Nanocrystalline diamond film for biosensor applications

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ABSTRACT

In this study, we have developed a novel capacitive biosensor based on interdigitated gold nanodiamond (GID-NCD) electrode for detection of C-reactive protein (CRP) antigen. CRP is one of the plasma proteins known as acute-phase proteins and its levels rise dramatically during inflammatory processes occurring in the body. It has been reported that CRP in serum can be used for risk assessment of cardiovascular diseases. The antibodies immobilization were confirmed by fourier transform spectroscopy (FTIR) and contact angle measurements. In this capacitive biosensor, nanocrystalline diamond acting as a dielectric layer between the electrodes. The CRP antigen detection was performed by capacitive/dielectric-constant measurements. Our results showed that the response of NCD-based capacitive-based biosensor for CRP antigen was dependent on both concentration (25–800 ng/ml) as well as frequency (50–350 MHz). Furthermore, using optimized conditions, the biosensors developed in this study can be potentially used for detection of elevated level of risk markers protein in suspected subjects for early diagnosis of disease.

1. Introduction

A biosensor is a device designed to detect or quantify a biochemical molecules and it has been widely used as powerful analytical tools in medical diagnostics, food industry, environment, security and defense, etc. It includes proteins detection, nucleic acid or DNA sequencing or monitoring antigen–antibody interaction. In principle, it is generally fabricated by immobilizing a biological receptor material, for instance antibody and antigen, DNA, on the surface of a suitable transducer that converts the biochemical signal into quantifiable electronic signals. Capacitance measurement could be a useful tool in immunoassay [1]. The measuring principle of capacitive affinity biosensors was based on changes in dielectric properties, charge distribution, dimension and shape, when an antibody/antigen complex formed on the surface of an electrode [1]. Capacitive affinity biosensors can be constructed by immobilizing recognition elements in thin layers between the electrodes (or onto dielectric/substrate material, NCD in this study) and measuring changes in the dielectric/surface properties when an analyte binds. For providing larger sensor surface, conductors can be made into a pattern of interdigitated fingers.

In this study we are proposing an integrated solution for the detection and quantification of proteins to offer advantages of higher sensitivity, capability of single or multiple detection capability, easy to use, ease of signal processing with better sensor-signal integrity, smaller in size, compatibility to be integrated into a micro/electronics system, and much reduced system cost. The advantages are attributed to the use of gold electrodes on nanocrystalline texture for immobilization substrate (diamond), micron-sized capacitive transducer for detection and quantification and CVD-grown diamond as the dielectric layer between the electrodes and also substrate for antibody-antigen immobilization. CVD-diamond is a promising material because of its biocompatibility, durability, chemical inertness and its carbon composition. The high strength of C–C bonds as well as the established biocompatibility makes diamond a particularly attractive substrate for biosensor applications [2]. Therefore, nanocrystalline diamond film was layered on silicon support followed by gold interdigitated fingers.

C-reactive protein is one of the inflammation markers in human serum [3] and its level elevates to several thousand folds because of inflammation induced by infection or injury that leads to cardiovascular disease risk. Recent research suggests that patients with elevated basal levels of CRP are at an increased risk of diabetes [4,5] hypertension and cardiovascular disease [6,7]. Therefore, CRP is a potential biomarker to which biosensors for its detection are in demand.

In this paper, to our knowledge, for the first time, a new capacitive immunosensor was developed, based on a gold interdigitated electrodes fabricated on nanocrystalline diamond (GID-NCD) surface to detect CRP. Using CRP antibody as the model ligand/substrate, a direct detection of CRP by capacitance/dielectric measurements was demonstrated using a heterostructure of Au/nanocrystalline diamond covalently bound with CRP antibodies. When such CRP antibody immobilized heterostructure interacts with CRP antigen, the interaction of antibody with the antigen leads to the change in thickness of the dielectric layer and induces change in capacitance which can directly be related to detect the antigen.

