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Label-free capacitive biosensor for sensitive detection of multiple biomarkers using gold interdigitated capacitor arrays

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ABSTRACT

In this study, a highly sensitive and label-free multianalyte capacitive immunosensor was developed based on gold interdigitated electrodes (GID) capacitor arrays to detect a panel of disease biomarkers. C-reactive protein (CRP), TNFα, and IL6 have strong and consistent relationships between markers of inflammation and future cardiovascular risk (CVR) events. Early detection of a panel of biomarkers for a disease could enable accurate prediction of a disease risk. The detection of protein biomarkers was based on relative change in capacitive/dielectric properties. Two different lab-on-a-chip formats were employed for multiple biomarker detection on GID-capacitors. In format I, capacitor arrays were immobilized with pure forms of anti-CRP, -TNFα, and -IL6 antibodies in which each capacitor array contained a different immobilized antibody. Here, the CRP and IL6 were detected in the range 25 pg/ml to 25 ng/ml and 25 pg/ml to 1 ng/ml for TNFα in format I. Sensitive detection was achieved with chips co-immobilized (diluted) with equimolar mixtures of anti-CRP, -IL6, and -TNFα antibodies (format II) in which all capacitors in an array were identical and tested for biomarkers with sequential incubation. The resulting response to CRP, IL6, and TNFα in format II for all biomarkers was found to be within 25 pg/ml to 25 ng/ml range. The capacitive biosensor for panels of inflammation and CVR markers show significant clinical value and provide great potential for detection of biomarker panel in suspected subjects for early diagnosis.

1. Introduction

In recent years, there is increasing interest and demand for automated high-throughput multianalyte detection in clinical diagnosis. There are many immunoassay methods that can realize ultrasensitive (Tang et al., 2008; Yang et al., 2009), label-free (Okuno et al., 2007), simple (Haab, 2005), high throughput (Tothill, 2009; Wang et al., 2008) and detection of a single protein have been well developed. Although, most protein biomarkers are not highly specific to a particular disease, for example, cardiovascular risk (CVR) is associated with more than one biomarker for its incidence. The biomarker panel includes C-reactive protein (CRP), TNFα, and IL6 that are shown to have strong and consistent relationships between markers of inflammation and future CVR events (Martin-Ventura et al., 2009). Thus, the development of a multianalyte immunoassay for panels of biomarkers holds significant importance for diagnosis of disease (Hill and Martins, 2006). Some studies have shown that the measurement of a biomarker panel can avoid false-positive or -negative results, thus improving the diagnostic value of the biomarkers (Wilson and Nie, 2006).

Multianalyte detection offers the advantages of shortened analysis time, simplified analytical procedure, minimum sampling volume, improved test efficiency, and cost effectiveness as compared to parallel, single-analyte assays. In recent years, optical immunosensor arrays, have been used for detection of multianalyte protein biomarkers (Hill and Martins, 2006; Stoeva et al., 2006). Other techniques that includes chemiluminescent (Pei et al., 2010), radioisotopes (Gow et al., 1986), colorimetric (Wang et al., 2009), piezoelectric (Hasenbank et al., 2008) bead based microarrays (Shariatmadar et al., 2005), and quantum dot and nano-particles bioconjugate multianalyte assays have been reported (Jokerst et al., 2009). A majority of these analytical approaches require adequate transducing elements, such as enzymes, fluorescent dyes, to generate a physically readable signal from this recognition event. However, these methods involve time consuming labeling procedures, trained users and sophisticated/expensive experimental tools and techniques. Electrochemical immunosensors have also been reported to detect multiplexed protein biomarker detection (Bhavsar et al., 2009; Lai et al., 2009; Polsky et al., 2008; Tang et al., 2010; Wu et al., 2008).

