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Highly Sensitive Label Free Biosensor for Tumor Necrosis Factor

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***Abstract*—**We present a label-free biosensor based on electrochemical impedance spectroscopy for the detection of pro-inflammatory cytokine Tumor Necrosis Factor (TNF-α). Secretion of TNF-α has been correlated to the onset of various diseases including rheumatoid arthritis, Crohn's disease etc. Gold electrodes were patterned on a silicon substrate and self assembled monolayer of dithiobis-succinimidyl propionate was used to develop the biosensor which achieved a detection limit of ~57fM. A linear relationship was also observed between increasing TNF-α concentrations and charge-transfer resistance within a dynamic range of 1pg/ml – 1ng/ml.

***Keywords*—**Tumor necrosis factor, electrochemical impedance spectroscopy, label free, self assembled monolayer

I. Introduction

PATHOGEN specific T-cells are a hallmark of specific immunity and are crucial for the protective effect of a vaccine.When a vaccinated individual encounters a pathogen, these "educated" or "memory" T-cells respond rapidly by secreting cytokines that orchestrate an immune system fightback. Cytokines are a family of regulatory proteins which are mostly produced by activated microphages and trigger immune-mediated inflammatory responses within the body via intercellular signaling. In healthy individuals, cytokines tend to be low (usually in pg/ml concentrations). However, at the onset of a pathological condition such as dengue, arthritis etc. these levels get elevated ten to hundredfold, triggering local cell apoptosis.

Efforts are being made to utilize cytokines as therapeutic and vaccine targets [1]. However, more research is needed to accurately assign specific profiles to specific diseases and to use cytokine-secreting cells as prognostic markers. So far, studies have mostly been performed at a small scale because of complicated sample preparation and manual operation steps [2]. Additionally, the time required to perform flow cytometry [3] makes it impossible to analyze hundreds and thousands of samples. Yet, information about the nature of cytokine-secreting cells could be of immense diagnostic value.

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Measurement of serum cytokines can also provide information about the magnitude of the overall immune activation in a patient. Recently, it was demonstrated that there is indeed a correlation between serum and cell-secreted cytokines [4]. Bead-arrays are primarily used to measure the concentrations of cytokines in the serum [5]. However, serum cytokines do not provide any information about specific protection if the source of the cells is unknown.

Whereas the protective or pathological function of these cell subsets have mostly been studied in mice, a comprehensive T-cell analysis has become a standard readout to test the efficacy of vaccines, including dengue vaccine candidates [6,7].

Conventionally, cytokines are measured using ELISA. Such fluorescent labeling systems are highly-sensitive but are often expensive and time consuming contributing to the difficulty of diagnosing the disease quickly.

Immunological research has seen an enormous progress in the identification and functional characterization of immune cell subsets and T-cells in particular, using mostly multiparametric flow cytometry as the method of choice. Currently, the only routine analytical assay based on cell type-specific cytokines is the Quantiferon test, which is used to diagnose TB infection by detecting TB-specific T-cells [8].

Only selected clinical laboratories are able to perform this time-consuming and labor-intensive test. In clinical practice, one of the persistent problems with inflammatory therapy has been a lack of simple measuring tools which give an accurate picture of inflammatory stress.

Label-free immunosensors, in which interactions between antibody and antigen are directly monitored without the presence of fluorescent labels offer increased advantages of low cost, speed and simplicity over conventional methods. Electrochemical impedance spectroscopy (EIS) is a label free Faradaic impedance technique that is performed in the presence of a redox probe. Various other biomolecules such as proteins, nucleic acids etc. have been detected with EIS [9-13].

Excellent reviews on EIS technique and its applications are available elsewhere [14, 15]. EIS techniques combined with self assembled monolayer (SAM) have gained increased attention [16, 17, 18] as SAM allows a closer binding of biomolecules enabling EIS to detect the smallest of capacitance or resistance changes [19], resulting in improved detection. The advantages of EIS-based sensors over labeled approaches lie in their simple labeling protocol, high sensitivity and potential of mass fabrication for low cost diagnostic and therapeutic purposes in remote settings.