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2. Experimental details

2.1. Reagents and materials

Monoclonal antibodies and purified antigen, C-reactive protein were purchased from Fitzgerald Industries International (Concord, MA, USA). 3-Mercaptopropionic acid, N-(3 dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), and N hydroxysuccinimide (N-hydroxy-2,5-pyrrolidinedione, NHS) were obtained from Sigma-Aldrich (Steinheim, Germany). PBS and Tween 20 were purchased from Sigma (USA). All other reagents and solvents were of analytical grade and the doubly distilled water was used throughout the experiments.

2.2. Synthesis, cleaning and hyderogenation of nanocrystalline diamond film (NCD)

Nanodiamond films were formed with the process of the gases of $CH_4/H_2/N_2$ in a Microwave Plasma Enhanced Chemical Vapor Deposition (PECVD), employing growth rate reduction conditions and using an ASTeX® reactor equipped with a 2.45 GHz microwave generator. Pre-treatment of the Silicon (1 0 0) substrate consisted of mechanical polishing of the surface using a 2.5-μm diamond powder, and ultra-sonication with a 5- to 20-nm diamond powder in acetone solution to augment diamond nucleation. A gas mixture of $CH_4/H_2/N_2$ with flow rates of 15/8/190 sccm, respectively, was introduced into the CVD system at a pressure of 20 Torr, with the substrate temperature being 800 °C, and microwave power at 550 W. The growth rate of the diamond deposition process was controlled to be 0.1 μm/h to achieve nm-scaled grains of diamond.

2.3. Fabrication of gold/interdigitated electrodes

Gold/interdigitated electrodes were fabricated on nanocrystalline diamond surface using image reversal technique. In this process the metal layers were patterned using the dual tone photoresist AZ 5214 E. Very thin tungsten layer of 50 nm was DC sputter deposited on the diamond-Si film, which was used to improve the adhesion of gold on substrate. Then 500 nm of gold was deposited using DC sputter deposition. Following this step, the gold layer was patterned by image reversal with the mask. Length of each electrode was 750 μm with a width of 25 μm. The distance between two electrodes was 25 μm. A 40 μm deep SU8 wells were patterned over the interdigitated structure for easing the antibody immobilization on the sensor structure.

2.4. Immobilization of C-reactive protein

For preparation of self-assembled monolayer (SAMs), a clean GID-NCD was immediately immersed in a 10 mM mercaptopropionic acid (MPA) solution at room temperature for 24 h before being thoroughly rinsed with distilled water and dried over pure nitrogen gas. As a result, SAM of MPA (SAMPA) was formed at room temperature by spontaneous adsorption of alkanethiol on gold surface by the reaction of sulfide.

Human CRP antibody was then immobilized on SAMPA through covalent binding. First, the carboxylic groups of SAMPA were activated by adding 0.05 M of EDC and 0.03 M of NHS in phosphate buffer for 5 h. The amine activated GID-NCD electrode was incubated by adding 20 μl of 100 μg/ml CRP antibodies for 1 h at room temperature. The GID-NCD electrode surface immobilized with CRP antibodies was washed with PBS buffer followed by double distilled water. The free/unoccupied carboxyl groups on GID-NCD electrode surface was blocked by adding 100 mM ethanolamine buffer (pH 8.0) and incubated for 2 h at 4 °C followed by washing with PBS buffer and distilled water and finally dried. A schematic diagram of covalent coupling of CRP-antibody on GID-NCD surface is shown in Fig. 1. For CRP-antigen detection, a series of CRP-antigen concentration (0–1000 ng/ml) in 20 μl volumes was dropped on the electrode and incubated for 1 h.