Capacitive immunoassays are promising alternatives to existing immunochemical tests for the development of hand-held devices which can be used for point-of-care applications. The attraction of affinity-based capacitive sensors is that they are able to determine the analyte directly in a sample with no or very little sample

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preparation. The sensing principle of these sensors is based on changes in dielectric properties, charge distribution, and/or conductivity change that is brought on by antibody–antigen complex formed on the surface of the electrodes. Capacitive affinity biosensors can be constructed by immobilizing recognition elements, such as antibodies on the electrodes, 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. The capacitance between the interdigitated electrodes can then be described by the basic capacitance equation:

$$C = \frac{2n\varepsilon\varepsilon_0 A}{d} \quad (1)$$

where ε is the dielectric constant of the medium between the plates, ε_0 is the permittivity of free space, A is the area of the electrodes and d is the distance between the two electrodes, n being the number of electrodes and finally the factor 2 in equation represent each electrode forming two capacitors. Thus, when there is a change in the dielectric properties of the material between the electrodes, a change in the capacitance will occur and it is correlated to the bound antigen molecules and amount captured by antibodies on the surface, as well as between the electrodes. There have been some reports on label-free capacitive biosensors with or without redox mediator (faradaic or non-faradaic) (Carrara et al., 2009; de Vasconcelos et al., 2009; Quershi et al., 2009; Saravan et al., 2008; Zor et al., 2009). It is observed that the capacitance behavior of most sensors often are prone to large standard deviation, poor reproducibility from electrode to electrode, non-specific signal and the interface behavior is not uniform as that of an ideal capacitor.

In this study, to our knowledge, for the first time, a new label-free multianalyte capacitive immunosensor was developed based on gold interdigitated electrodes (GID) fabricated on SiO_2 surface (capacitors) to detect a panel of disease biomarkers that include CRP, TNFα, and IL6 that potentially determine the CVR. The fact that the present study is different from the previous reports in several important considerations. These include (a) efficient covalent immobilization of pure/mixture of antibodies directly on an optimized GID electrode geometry compared to epoxy-silanization (Saravan et al., 2008), which is prone to less sensitivity, and (b) use of a less-expensive SiO_2 background of the capacitors with high sensitivity compared to that reported with nanocrystalline diamond (NCD) background (Quershi et al., 2009). We here, investigated a simple, label-free, and sensitive multianalyte capacitive immunoassay that can be potentially applied for developing hand-held devices for point-of-care applications.

2. Materials and methods

2.1. Reagents and materials

Monoclonal antibodies and purified antigens for C-reactive protein, TNFα, and IL6, and Tween 20 were purchased from Sigma (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 (Germany). All other reagents and solvents were of analytical grade and the doubly distilled water was used throughout the experiments.

2.2. Patterning GID array electrodes on SiO_2 surface for fabrication of capacitor arrays

GID array electrodes were patterned on SiO_2 surface using image reversal technique. In this process, the metal layers were patterned using the dual tone photoresist AZ5214E. A 2 μm thick AZ5214E photo resist was patterned with the help of a mask for a lift-off process. Following this step, a very thin tungsten layer of 50–60 nm size was layered to improve the adhesion of gold on the SiO_2 film by DC sputter deposition and about 200–210 nm thick gold layer was deposited. The lift-off process was performed in pure acetone as a solvent. The length of each electrode was 800 μm and a width of 40 μm with a distance between two electrodes was 40 μm. As a result, each GID array on a capacitor contained 24 GID fingers.

2.3. Immobilization of antibodies (anti-CRP, -TNFα, and -IL6) on GID electrodes

The immobilization of antibodies on GID electrode surface of capacitors was carried out using two different lab-on-a-chip formats; (I) each GID-capacitor was immobilized with pure antibodies in an array and (II) each GID-capacitor was co-immobilized with multiple antibodies in equimolar ratios in arrays (Fig. 1a and b). First, all capacitor arrays were subjected to plasma cleaning and immersed in an ethanolic solution of 10 mM of β-mercaptopropionic acid (MPA) at room temperature for 24 h. The SAM layer of MPA on the GID electrodes was thoroughly rinsed with distilled water and dried over pure N_2 gas. The free carboxyl groups of MPA on the surface of GID electrodes of capacitor arrays were activated by incubating with a mixture of 0.05 M of EDC and 0.03 M of NHS in distilled water for 5 h. The surface activated GID-capacitors arrays were then divided into two groups for formats I and II, respectively. In format I, each activated GID electrode array was covalently coupled with specific pure form of 20 μl of 100 μg/ml antibodies (anti-CRP, -TNFα, -IL6), respectively in PBS for 1 h at room temperature (Quershi et al., 2009). Where as in format II, the anti-CRP, -TNFα, and -IL6 antibodies were mixed in the ratio of 1:1:1 and co-immobilized covalently on activated GID electrodes by incubating 20 μl of 100 μg/ml of the antibody mixture. Here, each GID electrode in a capacitor array received equal amounts of anti-CRP, -TNFα, and -IL6 antibodies.

