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Feasibility in the development of a multi-marker detection platform

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ABSTRACT

A feasibility study for a label-free, multi-marker single sensor using electrochemical impedance spectroscopy (EIS), imaginary impedance, and a signal decoupling technique is reported. To our knowledge, this is the first reported attempt of using imaginary impedance for biomarker detection and multi-marker detection. The electrochemical responses of purified low and high density lipoproteins (LDL and HDL, respectively) were first individually characterized through the immobilization of their molecular recognition elements (MREs) onto gold disk electrodes (GDEs). The co-immobilization was performed by immobilizing the MREs of both LDL and HDL on the same GDE, which was then used to detect LDL and HDL simultaneously in mixed solution. Previous individual purified responses were then used to de-convolute the mixed response, when the two biomarkers were detected in mixed solutions. The optimal frequencies of LDL and HDL were found to be 81.38 Hz and 5.49 Hz, respectively, which shifted to 175.8 Hz and 3.74 Hz under co-immobilized conditions. After comparing the electrochemical signal in complex and imaginary impedance, imaginary impedance was found to be more suitable for multi-marker detection purposes. Since imaginary impedance is related to capacitance, electric displacement, relative permittivity, and effective capacitance were derived to elucidate the theory of optimal frequency. This work shows that EIS has the potential for multi-marker detection and can be extended to monitor other complex diseases such as diabetes mellitus for better management and diagnostic purposes.

1. Introduction

The development of multi-marker assays in place of single-marker assays is continuously rising as many studies have revealed the benefit of monitoring multiple biomarkers in disease diagnosis, prognosis, and management (Boer et al., 2011; Sullivan et al., 2011; Wang et al., 2007, 2006). For example, in the case of diabetes mellitus, measuring insulin, glucose, and glucagon provides a more comprehensive understanding of a patient's state of health than glucose alone, which then provides more accurate information for insulin administration (Adamson et al., 2012). Currently, one of the most common mechanisms for multi-marker detection employs multi-sensor arrays (Wang, 2006; Wiesner, 2004), but detecting multiple biomarkers using a single electrochemical sensor has not yet been demonstrated. Recently our group has demonstrated that EIS has the potential for multi-marker detection (Adamson et al., 2014, 2012; La Belle et al., 2013, 2011; Nandakumar et al., 2011). EIS offers various advantages for biosensing, including improved sensitivity, label-free detection and speed (<90 s) (Ronkainen et al., 2010). It measures the resistance and capacitance of an electrochemical system with variable AC signal. The AC signal consists of a varying potential and a wide range AC frequency sweep. When varying AC signals are applied to the sample of interest, a current response is generated. The current response is measured over the range of frequencies encompassed by the sweep and is then used to calculate the real, imaginary, phase angle, and complex impedance. Mathematically, the complex impedance is defined by the equation below:

$$Z(j\omega)=\frac{U(j\omega)}{I(j\omega)}=Z_r(\omega)+jZ_i(\omega) \quad (1)$$

Where, $Z(j\omega)$ is the complex impedance,ω the angular frequency (which is equivalent to $2\pi f$ where f is the input frequency), $U(j\omega)$ the applied potential, $I(j\omega)$ the current response, $Z_r(\omega)$ the real impedance, and $jZ_i(\omega)$ the imaginary impedance.

After investigating the correlation between the complex impedance and target concentration, the concept of optimal frequency was developed (Adamson et al., 2014, 2012; La Belle et al., 2013, 2011; Nandakumar et al., 2011). The optimal frequency of a biomarker is the AC frequency at which the resulting impedance best represents the interaction between the biomarker and its MREs. The optimal frequency is determined by optimizing the responsivity and R-square values (RSQ). It offers an orthogonal means for target detection in addition to the specific interaction between target and their MREs. By determining the optimal frequencies of the biomarkers of interest, it is

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proposed that each biomarker can be detected at its optimal frequency simultaneously on a single sensor platform, envisioning the possibility of a multi-marker detection platform technology. The idea of an optimal frequency is also supported by other groups (Boonyasit et al., 2016; Esfandyarpour et al., 2016; Hui et al., 2013; Rinaldi and Carballo, 2016) and is becoming a future point of interest for EIS (Yun et al., 2016).

However, using complex impedance to determine the optimal frequency and measure the target concentration of multiple biomarkers simultaneously has a major limitation: signal overlap. To address this issue, we propose a novel approach to determine a biomarker's optimal frequency by using imaginary impedance. We correlate imaginary impedance to target concentrations and to determine optimal frequencies. Since imaginary impedance correlates to capacitance, we attempt to expand the theory of optimal frequency in terms of effective capacitance and constant phase element. We also report a novel algorithm that decouples the convoluted signal when two biomarkers are co-immobilized onto a single sensor. As a verification of the technique, we demonstrate a preliminary investigation of the feasibility of the approach to simultaneously detect LDL and HDL. The two biomarkers are key biomarkers for coronary vascular disease (CVD), which is the leading cause of death in the United States with over 800,000 deaths per year (Yang et al., 2012). The National Cholesterol Education Program recommends the use of LDL and HDL as risk indicators for the development of CVD (Expert Panel on Detection, E., 2001). Furthermore, the LDL/HDL ratio is an excellent predictor of coronary heart disease risk and an outstanding monitor for the effectiveness of lipid lowering therapies (Fernandez and Webb, 2008). A multi-marker sensor that can detect LDL and HDL simultaneously would greatly benefit the efficiency of diagnosing CVD and serve as a precursor to other multi-marker electrochemical sensors employing antibodies as MREs.

