three-electrode electrochemical cell comprised of fabricated gold working electrode (0.36 $cm^2$), spiral platinum wire counter electrode (1.25 $cm^2$), and a homemade electroplated open Ag/AgCl quasi reference electrode, using BioLogic Inc. SP-200 potentiostat, and analyzed with EC-Lab™ software. Measurements were performed in 300 μl solution containing 100 mM $KNO_3$, 5 mM HEPES buffer pH 7.5, and 5 mM $[Fe(CN)_6]^{-3/-4}$, at open circuit potential with 10 mV modulation voltage, within the frequency range of 100 kHz-100 mHz.

## 3. Results and Discussion

Electrochemical impedance spectroscopy is an analytical method sensitive to surface phenomena and changes of bulk properties. In Faradaic EIS, a redox probe is usually added into the system and reacts at the solution-electrodes interface. Electrons resulting from the redox reaction pass across the interface and enable detection of changes in the effective serial resistance, $R_{ct}$ [12]. The changes in $R_{ct}$ are mostly attributed to physical blocking of electrode's surface by the bound analyte molecules, and to changes in surface charge which can attract or repulse the redox probe, affecting reaction kinetics. It is expected that impedance based biosensors will be most sensitive to analytes which are larger and/or have significant different charge state compared to the binding probe molecule. Fig. 1 presents impedance measurements of avidin modified electrodes before and after the addition of biotin at different concentrations. Significantly different $R_{ct}$ values were recorded for the different biotin concentrations as

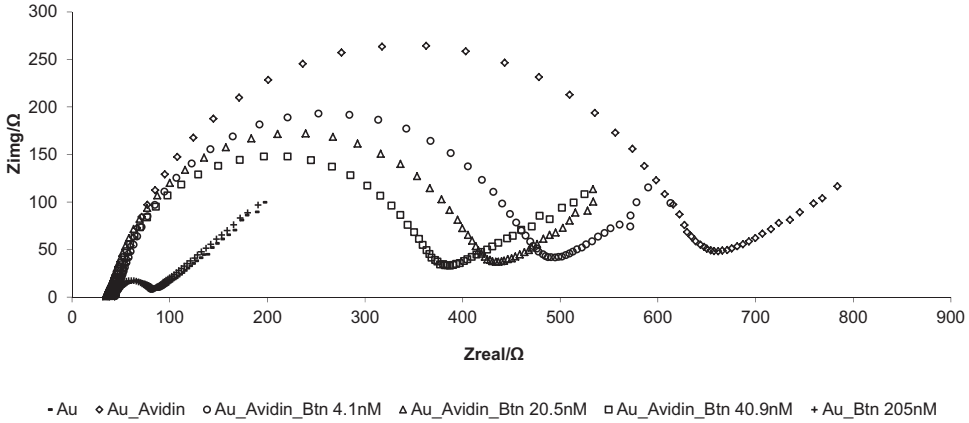


**Fig. 1.** Nyquist plot of the avidin-modified electrode exposed to different concentrations of biotin. Measurements were performed in 100 mM $KNO_3$, 5 mM HEPES buffer (pH 7.5) and 5 mM $[Fe(CN)_6]^{-3/-4}$. $E_{DC}$ = 0 mV *vs* OCP, $E_{AC}$ = 10 mV, frequency range 100 kHz -100 mHz (Btn = biotin).