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Detection of tumor necrosis factor (TNF-α) in cell culture medium with label free electrochemical impedance spectroscopy

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ABSTRACT

Here we present an electrochemical impedance spectroscopy (EIS) based biosensor to detect TNF-α as part of an integrated platform to detect toxicity caused by inflammatory responses in cell culture systems. EIS based biosensors offer label free and low cost solution over conventional methods such as ELISA and have the potential to be integrated with cell culture systems for real time cell monitoring. The biosensor was fabricated on a silicon substrate with an array of gold electrodes which was functionalized with a self assembled monolayer of dithiobis-succinimidyl propionate (DSP). Results indicated that the biosensor was more sensitive (∼57 fM) than ELISA (∼890 fM), and achieved a proportionally decreasing response while detecting TNF-α (1 pg/ml to 100 pg/ml) in culture media. Ethanol amine was used to reduce non specific binding which was confirmed with interferon-γ (IFN-γ) in a competitive assay. Dose response curves were elicited by introducing lipopolysacchride (LPS) in Jurkat cell culture and the supernatant showed an exponential increase in TNF-α production, as detected by the biosensor. Time course results indicated that the biosensor reached a saturation limit after 24 h. Overall, the biosensor has the potential to provide a minimally invasive solution to assess inflammatory responses in real time in integrated cell culture systems.

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1. Introduction

Cell and tissue cultures are common techniques to grow cells in vitro to investigate their growth and/or product yield metrics as a function of pH, oxygen, medium composition, etc. [1,2]. This in vitro technique presents an excellent tool to understand cellular toxicity (cytotoxicity) over in vivo methods because it offers advantages such as defined cell types, an extracellular environment (for precise manipulation) and direct observation. Such in vitro cytotoxicity assays rely heavily on cell viability by using vital dyes such as Trypan blue (which enters dead cells only), Neutral Red (which is actively taken up by living cells) etc. or on the detection of apoptotic markers such as Annexin V, Caspase-3 etc. [3,4].

More recently, researchers are starting to investigate cellular stresses and inflammatory responses as methods to evaluate cytotoxicity via the detection of specific biological markers [4]. In particular, the release of cytokines by cells has been studied as a marker of cellular immune responses to cytotoxicity [5,6]. Cytokines represent a class of regulatory proteins which are mostly produced by activated microphages and trigger immune-mediated inflammatory responses via intercellular signaling. They act as important physiological and pathological markers for the prognosis and diagnosis of various diseases including rheumatoid arthritis (RA), alzheimer's, cancer [7–10] etc. For example, the release of multiple cytokines is proven to be correlated with the symptoms of malaise and fatigue experienced by RA patients [11], giving their detection a high diagnostic value.

Secretion of pro-inflammatory cytokines such as TNF-α, IFN-γ have been correlated to the onset of various diseases including rheumatoid arthritis, Crohn's disease etc. because of their involvement in a cascade of systemic inflammatory reactions. Tumor Necrosis Factor (TNF-α) is a cytokine capable of activating multiple signal transduction pathways and controlling the expression of numerous genes. The primary role of TNF-α (∼17.5 kDa) is to regulate immune cells. However, it is also involved in triggering a cascade of systemic inflammatory reactions [12] and has been suggested to stimulate the production of enzymes that degrade cartilage, inhibit the production of new cartilage and contribute to the local demineralization of bone by activating osteoclasts (cells which break down bones) [13]. Studies using anti-TNF-α agents have shown that they can slow or even prevent the progression of bone and cartilage damage in RA and Crohn's disease patients [14,15]. Therefore, in order to develop a better understanding of immune-mediated disorders, it has become important to detect TNF-α production and inflammatory stresses related with it.

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In individuals, TNF-α levels tend to be low (usually in pg/ml concentrations), which at the onset of a pathological condition get elevated and trigger local cell apoptosis, thus requiring highly-sensitive assays to monitor them. Sensitive detection of low levels of cytokines in biological medium can be achieved with enzyme-linked immunosorbent assays (ELISA). However, ELISA tests are often expensive, time consuming, and require several sample preparation steps. Real-time detection of cytokines has been investigated with sensing instruments such as the surface plasmon resonance (SPR) and waveguide grating sensors [16–18] which are sensitive to changes in the refractive index due to biomolecule adsorption at the sensor surface [19–21]. However, most of these techniques are based on optical methods which require expensive equipment and are not ideal for integration or as low cost solutions.