2.5. Detection of CRP-antigen and characterizations

Scanning electron (JSM-5600LV-SEM) and optical micrographs of the nanocrystalline diamond surfaces were studied. IR spectrum of surface activated GID-NCD was taken using a THERMO (Nicolet) 6700 Model FT-IR spectrometer. The wet ability of surfaces was characterized by measuring the water contact angle (CA; contact-angle measurement system). Dielectric parameters (impedance/capacitance) were measured in the frequency range 50 MHz–1 GHz using Network Analyzer (Karl-Suss PM-5 RF Probe Station and Agilent-8720ES). Network analyzer was calibrated using SOLT (short-open-load-through) method. Dielectric constant and conductivity were calculated from measurements of the sample capacitance and resistance. Dielectric constant and conductivity values were extracted from the measurements at certain frequencies (f) of 50 to 400 MHz. Capacitance measurement were taken

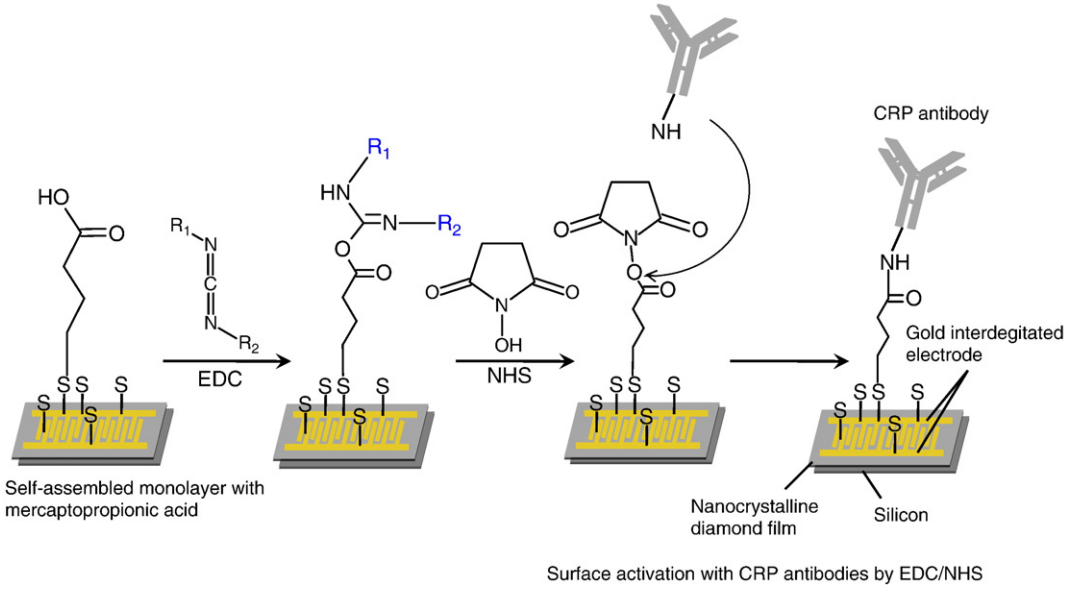

Fig. 1. Schematic diagram of covalent immobilization of human CRP-antibody on to the sensor surface.

at each step of: (a) Blank sensor surface, (b) after SAM formation, (c) after surface activation, (d) after blocking of CRP-antibody immobilization, and (e) after capturing of different concentrations (25, 100, 500, 800 and 1000 ng/ml) of CRP-antigen by antibodies on sensor platform surface.

3. Results and discussion

3.1. SEM and optical micrographs and FTIR characterization

Fig. 2 shows scanning electron micrographs of the diamond film on silicon substrate. It shows that diamond surface is quite homogenous, flat and nonporous. Nanoscaled surface texture of the PECVD grown diamond is presented in Fig. 2(a) and ~1.5 μm in thickness nanocrystalline diamond on silicon substrate is also presented in Fig. 2(b).

Fig. 2(c) shows optical micrograph of sensor platform. Thickness of sputtered gold was about 500 nm on the diamond film and homogenously well distributed on the surface.

To show the presence of amine groups on sensor surface, FTIR spectrum of the GID-NCD surface was recorded after the formation self assembled monolayer as shown in Fig. 2(d). The absorption peak at 2200–2400 cm^{-1} (2360 cm^{-1}) wave number range indicates the stretch vibration of Si–H groups. The adsorption peaks in the range of 1750–1500 cm^{-1} are the deformation of vibrations of N–H bonding. The less intense peak at 1450 cm^{-1} shows the presence of normal amine. The peak at 1160 cm^{-1} shows the C–N stretch.

