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Electrochemical immunosensor for highly sensitive and quantitative detection of tumor necrosis factor-α in human serum

Ajay Kumar Yagati, Min-Ho Lee, Junhong Min *

School of Integrative Engineering, Chung-Ang University, Heukseok-dong, Dongjak-gu, Seoul 06974, South Korea

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ABSTRACT

Highly sensitive and selective biosensors for accurate determination of specific protein biomarkers at low levels in serum are a prerequisite for the present healthcare systems. Therefore, here we developed a label-free impedimetric method for tumor necrosis factor-alpha (TNF-α) detection using reduced graphene oxide (RGO) with gold nanoparticles (AuNP) on an indium tin oxide (ITO) microdisk electrodes. The detection mechanism relies on resistance change occurs due to $[Fe(CN)_6]^{3-/4-}$ redox probe movement towards the conductive channels of the AuNP-RGO films gated by the recognition of the target biomarker by its anti-TNF-α antibody. The conductivity of the AuNP-RGO structures enhanced by 10-fold in comparison with bare electrode chips. The observed resistance changes at 2 Hz ($\Delta R_{\text{at 2Hz}}$) enabled the quantification of various concentrations of TNF-α in human serum ($C_{\text{HS-TNF-}\alpha}$). The antibody-adsorbed electrode showed a good increment of resistance change (ΔR) with additions of antigen concentration. The sensor possesses a linear range of 1–1000 pg/ml had a detection limit of 0.67 pg/ml (38.51 fM) and 0.78 pg/ml (44.83 fM) in PBS and human serum, respectively.

1. Introduction

Tumor necrosis factor-alpha (TNF-α, 17 kDa) is one of the most potent multifunctional pro-inflammatory cytokine playing a pivotal role in apoptosis and cell survival which linked to many infectious and inflammatory diseases [1]. Elevated levels of TNF-α leads to rheumatoid arthritis, psoriasis, diabetes, and cancer [2–4] and thus, quantification of TNF-α is necessary to estimate and to develop early disease diagnosis systems. However, sensitive and selective determination of TNF-α remains a significant challenge owing to its presence at a low concentration level in the human body (10 to 30 pg/ml) [5]. Typical methods for TNF-α detection include enzyme-linked immunosorbent assays (ELISAs) [6], bioassays [7], and fluorescence immunoassays [8]. Although these methods are robust and well-established but suffer from drawbacks such as the lack of reusability of the kits, being expensive, multi-step sample preparation methods and labeling secondary antibodies with fluorescent or chemiluminescent agents which make the assay less suitable for real-time measurement [9]. In this context, newer platforms are highly desired coupled with enhanced analytical capability for low-level detection of biomarkers.

Electrochemical methods have excellent features emerging as an alternative way to the other methods mentioned above for the detection of TNF-α. However, traditional electrodes exhibit poor response towards analyte detection due to slow electrode kinetics, electrode fouling, low sensitivity and reproducibility [10,11]. Also, the reduction in the size of the active electrode area results in a decrease in current levels between nano to pico current level. Therefore, attempts have been made to modify the electrode surfaces with selective recognition elements to improve the current/impedance level to enhance the sensor performance in the analyte detection.

Graphene, a single atom thick 2D carbon nanosheet possesses excellent electrical, chemical, and optical properties, thermal conductivity, high electron mobility, and stability [12]. Graphene can easily integrate into complex sensors through various fabrication procedures. However, the use of graphene alone suffers from disadvantages such as irreversible self-agglomerations which occur by van der Waals and π-π stacking interactions, less colloidal stability, reduced reliability, and non-specificity [13]. Therefore, the addition of gold nanoparticles (AuNP) with graphene acts as nano-spacer and conductor which minimizes these limitations and provides additional synergistic properties such as higher effective surface area, enhanced electrocatalytic activity, excellent thermal stability, electrical conductivity, biocompatibility, and water solubility [14].