2.4. Detection of pure and multiple antigens

Initially, a series of antigen concentrations (0–100 ng/ml) were prepared in PBS buffer. The capacitors were then incubated for 1 h with different antigen concentrations in 5 μl volume each for formats I and II. The dielectric parameters (impedance/capacitance) were measured before and after the antigen treatment. First, the dielectric parameters were measured with (a) blank capacitor, (b) capacitor after SAM formation, (c) after surface activation, (d) after antibody immobilization, and compared the results with (e) after capturing of different concentrations of antigen on antibody functionalized capacitors. The dielectric parameters (impedance/capacitance) were measured in the frequency range 50 MHz to 1 GHz using Network Analyzer (Karl-Suss PM-5 RF Probe Station and Agilent-8720ES) and the data were extracted at an effective frequency range. Network analyzer was calibrated using SOLT (short-open-load-through) method. Dielectric constant was calculated from measurements of the sample capacitance using basic Eq. (1).

A series of capacitor arrays were used in formats I and II for detection of different concentrations of pure and multiple antigens, respectively (Fig. 1). All experiments were performed in at least duplicates for error analysis. Detection process for format I was performed by incubating specific antigens (CRP, TNFα, and IL6) on GID electrode regions of respective capacitor arrays each immobilized with pure antibodies.

In case of format II, detection of multiple antigens was performed by sequential incubation of multiple antigens (CRP, TNFα, and IL6) on capacitor arrays each immobilized with equimolar ratios of multiple antibodies (anti-CRP, -TNFα, and -IL6). In the first step, the chips were incubated with different concentration

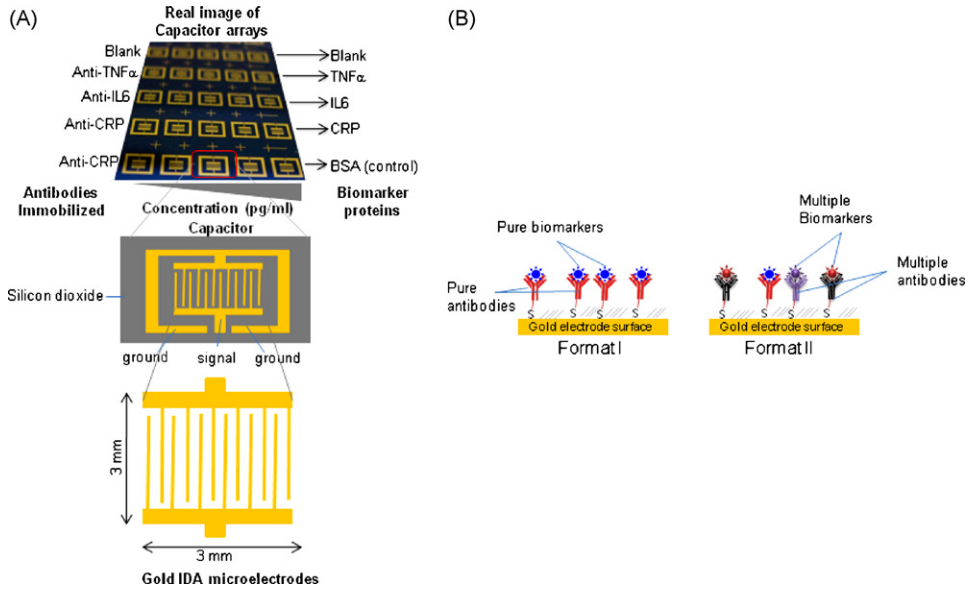

Fig. 1. Schematic representation of (a) GID-capacitor array chip and (b) GID region of each capacitor immobilized with pure (format I) and multiple antibodies (format II) and capturing of protein biomarker target/s.

of CRP for 1 h and the chips were washed, dried and measured the capacitance and dielectric properties. This process was repeated in the second and third steps with the same chips by incubating with TNFα and IL6 antigens, respectively and the change in capacitance/dielectric properties after each step was recorded.

