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Label-free detection of C-reactive protein using a carbon nanofiber based biosensor

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ABSTRACT

We report the sensitive detection of C-reactive protein (CRP), a biomarker for cardiac disease, using a carbon nanofiber based biosensor platform. Vertically aligned carbon nanofibers were grown using plasma enhanced chemical vapor deposition to fabricate nanoelectrode arrays in a 3×3 configuration. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used for the CRP detection. The CV responses show a 25% reduction in redox current upon the immobilization of anti-CRP on the electrode where as a 30% increase in charge transfer resistance is seen from EIS. Further reduction in redox current and increase in charge transfer resistance result from binding of CRP on anti-CRP immobilized surface, proportional to the concentration of the CRP target. The detection limit of the sensor is found to be ~90 pM or ~11 ng/ml, which is in the clinically relevant range. Control tests using non-specific myoglobin antigen confirmed the specificity of the present approach.

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1. Introduction

Detection and quantification of biomarkers for acute myocardial infraction (AMI) is critical in the diagnosis of cardiovascular diseases. Traditional diagnosis methods for cardiovascular diseases are time consuming and expensive since they are primarily conducted at central clinical laboratories. Faster and inexpensive diagnosis through point-of-care, lab-on-a chip type systems is highly desirable (Mohammed and Desmulliez, 2011). Cardiac troponin (cardiac troponin T (cTnT) and cardiac troponin I (cTnI)), CK-MB (one of the three isoenzyme forms of creatine kinase (CK)) and myoglobin are known as the definitive biomarkers and their elevated concentration in blood remains maintained from few hours to several days upon the occurrence of AMI (Yang and Zhou, 2006). Biomarkers specific to systemic inflammation (SI), a state that occurs much earlier to myocardial necrosis, include C-reactive protein (CRP), myeloperoxidase (MPO), P-selectine etc. and these are known as the biomarkers for earlier detection of acute coronary syndrome (Yang and Zhou, 2006). Among these, CRP, an acute phase protein, is very sensitive and specific to SI, which is synthesized in liver and secreted into blood stream causing infection or inflammation (Du Clos, 2000). The blood plasma concentration of CRP, which increases rapidly several orders during the SI state, can therefore predict future chances of AMI and hence, can help in risk stratification and implementing appropriate and timely therapeutic procedures (Tsai et al., 2007). A rapid and early diagnosis of cardiovascular disease should therefore involve the testing for a definite biomarker (cardiac Troponin, CK-MB or myoglobin) in combination with an early biomarker (C-reactive protein) (Apple et al., 2005).

C-reactive protein has thus become an important candidate for the early detection of cardiovascular events and for setting up preventive measures to reduce the number of deaths due to AMI. There are three different levels of CRP concentrations, suggested by the American Heart Association and the United States Center for Disease control, in human blood serum that evaluates the cardiovascular disease risk: CRP concentration less than 1 μg/ml represents a low risk state, concentration between 1 and 3 μg/ml is considered as average risk and any concentration above 3 μg/ml represents high risk (Bryan et al., 2013; Kushner and Sehgal, 2002). Various studies have proved CRP as a strong predictor of life threatening events such as AMI, giant cell arteritis, arterial disease, stroke and cardiac arrest causing sudden death (Kervinen et al., 2001). Its concentration level in blood rises from a normal level of ≤ 5 μg/ml to > 100 μg/ml in case of systemic infections (Tsai et al., 2007). The reliable means of detection and quantification of CRP concentration thus becomes important for accurate implementation of therapeutic interventions before and after AMI occurrence for monitoring the status of AMI patients. Besides the traditional expensive and time-consuming methods of CRP testing (Roberts et

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al., 2000), quantification of CRP using surface plasmon resonance based biosensors (Hu et al., 2006), optical and acoustic biosensors (Luchansky et al., 2011; McBride and Cooper., 2008), enzyme-linked immunosorbent assay (ELISA) (Leung et al., 2005), fluorescence based biosensors (Wolf et al., 2004), magnetic biosensors (Tsai et al., 2007; Martin et al., 2007), electrochemical biosensors based on gold electrode (Bryan et al., 2013; Hennessey et al., 2009), magnetic nano-particles (Tsai et al., 2007) etc., has been demonstrated with a wide range of sensitivity, specificity, cost, turn around time (TAT), immunoassay complexity, flexibility for multiplexing, and detection limit (Qureshi et al., 2012).

Among the available techniques, electrochemical based biosensors have been one of the most successful in offering less expensive, faster, simpler, portable, accurate, flexible for multiplexing and label free detection of a wide range of proteins, nucleic acids, enzymes and other biomolecules. In addition, with the advancements in bioelectronics and biosensors technology using materials such as carbon nanotubes (CNTs) (single and multi-walled) (Jacobs et al., 2010), carbon nanofibers (CNFs) (Li et al., 2012; Vamvakaki et al., 2006) graphene (Kuila et al., 2011), gold nano-wires, nano-structures (Das and Kelley, 2011) and other inorganic wires (Meyyappan and Sunkara, 2010), the sensitivity and detection limits for proteins, nucleic acids etc. have improved tremendously in recent years. Among carbon nanostructures, vertically aligned carbon nanofibers (VACNFs), where each fiber is an individual freestanding nanostructure and acts as a nanoelectrode, have been used successfully to construct biosensors (Li et al., 2005; Cassell et al., 2009; Koehne et al., 2009; Arumugam et al., 2009, 2010; Siddiqui et al., 2010; Periyakaruppan et al., 2011) including detection of cardiac troponin (Periyakaruppan et al., 2013). Due to their small diameter, robustness, high conductivity, biocompatibility and ease of surface modification, VACNF based nanoelectrode arrays (NEAs) serve as a biosensor platform. These VACNFs are typically grown by plasma enhanced chemical vapor deposition (PECVD) wherein the inherent vertical electric field on the substrate platform helps to orient the carbon nanofibers vertically. The VACNFs can be randomly placed on the wafer like a forest or can be precisely positioned at predetermined spots by patterning the growth catalyst on the wafer (Arumugam et al., 2009, 2010; Siddiqui et al., 2010). The nanoelectrode array can be employed with conventional electrochemical techniques such as cyclic voltammetry (CV) (Nicholson, 1965), differential pulse voltammetry (DPV) (Brown and Anson, 1977), and electrochemical impendence spectroscopy (EIS) (Brown and Anson, 1977). Here we use a nanoelectrode array with patterned VACNFs for the detection and quantification of C-reactive protein using both CV and EIS and obtain a detection limit of 90 pM.