In recent years, various biosensing methods have been evolved for the detection of protein biomarkers, however many of these developed methods require complicated transducing elements for instance labeling with fluorescence dyes, or enzymes to obtain a signal from the binding events [15]. With increasing interest in point of care (POC) devices there a need for fast, real-time and reliable diagnosis with the capability of detecting biomarkers at lower levels. To address this issue, recently various types of transducers coupled with different types analytical methods were introduced to develop sensitive detection of TNF-α

* Corresponding author.
E-mail address: junmin@cau.ac.kr (J. Min).

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[15–22]. We have recently published preliminary studies on impedance sensor for TNF-α detection in PBS buffer medium using RGO-NP electrodes [23]. However, this method has several drawbacks such as (*i*) it lacks the measurements of TNF-α in real serum samples; (*ii*) not a reproducible method for developing stable RGO-NP platform; (*iii*) no stepwise construction and characterizations of the biosensor and (*iv*) has many flaws related to its sensing part. To address these problems, here we adopted plenty of optimization steps and methods to achieve stable and reproducible transducers coupled with sensing analysis to provide full practicability towards PoC applications.

Thus present study focusses on the stable AuNP decorated RGO on ITO disk electrodes through electrodeposition procedures and their application towards for ultrasensitive detection of TNF-α in human serum. The RGO conjugated with AuNPs showed enhanced electrochemical properties and allowed good electrochemical response with sensitivity towards TNF-α. Amine coupling reactions enabled the binding of TNF-α antibody (Ab-TNF-α) with underlying AuNPs/RGO electrodes. Comprehensive surface analysis for AuNP-RGO was carried out by analytical tools to prove the formation of composite on the electrode surface. Furthermore, the efficacy of the developed sensor has been tested for the determination of TNF-α in human serum to show the applicability of this method to real matrices.

2. Experimental details

2.1. Materials and reagents

Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), N-(3-dimethyl aminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-mercaptopropionic acid (3-MPA), 2-(N-morpholino)ethane sulfonic acid (MES), and polydimethylsiloxane (PDMS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Graphene oxide (5 mg/ml; dissolved in water) obtained from a graphene supermarket (UniNanoTech, Korea). Recombinant TNF-α was purchased from Enzo Life Sciences, Inc., and TNF-α monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Bovine serum albumin (BSA), phosphate buffer solution (NaH_2PO_4, 10 mM, pH 5.7), phosphate-buffered saline (PBS) and C-reactive protein (CRP, human) were purchased from Sigma-Aldrich. Volumetric standard HCl and excess pure NaOH obtained from OCI Company (Korea). Human serum was collected with informed consent from patients at Yonsei Severance Hospital (Seoul, Korea). Buffer solutions were prepared using ultrapure deionized water (18.2 MΩ/cm) supplied by a Milli-Q system. All other reagents were of analytical reagent grade or the highest purity available and used without further purification unless indicated otherwise.

2.2. Preparation of ITO MDEA and electrodeposition

The procedure for the fabrication of ITO MDEA on slide glass (7.5 × 2.5 cm) substrates is presented as follows. Spin-coating an epoxy-based photoresist (SU-8 3005, Microchem, MA, USA) and with standard photolithography procedures, enabled to produce ITO disk-shaped working electrodes (Ø = 500 μm) with a large counter electrode (CE). The development of electrode fabrication resulted in an ITO pattern on a glass substrate consisting of eight working electrodes with a common counter electrode, along with transmission lines and connecting pads shown in Fig. 1a. PDMS chamber was attached to assist with the electrochemical depositions and to preserve the solvents for immunoreaction. The fabricated electrodes mounted on chip adapter for testing was shown in Fig. 1(b).