3.2. Contact angle (CA)

It is known that if the liquid is very strongly attracted to the solid surface (for example water on a strongly hydrophilic solid surface) the droplet will completely spread out on the solid surface and the contact angle will be close to 0°. Less strongly hydrophilic solids will have a contact angle up to 90°. Contact angle measurement was measured on self assembled monolayer formed on the GID-NCD surface before and after the antibody treatment.

The contact angle measurement for SAM of the film showed 73°. After antibody immobilization, the contact angle was observed to be 60°. It is clear from this result that after antibody immobilization on the surface, the surface turned to be hydrophilic in nature, which evidently showed that the antibodies were immobilized on the sensor surface.

3.3. Dielectric measurements

Fig. 3(a) shows the variation of the dielectric constant as a function of different concentrations of CRP antigen at different frequency. It was observed from the figure that dielectric constant passed through dielectric dispersion [8] and decreased with frequency. The Fig. 3(b) also shows that conductivity is increased with the increased amount of CRP antigen concentration at a constant frequency.

Protein molecules, such as CRP-antigen and CRP-antibodies are composed of one or more polypeptide chains folded in a complex and fractural geometry. Polypeptide bonds, amino acid side-chains and solvent molecules are organized at different levels to control

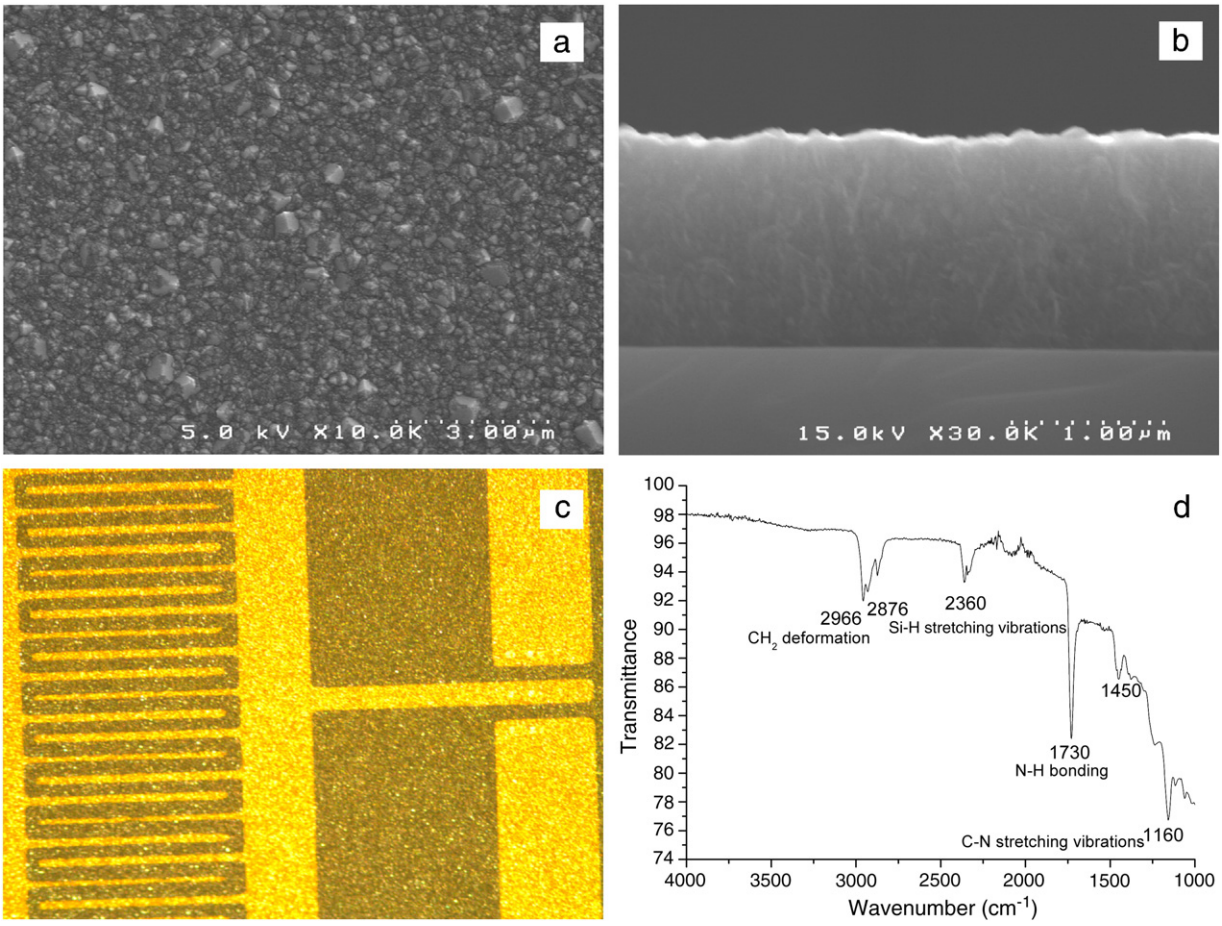

Fig. 2. Scanning electron micrographs of (a) the NCD film on silicon substrate and (b) cross-sectional image; optical micrograph of (c) GID-NCD surface (dimension of the electrode was 750 μm × 25 μm) with spacing between two electrodes was 25 μm; and (d) FTIR spectrum of the sensor surface after the formation self assembled monolayer.

secondary and three-dimensional structure of proteins through the formation of peptide bonds as shown in the reaction (Fig. 3c).