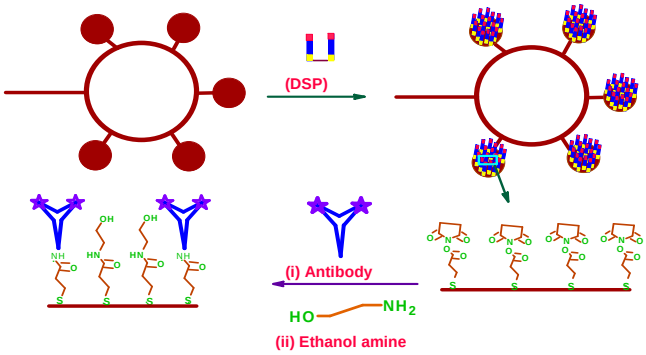

Fig. 1 Detailed steps of EA/anti-TNF-α/DSP/Au electrode functionalization process. Ethanol amine was used to prevent non-specific adsorption

Here we present a cheap and highly sensitive label-free immunosensor to detect TNF-α. Array of gold electrodes were fabricated on a silicon substrate with common photolithography processes. A self assembled monolayer of DSP was utilized to immobilize TNF-α antibodies and ethanol amine was used to prevent non-specific binding. Nyquist plots of EIS spectra indicated a proportional increase in charge-transfer resistance with increasing TNF-α concentration in the physiological range of 1pg/ml – 1ng/ml. A detection limit of ~57fM was achieved with minimal non-specific binding.

II. Procedure

A. Materials and Chemicals

Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), ethanol amine (EA) and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich-USA. Human anti-TNF-α, target protein TNF-α and ELISA kit were purchased from Biolegend. Phosphate-buffered saline (PBS) and dithiobis-succinimidyl propionate (DSP) were purchased from Invitrogen and Proteochem respectively.

B. Electrode fabrication

An array of circular gold microelectrodes with sensing areas having a diameter of 110 µm each were fabricated on an 8" silicon wafer using standard photolithography techniques. Ti/Au (0.1µm/1.0µm) layer was deposited using an electron-beam evaporator (Temescal Inc.). The sensing microelectrodes, bond pads, and connecting route lines were patterned by a series of processes such as photoresist spin coating (PFI-A26, Sumitomo Chemical Co., Ltd.), photolithography through a chrome mask in EVG 5200 Mask Aligner (EVG Group) and development in Shipley MF-319 developer (MicroChem Corp.). The unpatterned metal regions were etched with Au etchant (Au-600, CLC) and Ti etchant (Ti-890, CLC). Photoresist was then stripped in solvent and the wafers were cleaned in H_2SO_4:H_2O_2 solution (4:1) at 125°C to remove residual contamination. Subsequently, the wafer was passivated with a moisture barrier layer SiO_2/SiN (0.8µm/0.2µm) through plasma enhanced chemical vapor deposition (PECVD).

C. Immobilization of antibodies

Electrodes were pre-cleaned with acetone, ethanol, and copious amounts of de-ionized (DI) water. Afterwards, they were cleaned with piranha solution (H_2SO_4:H_2O_2; 7:3) for 5mins followed by rinsing in DI. For DSP SAM formation, 2 mg/ml solution of DSP in DMSO was prepared and reduced with 5mM TCEP for 15mins at 37°C. Chips were then incubated in DSP solution for 1hr at room temperature. After SAM formation, electrodes were rinsed with DMSO to remove any unbound DSP followed by rinsing in DI. The DSP modified electrodes were utilized immediately for immobilization of TNF-α antibodies (anti-TNF-α) after their preparation. The antibodies were covalently attached to DSP SAM by incubating the active area of electrodes in 50µl of 50µg/ml antibodies in phosphate buffer saline (PBS) solution (1x, pH 7.4) for 1hr at room temperature. Covalent binding occurs via reaction between the amino group on the antibody and reactive succinimidyl group on DSP. The electrode (anti-TNF-α/DSP/Au) thus formed was washed thoroughly with 1% EA in PBS to block remaining DSP groups and to prevent any non-specific adsorption (Fig. 1). Prepared electrodes were stored at 4°C when not in use and were characterized using electrochemical impedance spectroscopy during experimentation.