Dielectric constant values were calculated from the data measurements at the effective frequency (f) range from 50 to 173 MHz for plotting under standard assay conditions. The data values relative to the controls (signal obtained with blank, after SAM formation and after antibody immobilization) and the changes in capacitance or dielectric properties were compared. The differences in the dielectric constant values relative to their respective controls were plotted. A negative control experiment was also conducted under standard conditions using bovine serum albumin (BSA) as a non-specific protein. Average values of dielectric measurements obtained from duplicate experiments were plotted and the standard deviations were calculated that are shown as errors. Limit of detection was estimated based on intersection of the extrapolated linear midrange and final low concentration level segments of the calibration plot (Buck and Lindner, 1994). Inter- and intra-assay relative standard deviation (RSD%) was calculated from all the experiments and are found to be within 14%.

3. Results and discussion

In this study we report on the development of a label-free capacitive based biosensor for the detection of multiple biomarkers using GID-capacitor arrays. For this, three target protein biomarker candidates (CRP, IL6 and TNFα) were selected as models because of their presence at elevated levels in human serum has been correlated with cardiovascular disease risk (Martin-Ventura et al., 2009). Multiple protein biomarker detection was performed mainly by two formats (I and II) as described in Section 2. The detection of multiple protein biomarkers using an array of GID-capacitors immobilized with pure and multiple antibodies on SiO_2 wafers is as shown in the schematic diagram (Fig. 1a and b). Both formats are able to detect multiple biomarkers.

In format I, four arrays each contained five capacitors on a SiO_2 chip with a 3 mm × 3 mm dimension of each GID on capacitor surface were immobilized with pure anti-CRP, anti-IL6 and anti-TNFα antibodies, respectively (Fig. 1a). The chip was incubated with a series of pure CRP, IL6 and TNFα concentrations on capacitor arrays, respectively for 1 h. A separate chip immobilized with anti-CRP antibodies was incubated with a non-specific protein (BSA) to ascertain that no non-specific binding occurred during the assays. The linearity of dielectric response against different concentrations for each of the biomarker proteins at varying frequencies showed a detection range from 25 pg/ml to 25 ng/ml (Fig. 2a–c). This detection limit using the capacitors composed of GID arrays on SiO_2 background was enhanced compare to the previously reported studies (de Vasconcelos et al., 2009; Quershi et al., 2009). The difference and the sensitivity in response of the protein biomarkers under the applied frequency probably be strongly dependent on the nature of protein as well as the geometry of metal electrodes (Dobrikova et al., 2007; Song, 2002) (Fig. 1a and b; Supplementary Fig. S1a and b). The response of the sensor was consistent in the range 150–173 MHz frequency for the biomarkers tested under standard conditions and therefore, this range was chosen for the analysis. The dielectric response pattern against pure CRP, IL6 and TNFα varied with respect to concentration as well as frequency. Further, there was no non-specific response as evidenced by incubation of a non-specific protein (BSA). The remarkable sensitivity achieved toward binding of protein biomarkers on antibody functionalized capacitors was detected in picogram-levels. The assay had detection limit of 25 pg/ml with a dynamic range of up to 25 ng/ml at varying frequencies from 50 to 173 MHz for CRP and IL6 (Fig. 2a–c and Supplementary Fig. S2). However, it was found that the values of dielectric changes (capacitance) for the tested biomarker proteins varied probably because of the nature of the proteins, size and charge on their surface combined with area and geometry of gold interdigitated electrodes that greatly influenced the dielectric properties (Pethig and Kell, 1987). The dielectric responses at higher levels of TNFα above 1 ng/ml showed no significant change in dielectric properties probably because of the limited binding sites on GID-capacitor surface or the change in dipole moment and distinct charge distribution on TNFα protein.

