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Multiplexed electrochemical immunosensor for label-free detection of cardiac markers using a carbon nanofiber array chip

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ABSTRACT

We present an electrochemical multianalyte or multiplexed immunosensor for simultaneous label free detection of cardiac markers panel, comprising of C-reactive protein, cardiac troponin-I and myoglobin. The multi-electrode biosensor chip contains nine identical but electrically isolated microelectrodes arranged in a 3 × 3 array configuration. Each electrode contains carbon nanofiber nanoelectrodes grown vertically using plasma enhanced chemical vapor deposition. A hydrophobic photoresist layer, lithographically etched on the chip, exposes the electrodes and helps to selectively immobilize the antibody probes for the three target cardiac biomarkers using carbodiimide chemistry. The real-time label free detection of the three cardiac markers from a mixture is demonstrated with high sensitivity and selectivity. Detection in complex protein mixtures in human blood serum does not show any false positives from non-specific protein adsorption. The results show that the present sensor can serve as a miniaturized, low cost lab-on-a-chip system for the detection of various biomarkers in healthcare, environmental monitoring and security applications.

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1. Introduction

Rapid and accurate diagnosis of acute myocardial infarction (AMI) can help in devising appropriate and timely surgical procedures or cardiovascular disease therapy for heart patients, thereby avoiding life-threatening situations such as heart attack or heart failure [1–5]. An appropriate approach for cardiac risk management can be an independent monitoring of the blood concentration of standard cardiac markers before and after an AMI event and likewise determining the blood concentration levels of some of the early cardiac markers useful for diagnosis/prognosis of a heart patient [6,7]. An alternative approach is the simultaneous determination of blood concentration of biomarkers specific to AMI and systemic inflammation (SI) - a state that occurs much earlier to myocardial necrosis; this can be done using an array of electrodes or devices with each containing respective bio-recognizing elements (capture antibodies, for example) [8]. The blood concentration of standard biomarkers such as cardiac troponin T & I (cTnT and cTnI), creatine kinase (CK-MB) and myoglobin (Mb) remain elevated after the occurrence of AMI whereas the concentration levels of early markers such as cardiac reactive protein (CRP), myeloperoxidase (MPO), natriuretic peptide, both B-type natriuretic peptide (BNP) and N-terminal ProBNP, and P-selectine in blood plasma rise on the occurrence of symptoms of SI [6–9]. The simultaneous detection of certain specific cardiac biomarkers thus becomes important for the accurate detection or monitoring of cardiovascular disease, and the choice of the cardiac biomarkers for simultaneous detection can be made from the list given above.

There are several examples of immunoassays developed for the accurate and sensitive detection of a single cardiac protein with limits of detection (LOD) down to ng/mL to pg/mL [10–18]. Recently, electrochemical analysis for the simultaneous detection of different targets using several parallel single-analyte immunoassays or the simultaneous amperometric determination of multianalytes using multiplexed assays has shown impressive potential [19–21]. The real time detection of analytes can be performed using either a label-free electrochemical impedimetric immunosensor [22] or a labeled amperometric immunosensor that uses labeled antibodies: the labels include florescent conjugated enzymes, metal ions, magnetic or non-magnetic nanoparticles as capture probes with enzymatic amplification features [23,24]. For example, simultaneous electrochemical multianalyte immunosensors using single label or multiple labels have recently been demonstrated [25–30]. The use of labeled antibodies for each type of antigens and florescent detection method add to the complexity of integrating such a device in hand-held point-of-care-testing (POCT) systems.

Alternatively, a wide range of label-free array based immunosensors for the simultaneous detection of cardiac biomarkers has been reported. Arntz et al. described an immunosensor for early and rapid label-free real-time detection of CK-MB and Mb using an array of seven microfabricated cantilevers [31]. The relevant capture antibodies were immobilized covalently on distinct cantilevers, and

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independent detection of dual cardiac markers was obtained through the cantilever deflection induced by the stress generated upon antigen–antibody interaction. Deflection sensitivity for Mb was reported to be below 20 μg/mL. Differential measurements between the sensors and reference cantilevers were used to reduce the effect of thermal drifting as well as cantilever instabilities generated through the reagent injection system; nevertheless, the non-specific adsorption can still generate false positive signals and the use of differential signal measurements adds to the sensor complexity. Zhang et al. reported an integrated system consisting of an array of silicon nanowires interfaced with readout circuit that can perform label-free simultaneous detection of cTnT, CK-MM and CK-MB in serum with high sensitivity (100 fg/mL) and selectivity [32]. Most of the above-mentioned biosensor platforms either involve expensive and complex instrumentation or sophisticated numerical algorithms limiting their use in research laboratory settings.

In the present work, an array based immunosensor for the simultaneous detection of cardiac marker panel - comprising of an early marker CRP and two established markers, Mb and cTnI - is reported. The biosensor uses a nanoelectrode array (NEA) of vertically aligned carbon nanofibers (VACNFs), and the tips of these nanofibers are functionalized covalently with the bio-recognizing elements (cardiac capture antibodies). The target markers are electrochemically detected from the mixture solution, thus developing the sensor into a lab-on-a-chip offering advantages such as low sample volume, multiplexing capability, potential low cost per test through wafer-scale fabrication and simple electrochemical detection strategy with improved sensitivity and specificity. The sensor performance in terms of target antigen quantification, electrode cross talk and specificity is presented and also compared against devices reported previously in the literature. The physiological and disease state concentrations of these three cardiac biomarkers are well known. The CRP concentration is >3 μg/mL in human serum several hours after an AMI event [33,34]. The normal serum concentration of cTnI is below 0.4 ng/mL and any value above 2.0 ng/mL represents high risk [35,36]. The normal myoglobin concentration is 100–200 ng/mL, which can reach levels of 420–2000 ng/mL in serum after the AMI event [37]. The presented approach is able to meet these clinical levels while satisfying all the other metrics mentioned above.