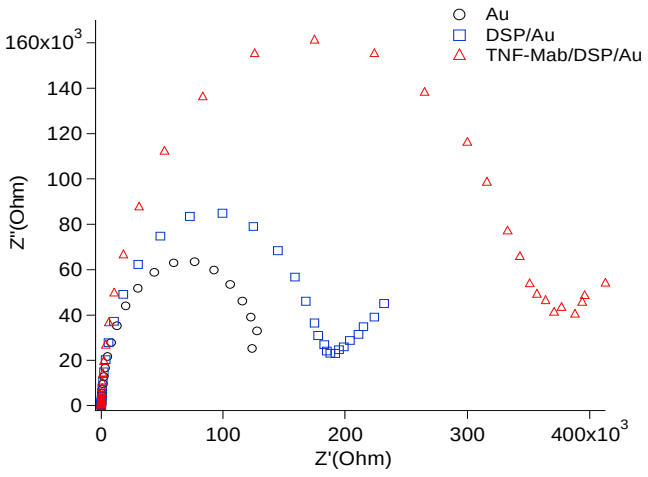

Fig. 2 Nyquist plots of (i) blank gold electrode (Au) (ii) DSP/Au electrode (iii) TNF-α monoclonal antibody/DSP/Au electrode. Diameter of the semicircle represents the charge-transfer resistance (R_{ct}) on the electrode surface. Increase of R_{ct} with each process i) 140 kΩ, ii) 185 kΩ and iii) 370 kΩ) indicates the retardation of the charge transfer process and therefore the successful modification of the surface

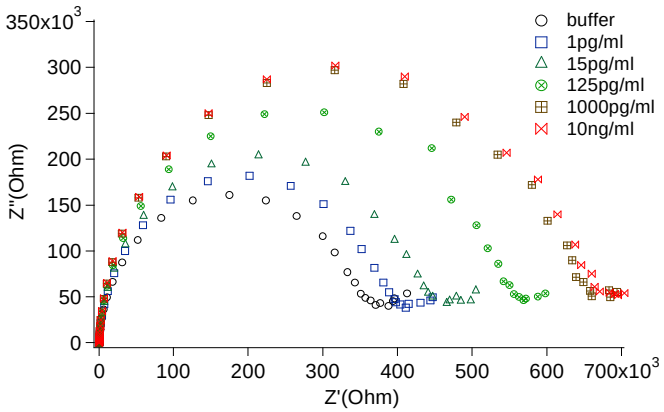

Fig. 3 Nyquist plots of EIS spectra on EA/anti-TNF-α/DSP/Au electrodes for buffer and 4 different concentrations of TNF-α in the range of 1pg/ml – 10ng/ml. A saturation effect was seen at 10ng/ml

D.EIS measurements

Autolab PGSTAT302 was used to record the changes in impedance. Concentrations of TNF-α from 1 pg/ml to 10ng/ml were prepared in 1x PBS containing 5mM ferrocyanide and 5mM of ferricyanide i.e. 5mM $Fe(CN)_6^{3-/4-}$ as a redox probe. Electrodes were stored in PBS before use in EIS analysis. For AC sweep impedance measurements, initial potential of 0V, amplitude of 25mV and a frequency range of 1Hz – 1MHz was used.

III. Results and Discussion

Nyquist plots of impedance spectra on the gold electrodes after each modification (bare electrodes, after SAM functionalization and after antibody immobilization) were recorded and used for the characterization of electrode fabrication (Fig. 2). Diameter of the semicircle represents the charge-transfer resistance (R_{ct}) on the electrode surface. Increase of R_{ct} with each process indicates the retardation of electron transfer and therefore the successful modification of the surface. Immobilization of monoclonal antibodies on gold electrodes had the biggest increase in electron charge transfer.

Fig. 3 shows the Nyquist plots of EIS spectra obtained on the EA/TNF-α/DSP/Au electrodes for 5 different TNF-α concentrations (1pg/ml – 10ng/ml) indicating a detection limit of ~57fM. A saturation effect was seen at levels above 1ng/ml that can be attributed to the saturating density of antibody on the electrodes and the wide range under consideration. Several detection methods including SAM based impedimetric immunosensors [20, 21], fluorescent microwells [22] and photonics [23] have been used for the detection of various cytokines including TNF-α. Cesaro-Tadic et al. [24] developed a miniaturized immunoassay using an independent capillary system. Although highly sensitive, their design employed immunofluorescence which is a costly approach.