2. Materials and methods

2.1. Chemicals and regents

C-reactive protein (2.1 mg protein per ml (lowry)) from human plasma and anti-human C-reactive protein (anti-CRP, 54.7 mg protein per ml (Biuret)) antibody (produced in goat), anti-myoglobin (produced in rabbit, 1:500 protein dilution) as control antibody, myoglobin from human heart ($\geq$ 95%, SDS-PAGE, 2 mg protein per ml (lowry)) as control antigen, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, $\geq$ 99%) and N-hydroxysuccinimide sodium salt (sulfo-NHS, $\geq$ 98% (HPLC)) were purchased from Sigma Aldrich (Saint Louis MO). The stock CRP solution was stored at 4 °C whereas the anti-CRP, anti-myoglobin and myoglobin 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. Phosphate buffered saline (PBS, 10 mM, pH 7.4) was prepared by dissolving PBS sachet (from Sigma Aldrich) in de-ionized water and was filtered using a 0.22 μm membrane filter before every use. Sodium hydroxide (NaOH, 1 mM) solution, for electrochemical etching, was prepared by dissolving appropriate quantity of NaOH pellets (from Sigma Aldrich) in de-ionized water. For continuous use, the anti-CRP solution was stored at 4 °C in working aliquots. Potassium hexacyanoferrate (III) for probe molecule ($Fe[CN_6]^{3-/4-}$) and all other regents used in the study were of analytic grade.

2.2. Nanoelectrode array fabrication using e-beam patterned VACNFs

A 4-inch silicon (100) wafer with 500 nm thermal oxide layer consists of 30 devices (or chips). Each device contains nine identical micro pads (electrodes), each with a surface area of 40,000 μm^2, arranged in 3 × 3 array format as shown in Fig. S1(a) (Supporting Information). Each electrode is interconnected to an individual contact pad (1 mm) for external electrical contact. A brief account of the fabrication procedure is given here. A 200 μm thick chromium (Cr) layer was deposited by e-beam evaporation on optolithographically defined electrodes, contact pads and electrical interconnects. Then the electrodes (micro pads) were spun coated with 400 nm poly(methyl methacrylate) (PMMA) and patterned by using e-beam current (2 nA, 1950 μC/cm^2 at 100 keV). A 10 nm Cr adhesion layer followed by 30 nm nickel catalyst layer were further deposited on these e-beam patterned electrodes. VACNFs were grown from these patterned Ni catalyst particles (Arumugam et al., 2009) using a DC-biased PECVD system (Aixtron, Cambridge, UK). The growth recipe includes acetylene (C_2H_2, 125 sccm) feedstock for carbon source diluted with ammonia (NH_3, 444 sccm) at a pressure of 6.3 mbar, 700 °C and plasma power of 180 W. A 15 min deposition yields ~39,000 free standing VACNFs spaced 1 μm apart on each patterned electrode, where each fiber has a tip diameter of ~80 nm, base diameter of ~100 nm and an average height of ~1.5 μm with Ni catalyst particle on the tip. Passivation of CNF sidewalls and the underlying Cr layer was done by depositing a 3 μm silicon oxide (SiO_2) layer using CVD and employing tetraethylorthosilicate (TEOS) vapor precursor from a liquid source. Planarization of the top electrode surface and exposure of fibers tips were achieved by chemical mechanical polishing (CMP) in two steps, using 0.5 μm alumina (pH 4) for stock removal and 0.1 μm alumina (pH 4) for final polish. Contact pads for electric connection were exposed by optical lithography using a 2.5 μm thick Shipley 3012 resist. Finally the wafer was diced into 30 equal size (~14 mm squares) chips containing nine electrically isolated electrodes (3 × 3 format, 200 μm squares) interconnected with nine contact pads (1 mm squares) for electrical connections. Fig. S1(b) and (c) shows magnified scanning electron microscopy (SEM) (S-4000, Hitachi, USA) images of an individual electrode and VACNF tips embedded in oxide layer. Fig. S1(d) shows an AFM image of the electrode's surface topography with the line section showing the height profile and Fig. S1(e) depicts the 3D schematic of NEA, working electrode along with the exposed fiber tip (Fig. S1(f)).

2.3. Electrochemical characterization

All electrochemical measurements were carried out using a standard three electrodes electrochemical cell connected to a H-CHI660D Instrument, Electrochemical Workstation/Analyzer (CHI Instruments, Inc., Austin, TX). The instrument was interfaced to a personal computer and controlled by associated data processing/analyzing software, chi660d. The electrochemical cell consists of high quality platinum (Pt) wire as counter electrode, saturated calomel electrode (SCE, Accumet, New Hampshire, USA) as reference electrode and nine identical working electrodes of VACNF NEAs connected in a custom designed Teflon liquid cell. The Teflon