The variations in dielectric responses by complex proteins of different size, composition and structure can be attributed to the following reasons. In a complex protein, positive and negative charges are constituted from the ionizable side chains of acidic and basic amino acids present in a particular protein structure. A typ-

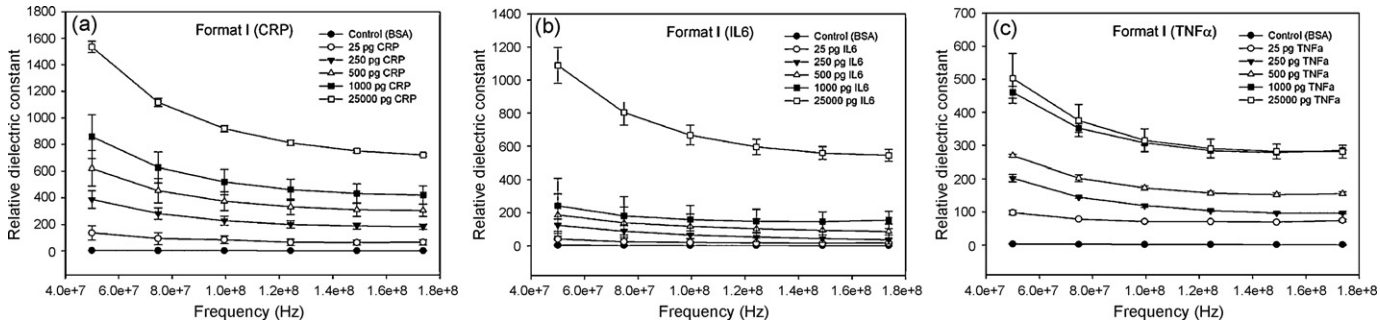

Fig. 2. Changes in dielectric responses to different concentration of pure protein biomarker targets: (a) CRP; (b) IL6, and (c) TNFα as a function of frequency in format I in which each capacitor array contained anti-CRP, anti-IL6, and anti-TNFα, respectively.

ical globular protein exhibits surface charges that constitutes an electric dipole (Supplementary Fig. S1a). The simplest molecular dipole of a monomeric protein consists of a pair of opposite electrical charges with magnitudes of $+q$ and $-q$ that are separated by a vector distance r (Supplementary Fig. S1b). The molecular dipole moment m is given by the equation $m = qr$. If a protein immobilized on a solid surface when allowed to bind its analyte, a protein–analyte complex is formed that will give rise to an increase in molecular size of a protein–analyte complex. This increase in size of a protein–analyte complex, therefore, leads to a relatively large permanent dipole moment, which can be calculated (Antosiewicz, 1995). The fact that amino acid composition, sequence, side chains, and their 3D structure vary from one protein to another. For example, human CRP is a pentameric protein in which each monomer consists of 187 amino acids (Oliveira et al., 1979) that differs from IL6, which is approximately five times smaller with only one polypeptide of ~184 amino acids (Orita et al., 1994). Such differences in protein composition bring about significant changes in surface properties that is specific to protein type (Kirkwood and Shumaker, 1952; Pethig, 1987; Pethig and Kell, 1987). We here exploited this property by monitoring changes on the surface of capacitor arrays brought on by formation of antibody–antigen complex.