The N–C bond in the peptide units has a partial double bond character, so that the six atoms C_α NHCO C_α are coplanar. In addition, the C O bond is itself polar, so that the peptide bond possesses a permanent dipole moment. Since each peptide unit possesses a permanent dipole moment, polypeptide chains take the form of strings of connected dipoles. The drop in values of dielectric constant with frequency was possibly because of the rotational relaxation of the protein molecules (β dispersion) [9]. It was also observed that the dielectric dispersion $\Delta\varepsilon'$ $(=\varepsilon'_s-\varepsilon'_\infty)$ for control is lower than the antigen treated sample (Table 1). The changes in the value of $\Delta\varepsilon'$ were attributed to change in shape and volume of protein molecules [10]. The changes in the values of $\Delta\varepsilon'$ are functions of changes in the dipole moment of the macromolecules which will consequently depend on the center of mass of the charge distribution and the molecules radius [10], it suggested that there are some biophysical process occurring within the protein molecules resulting from the interactions of the electric field which may causes rearrangement of its charge distribution and resulted change in properties. It is clear from the figure that the values of conductivity increased which accompanied by a decrease in the values of dielectric constant (Fig. 3a–b). Our results showed that the response of this capacitive based sensor for CRP-antigen protein was dependent on concentration in a range 25–800 ng/ml of CRP-antigen as well as frequency at a range 50–350 MHz. The concentration and frequency above 800 ng/ml and 350 MHz, respectively showed no increase in response by this sensor system (Fig. 3a). This was possibly because of saturation of antibody binding sites on the sensor surface. In addition, the sensor surface was bio-functionalized with a constant amount of CRP-antibody (100 μg/ml). It is clear that there are limited binding sites on CRP-antibodies and thus the limitation of CRP-antigen binding capacity.