On the other hand, 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. Electrochemical impedance spectroscopy (EIS) is a Faradaic impedance technique that is performed in the presence of a redox probe and gauges the frequency dependent sensor response. It is a label-free method to quantify antigen-antibody bindings on electrode surfaces by measuring their capacitance and interfacial charge-transfer resistance. Heiduschka et al. [22,23] were among the first groups to demonstrate an impedimetric immunosensor to detect virus. Various other biomolecules such as proteins, nucleic acids etc. have also been detected with EIS [24–28]. Excellent reviews on EIS technique and its applications are available elsewhere [29,30]. Recently, EIS techniques combined with self assembled monolayer (SAM) have gained increased attention [31–33] as SAM formation allows a closer binding of biomolecules to the electrode surface enabling EIS to detect the smallest of changes in capacitance or resistance [34], resulting in improved sensitivity. Several detection methods including SAM based impedimetric immunosensors [35,36], fluorescent microwells [37] and photonics [38] have been used for the detection of various cytokines including TNF-α. Cesaro-Tadic et al. [39] developed a miniaturized immunoassay for TNF-α using an independent capillary system. Although highly sensitive, their design employed immunofluorescence which is a costly approach. Ganesh et al. [38] used a photonic crystal to develop a fluorescence sandwich immunoassay but the sensitivity of their system was limited to 1 ng/ml. We previously utilized highly sensitive silicon nanowire approach to detect TNF-α [40]. However, silicon nanowires also remain costly, requiring specialized equipment and multiple processing steps, and are not appropriate as low cost biosensors.

Here we present a CMOS compatible, low cost and highly sensitive label-free immunosensor which can be integrated into cell culture systems to detect TNF-α. Array of gold electrodes were fabricated on a silicon substrate with common photolithography processes. A self assembled monolayer of dithiobis-succinimidyl propionate (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 change in charge-transfer resistance with changing TNF-α concentrations in the physiological range of 1 pg/ml–100 pg/ml in culture media. A detection limit of ∼57 fM was achieved with minimal non-specific binding which was confirmed by testing with a competitive assay of IFN-γ. Lipopolysaccharide (LPS) induced dose response curves were also recorded and showed an exponential increase in the secretion of TNF-α. For time dependent curves measured at 1 μg/ml, the biosensor showed a saturation effect after 24 h. The advantages of EIS-based sensors over labeled approaches lie in their high sensitivity and potential of mass fabrication for low cost diagnostic purposes.

2. Experimental details

2.1. 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.

2.2. 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 (0.1 μm/1.0 μm) 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 entire wafer (except Au electrodes) was passivated with a moisture barrier layer SiO_2/SiN (0.8 μm/0.2 μm) through plasma enhanced chemical vapor deposition (PECVD).

2.3. 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 5 min followed by rinsing in DI. For DSP SAM formation, 2 mg/ml solution of DSP in DMSO was prepared and reduced with 5 mM TCEP for 15 min at 37 °C. Chips were then incubated in DSP solution for 1 h at room temperature (25 °C). 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 50 μl of 50 μg/ml antibodies in phosphate-buffered saline (PBS) solution (1×, pH 7.4) for 1 h 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. Prepared electrodes were stored at 4 °C when not in use and were characterized using electrochemical impedance spectroscopy during experimentation.

2.4. EIS measurements

Electrochemical impedance spectroscopy (EIS) is a powerful and sensitive characterization tool for studying the charge transfer processes occurring at electrode-solution or modified electrode-solution interfaces. In the Nyquist plot of impedance spectra, the usually observed semicircle at higher frequencies corresponds to the electron-transfer resistance process (R_{ct}) and the linear portion seen at lower frequencies corresponds to the diffusion process i.e., Warburg impedance (Z_w). The magnitude of (R_{ct}) depends on the dielectric and insulating features at the electrode/electrolyte interface and can be used to interpret electrode/electrolyte

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