The capacitor arrays were also tested for detection of multiple biomarkers by format II, in which equimolar mixture of three distinct antibodies (anti-CRP + IL6 + TNFα) were immobilized on capacitor arrays. It was assumed that each GID-capacitor in an array received an equal number of binding sites for three protein biomarker targets (CRP + IL6 + TNFα), respectively. Comparison with format I in which GID-capacitors contained pure antibodies showed consistent increase in dielectric properties after individually incubating with pure biomarkers. Fig. 3 shows the dielectric responses of capacitor arrays in two formats (I and II) with respect to one of the concentrations of protein biomarker targets (CRP, IL6, and TNFα) (500 pg/ml) at a frequency of 173 MHz. The biomarkers were allowed to incubate sequentially on GID-capacitor arrays immobilized with antibodies (anti-CRP, -IL6, and -TNFα) in pure form (format I) and in 1:1:1 mixture (format II), respectively. The results showed consistent decrease in dielectric constant with respect to the dilution of binding sites in format II. The dielectric responses for protein biomarkers incubated on chips immobilized with pure antibodies showed considerably higher dielectric changes than same concentration tested on chips immobilized with diluted (three times) antibodies (Fig. 3). However, the levels of dielectric response varied among the three biomarkers. It was found that the dielectric constants were not consistent to the order in which diluted antibodies were immobilized on capacitor arrays. This was probably because of several factors that may have influenced on the distinct behavior of dielectric responses. For example, the nature of proteins (biomarkers/antibodies) such as their size and net charge on the molecules (Supplementary Information Fig. S2a and b). It is to be noted that chip–chip variations brought on by small variations due to fabrication errors combined with effect of the background SiO_2 is not to be ignored if the chips are from different batch (Quershi et al., 2009).

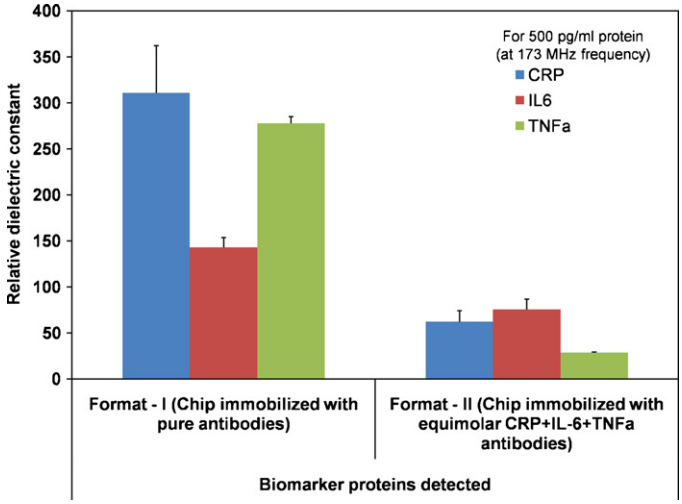

Fig. 3. Changes in dielectric response against 500 pg/ml concentration of protein biomarker targets (CRP, IL6, and TNFα) at a frequency of 173 MHz. The biomarkers are allowed to incubate sequentially on GID-capacitor arrays immobilized with antibodies (anti-CRP, -IL6, and -TNFα) in pure form (format I) and in mixture (1:1:1) multiple, respectively. The number of binding sites in format II contains three times lesser than that in format I.

The multiplexed detection was performed in three sequential reaction assays; (a) in step I, pure form of first biomarker (CRP) was incubated followed by capacitance/dielectric constant measurements, (b) in step II, the chips were regenerated that contained free antibody binding sites for IL6 and TNFα but were incubated with only IL6 and lastly, (c) in step III, the process was repeated for the third reaction but this time with pure TNFα protein (step III). The results of multiplexed detection of three protein biomarkers (IL6, CRP and TNFα) showed the concentration dependent increase in dielectric constants against the scanned frequencies for subsequently three times, respectively. Fig. 4 shows multiplexed detection for CRP, IL6 and TNFα at concentration range 25 pg/ml to 25 ng/ml. These results indicated that the enhanced detection range was achieved with multiplexed detection as compared to the detection with a single biomarker with pure antibodies, which showed dynamic range with a maximum of 25 ng/ml including for TNFα to which format I showed maximum response to only 1 ng/ml (Figs. 2 and 4). Limit of detection was also estimated based on intersection of the extrapolated linear midrange and final low concentration level segments of the calibration plot (Buck and Lindner, 1994). An estimated lower detection limit (LOD) by linear regression analysis for format II was found to be 32 pg/ml with a dynamic