2. Experiment

2.1. Sensor fabrication and characterization

The sensors consist of GDEs, silver/silver chloride reference electrodes, and platinum counter electrodes (CH Instrument, USA). The gold surface thickness of a GDE is approximately 2.5 mm. All EIS measurements were performed at room temperature using a CHI660C Electrochemical Analyzer from CH Instrument, USA. GDEs were polished with 100 figure-eight motions on Buehler felt pads using 3.0, 1.0, and 0.05 μm grit alumina oxide in distilled water (DI) followed by sonication in DI for 15 min. After sonication, the formal potential was obtained by performing cyclic voltammetry from −1.0 to 1.0 V in a solution of 100 mM potassium ferricyanide prepared in pH 7.4 phosphate buffer saline (PBS). EIS was then performed using the formal potential and a 5 mV AC sine wave sweeping from 1 Hz to 100 kHz to measure the bare impedance of GDEs, which helps determine GDEs' surface topography. After rinsing the GDEs with DI, 1 mM of 16-mercaptohexadecanoic acid (16-MHDA) in ethanol was incubated onto the GDEs for 1 h to form a self-assembly monolayer (SAM). Post-MHDA impedance was measured at the formal potential of each GDE for quality control. The carboxylate groups on the tail end of 16-MHDA were then activated by incubating the sensor with 40 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 20 mM sulfo-derivative of N-hydroxysuccinimide (NHS) for 1 h. After washing the sensor with DI, 5 mg/dL of the antibodies prepared in pH 7.4 PBS (LDL, HDL, or LDL and HDL combined) were then immobilized onto the sensor at room temperature for 1 h. For LDL and HDL co-immobilization, the antibodies were pre-mixed at a 1:1 ratio and the final concentration of each antibody was 5 mg/dL. The sensors were then washed with PBS following the immobilization and the remaining reactive sites were blocked with 1% ethanolamine for 30 min. After rinsing the sensors with PBS, they were stored at 4 °C until further use.

The reagents and solvents, 16-MHDA, EDC, NHS, and potassium ferricyanide were all obtained from Sigma-Aldrich, USA. PBS was purchased from VWR International, USA. LDL and HDL specific antibodies (not to cholesterol) were purchased from Academy Biomedical Company, USA.

2.2. Electrochemical characterization of LDL, HDL, and HDL-LDL co-immobilization

All sensors were brought to room temperature prior to testing. A Serial dilution made in PBS was used to prepare purified LDL and HDL samples. All samples were then well mixed with 100 mM potassium ferricyanide at a 1:1 ratio to form a total volume of 100 μL of each sample at 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, and 0 mg/dL. For LDL and HDL co-immobilization testing, the two markers were well mixed at a 1:1 ratio in a similar manner and the mixture has the concentration of 0–10 mg/dL for each biomarker. EIS was performed to measure each sample's impedance at each sensor's formal potential from 1 Hz to 100 kHz at 12 points per decade. The impedance at each frequency was correlated to the applied biomarker concentrations and the results were used to calculate sensitivity (slope) and specificity (RSQ). The slope and RSQ values were then plotted against the frequency to determine the frequencies at which the biomarker can be best detected (the optimal frequency).

2.3. Determination of optimal frequency: comparison between complex and imaginary impedance approach

EIS typically outputs 4 parameters: the real impedance (Z_r), imaginary impedance (Z_i), phase angle ($\varnothing$), and complex impedance (Z). Their relationships are shown below:

$$Z_r = |Z|\cos(\varnothing) \quad (2)$$

$$Z_i = |Z|\sin(\varnothing) \quad (3)$$

Where real impedance correlates to resistance and imaginary impedance capacitance and/or inductance. Nyquist plots are then plotted with real impedance (Z_r) on the x-axis and the negative of imaginary impedance ($-Z_i$) on the y-axis, producing a semi-circle curve shape. As targets enter the sensing area where MREs are immobilized, binding will occur and form the MRE-target complex. These complexes will obstruct the flow of electrons, causing a change in impedance that is concentration dependent.

Previously, complex impedance was used to determine the optimal frequency (Adamson et al., 2014, 2012; La Belle et al., 2013, 2011; Nandakumar et al., 2011). Complex impedance encompasses everything in the system, such as the Warburg (diffusion) resistance, charge transfer resistance, solution resistance, and double layer capacitance. By correlating the complex impedance at each frequency to the target concentrations, a slope and RSQ can be obtained at each frequency. The slope and RSQ values were then plotted against the frequency from 1 Hz to 100 kHz. The frequency with best slope and RSQ trade off was deemed the optimal frequency of the biomarker. Calibration curves can then be generated by correlating the complex impedance to target concentrations at the optimal frequency.

Complex impedance across the frequency spectrum is typically highest at low frequencies (< 1 kHz) and lowest at high frequencies (Fig. 2A). While this is not an issue for single biomarker detection, the abundance of signal from one biomarker's optimal frequency can overlap with the signal from another biomarker's optimal frequency, posing a great challenge for multi-marker detection.

On the other hand, as illustrated in Fig. 2B, imaginary impedance offers an additional parameter for the determination of optimal frequency: peak location. In contrast to complex impedance, imaginary impedance peaks at a specific frequency, forming a parabolic shape