2.3. Electrodeposition of RGO, AuNP, and AuNP-RGO on ITO MDEAs

The 0.5 mg/ml graphene oxide (GO) solution was prepared in 0.1 M sodium phosphate buffer (pH 7). GO was electrochemically reduced on ITO by cyclic voltammetry (CV) using a CHI 660E potentiostat (CH Instruments, TX, USA) with an Ag/AgCl electrode and patterned ITO was serving as reference and counter electrodes, respectively. The potential was scanned from 0 to −1.6 V for reducing GO on ITO electrodes, and the number of sweep cycles in potentiodynamic depositions was limited to two cycles. Effect of pH of GO solution was evaluated by preparing 0.5 mg/ml GO solutions under different pH 3, 5, 7, 9, and 11 towards electrodeposition. Linear sweep voltammograms (LSV) were recorded on ITO MDEAs from −0.6 to −1.4 V vs. Ag/AgCl to understand the reduction of GO under different pH conditions towards (*i*) RGO formation ability, (*ii*) potential shifts and (*iii*) structural defect analysis. Also, for AuNP formation on ITO and RGO/ITO, 0.25 mM $HAuCl_4 \cdot 3H_2O$ in 0.1 M $NaNO_3$ was prepared and electro-deposited through two potential sweep cycles from 0 to 0.8 V vs. Ag/AgCl to control the growth of the nanoparticles. Optical images were obtained for each electrode modification with nanomaterials as shown in Fig. 1(c–f). Further Fig. 1g shows in a glance of Experimental set-up for graphene oxide depositions under three-electrode configuration. More detailed experimental set-up for RGO-NP depositions and impedance measurements for the TNF-α sensor was given in Figs. S1 and S2 (Supporting information).

2.4. Immobilization protocol for impedimetric TNF-α sensor

The AuNP-RGO electrodes were incubated with 3-MPA (40 mM) for 1 h to allow the formation of a self-assembled monolayer. The MPA-modified electrodes were gently washed with 70% ethanol solution, DI water, and then dried in N_2 stream. The electrodes were immersed in 1:1 mixture of EDC (400 mM)/NHS (100 mM) in 100 mM MES buffer solution (pH 4.7) for antibody binding for 30 min at room temperature. The activated surface was then gently washed with 100 mM MES buffer to remove excess EDC/NHS from the surface, followed by rinsing with 10 mM PBS buffer at pH 7.0. Subsequently, 20 μl of Ab-TNF-α antibody solution (10 μg/ml) was drop-casted onto each electrode chip to covalently bind TNF-α antibody on the activated surface and then kept in a humid chamber for 1 h to prevent drying of the surface during binding. Afterwards, the electrodes were gently washed with 1× PBS to remove any unbound antibody molecules. TNF-α antibody was immobilized using a coupling reaction between the amino group of the TNF-α antibody and the EDC/NHS-activated MPA molecules on the modified electrodes. Finally, to avoid nonspecific adsorption, BSA (10 μg/ml) in PBS (10 mM, pH 7.4) was utilized, thus forming TNF-α antibodies/MPA/AuNP-RGO/ITO electrodes for detection of TNF-α (see Fig. 1(h)). After each washing step, the changes in impedance magnitude (|Z|) were observed to confirm the successful binding of the reaction agents and antigen-antibody reactions. Different amounts of TNF-α were prepared in PBS or human serum to obtain final concentrations of 0.1, 1, 10, 100, 500, and 1000 pg/ml respectively.

2.5. Measurements and characterization

The electrochemical conductivity of various electrodeposited ITO MDEAs was evaluated with impedance spectroscopy measurements in 10 mM ferricyanide/ferrocyanide ($[Fe(CN)_6]^{3-/4-}$) with 0.1 M KCl electrolyte. The input potential for electrochemical impedance spectroscopy (EIS) was 10 mV in amplitude with a frequency range of 0.1 to 10^5 Hz. The electrochemical properties of the electrodes were obtained by fitting with a modified Randles equivalent circuit using impedance fitting analysis software ZView (Scribner Associates Inc., Southern Pines, NC, USA). Field Emission Scanning Electron Microscopy (FESEM) images of the modified electrode surface were acquired using a Carl Zeiss Σ operated at 5 kV. Raman spectroscopy was conducted on UniRAM spectrophotometer (UniNanoTech, Korea) equipped with a mapping range XY of 75 × 50 mm and 1 μm in *Z*-axis. The spectra were acquired using a 532-nm excitation laser in the 500 to 2000 cm^{-1} range. X-ray diffraction patterns (XRD) of the samples were recorded using CuKα radiation (λ = 1.5406 Å) of a Rigaku X-ray diffractometer (Rigaku D/MAX-2500/pc).