Ganesh et al. [23] used a photonic crystal to develop a fluorescence sandwich immunoassay but the sensitivity of their system was very low (1ng/ml). All of these are costly methods requiring specialized equipment and multiple processing steps, and are not appropriate as low cost biosensors.

With increasing TNF-α concentration, diameter of the Nyquist plots was observed to increase as well, indicating that TNF-α binds to immobilized anti-TNF-α on the electrodes, producing a surface layer that decreases R_{ct} from solution to electrode surface in the presence of redox probe. A linear relationship was observed (Fig. 4) between changes in R_{ct} values and the log of TNF-α concentrations. Biosensor sensitivity can be increased at the expense of increasing electrode sizes and sensor area. However, we benchmarked our results against ELISA (Fig. 5) which yielded a detection limit of 15.6 pg/ml (~890 fM) indicating that our proposed biosensor design is already more sensitive than the conventional technique and works well within the physiological range.

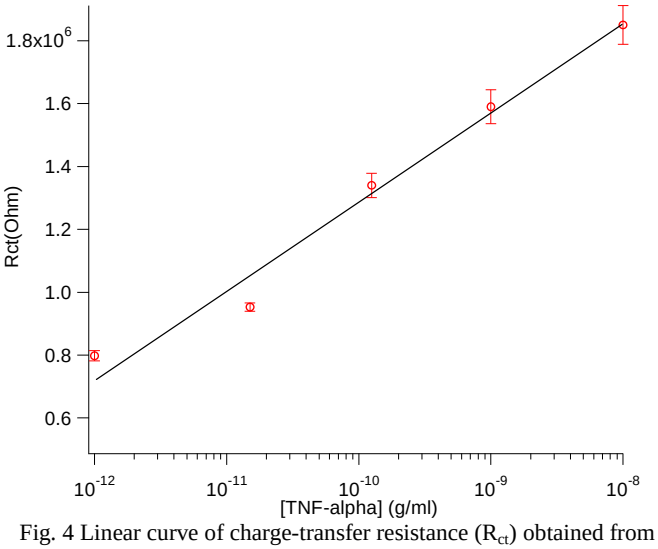

Fig. 4 Linear curve of charge-transfer resistance (R_{ct}) obtained from EIS studies for 5 different TNF-α concentrations over 3 chips

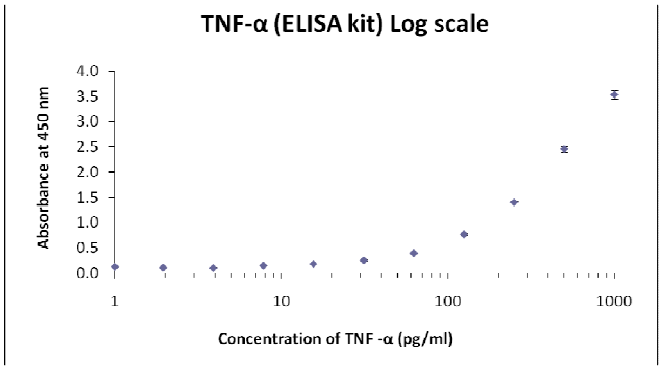

Fig. 5 Detection of TNF-α using ELISA kit. TNF-α was prepared at different concentrations (1 pg/ml – 1000 pg/ml) and was detected by ELISA. Optical density (OD) was read at 450 nM. Change in OD could be observed at 15.6 pg/ml and was undetectable at lower concentrations

To further understand and characterize complex cytokine networks and to make use of them as diagnostic and prognostic markers, a sensor system with the capabilities of simple operation, fast and quantitative detection of cell-specific cytokines secretion as well as with an affordable cost that can be used in large cohorts is indispensable.

IV. Conclusion

In this work, we fabricated a low cost, easy to fabricate and sensitive biosensor to detect TNF-α. TNF-α is a cytokine that plays an important role in cell signaling during inflammation. Self assembled monolayer of dithiobis-succinimidyl propionate (DSP) was functionalized on gold electrodes and non-specific binding was prevented with ethanol amine (EA). The biosensor showed excellent selectivity and detection limit (~57fM) compared to other cytokine sensor techniques. A linear relationship was found between the charge-transfer resistance and logarithmic value of the TNF-α concentrations in the physiological range (1pg/ml – 10ng/ml). We believe this biosensor may serve as a useful diagnostic and therapy monitoring tool for accurate monitoring of TNF-α

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