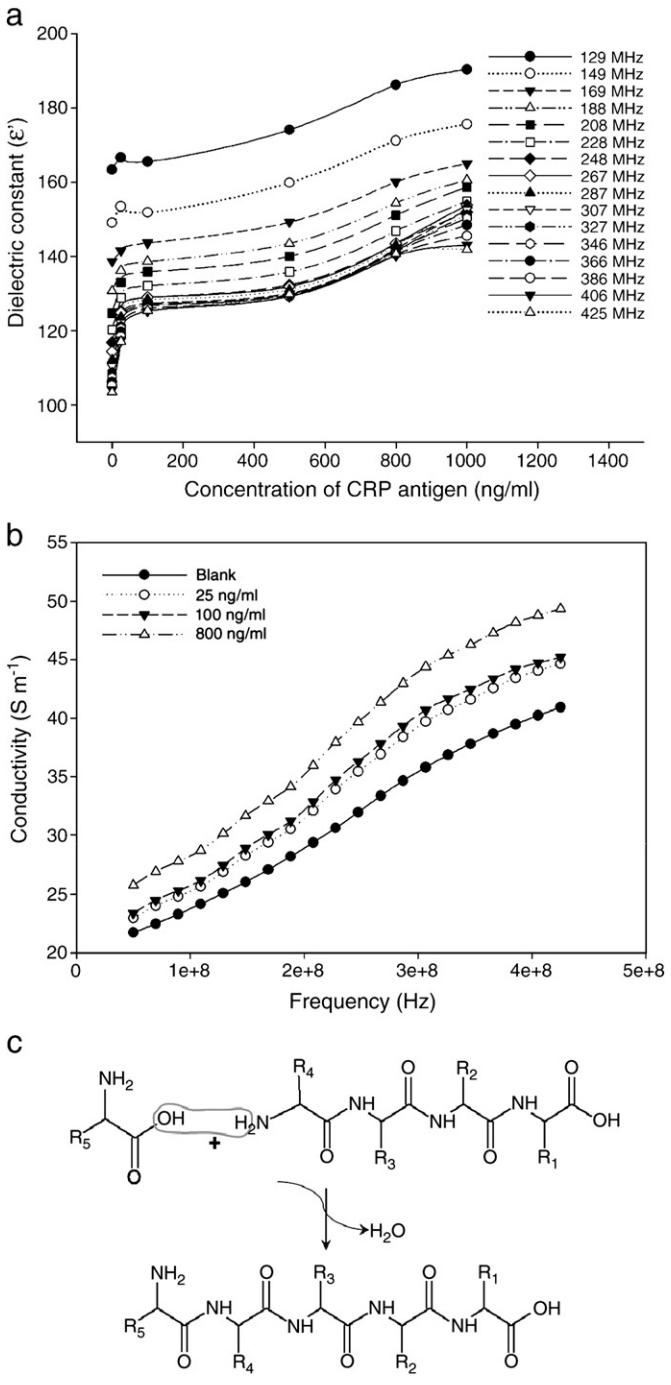

Fig. 3. (a) The variation of the dielectric constant as a function of the different concentrations of CRP antigen at different frequencies; (b) variation in the conductivity with different concentrations of CRP antigen at different frequencies as shown in the figure legend and (c) the condensation reaction between α-amino of one amino acid and α-carboxyl group of the other amino acid for the formation of peptide bond in a growing polypeptide chain of a protein. R1 to R5 represent the alkyl groups present on the amino acids.

Table 1
Values of dielectric dispersion for blank (no antigen but contains only antibodies on sensor surface) and tests with different concentration of CRP-antigen.

Parameter	CRP-antigen concentration (ng/ml)					
	0	25	100	500	800	1000
Dielectric dispersion ($\Delta\varepsilon'$)	5951.43	5186.56	5000.31	5120.99	5448.04	5096.36

4. Conclusion

A novel capacitive interdigitated gold electrodes/nanocrystalline diamond biosensor developed for the detection of CRP antigen cardiovascular risk marker. The response and sensitivity of this capacitive-based biosensor for CRP antigen was dependent on both concentration and applied frequency. The dynamic detection range using optimized conditions for a given antibody concentration (100 μg/ml) was found to be in the range 25–800 ng/ml of CRP-antigen. This range falls within the concentration levels of CRP-antigen in a cardiovascular disease risk conditions. The sensitivity can be greatly improved by manipulating the surface area of capacitive-sensor as well as the antibody concentration for immobilization.

The capacitive biosensor developed in this study is an inexpensive and versatile technique that can be potentially applied for detection of elevated CRP levels in suspected subjects for early diagnosis of cardiovascular disease. Finally using optimized conditions, the capacitive biosensor can also be applied for detection of various other diseases using novel biomarkers or probes for diagnosis or detection of environmental contaminants.

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