2. Experimental

2.1. Chemicals and regents

Troponin I (cTnI, 20 μg lyophilized powder) protein from human heart, monoclonal anti-Troponin I (anti-cTnI, 1 mg protein per mL (Biuret))) antibody produced in mouse, C-reactive protein (CRP, 2.1 mg protein per mL (lowry)) from human plasma, anti-human C-reactive protein (anti-CRP, 54.7 mg protein per mL (Biuret)) antibody (produced in goat), myoglobin from human heart (Mb, ≥95%, SDS-PAGE, 2 mg protein per mL (lowry)), anti-myoglobin (anti-Mb, 1:500 protein dilution) produced in rabbit, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, ≥99%), glycerol (for molecular biology, ≥99%), N-hydroxysuccinimide sodium salt (sulfo-NHS, ≥98% (HPLC)) were purchased from Sigma Aldrich (St. Louis, MO). Powdered skim milk as blocking agent was purchased from Saco Mix'n Drink (Middleton, WI).

The stock CRP solution was stored at 4 °C whereas the anti-cTnI, anti-CRP, anti-Mb and Mb solutions were stored at −20 °C. Highly pure de-ionized water (18.2 MΩ.cm) from super-Q Millipore system was used throughout the study. CRP-free human blood serum was purchased from Fitzgerald and used without modification. Phosphate buffered saline (PBS, 10 mM, pH 7.4) was prepared by dissolving PBS sachet (Sigma Aldrich, St. Louis, MO) in de-ionized water and was filtered using a 0.22 μm membrane filter (Millipore Durapore PVDF from Sigma Aldrich, St. Louis, MO) before every use. The 1× PBS solution contains the following 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4. Shipley i-Line photoresist (SPR220.7) and resist developer

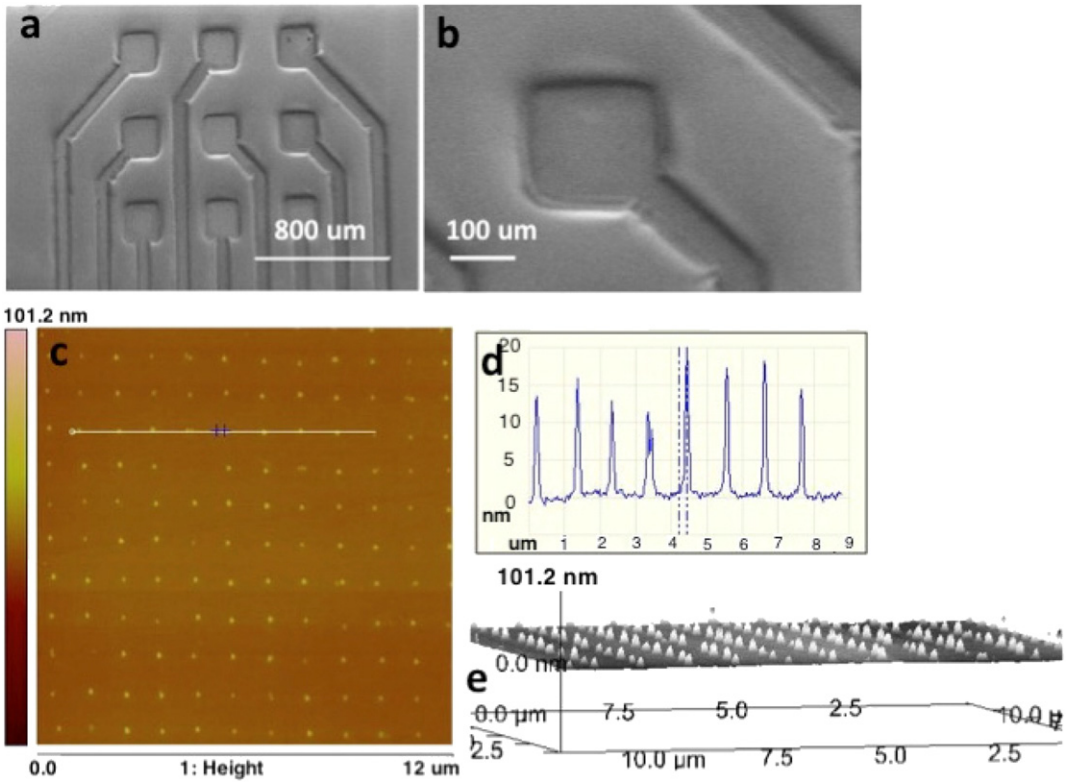

Fig. 1. Fabricated biosensor chip. a) SEM image of etched 3 × 3 array device chip exposing individual electrodes (scale 800 μm), b) SEM image of an individual electrode with resist layer coatings (scale 100 μm), c) 2D AFM scan of random surface (12 μm × 12 μm) of an electrode after etching, confirming the presence of exposed CNFs (bright dots), d) AFM line profile indicating CNF height in nm (vertical axis) and e) 3D AFM micrograph revealing the VACNFs protruding above the planer oxide surface.