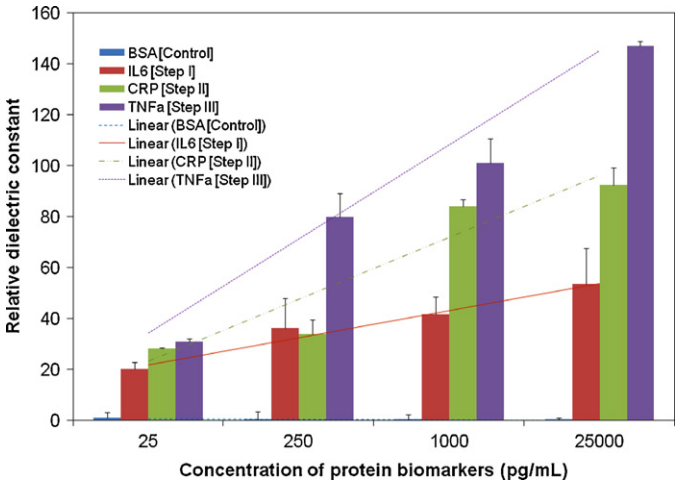

Fig. 4. Multiplexed detection of protein biomarkers on chips immobilized with equimolar mixture of anti-CRP, -IL6 and -TNFα antibodies (format II) and the concentration dependent increase in dielectric response within 173 MHz frequency are shown in the figure. The coloured lines shown are derived of linear fit for corresponding values for tested biomaker proteins and BSA protein was used as a negative control.

range of 25 pg/ml to 25 ng/ml within 173 MHz frequency for CRP, IL6 and TNFα.

The level of increased dielectric constants after binding with each biomarker in three sequential steps was consistent, and this can be attributed to accumulating protein layer on the GID-capacitor surface. However, it should be noted that the CRP did not follow this trend at a particular concentration (250 pg/ml). This combined with relatively lower dielectric constant of IL6 (Fig. 4) can be because of several reasons. For example, at a particular protein concentration (here CRP at 250 pg/ml) or for a particular protein (IL6 in this case), the increase in dielectric properties is not higher compared to other proteins such as TNFα. Whereas, the trend was different in format I in which the chips contained abundant antibody binding sites in which the dielectric response of TNFα was only upto 1 ng/ml (Fig. 2).

For multiplexed detection, initially, in step I, different concentrations of CRP were allowed to incubate on chips immobilized with anti-CRP/IL6/TNFα antibodies (1:1:1) and the same chips were incubated with IL6 and TNFα in steps II and III, respectively (Fig. 4). It may be impossible to determine all three biomarkers collectively in an unknown sample. Therefore, a two-step approach may be undertaken where the first step involves incubation with an unknown sample (suspected serum sample) on GID-capacitor chips with multiple recognition elements. The second step is the confirmation step where the same chips from step one can be tested with known standard protein biomarkers to determine the unknown biomarker as well as its concentration.

Initially, the capacitance/dielectric properties using a large number of capacitors were tested after immobilizing appropriate amounts of antibodies on a given area of GID electrodes. It was found that the immobilization of pure antibodies for detection of single target antigen often yielded low signal-to-noise ratio, and this method was suitable for concentrations over ng/ml levels as well as for some proteins such as TNFα. It was imperative to test with a series of concentration to determine appropriate antibody density after immobilization. Alternatively, an equimolar mixture of three antibody types was immobilized that can bind to three different antigens. This was followed by sequential incubation of pure antigens and measuring the change in the dielectric responses after each step. This allowed monitoring change in capacitance/dielectric properties brought on by formation of specific antigen–antibody complex. Our study demonstrated the feasibility of the capacitor array based biosensor for multianalyte detection using model biomarkers. Further, the sensor requires to be optimized for in-built processing to deal with the real samples that could allow separation of serum proteins, prevention of drying out of sample, and washing of non-specific proteins, such as by use of size separating microfluidics system.

4. Conclusions

Multiple detection of a panel of disease biomarkers for determining a particular disease risk is more reliable compared to the detection of a lone biomarker for the same disease. Therefore, we here demonstrate, for the first time, label-free detection of multiple biomarkers (CRP, IL6 and TNFα) for CVR using capacitor arrays made of GID electrodes immobilized with pure (format I) and mixture of antibodies (format II) for the detection of multiple biomarkers through capacitance/dielectric measurements. When the immobilized antibodies interacted with antigens, the interaction lead to the change in the dielectric layer on the capacitor surface and induced change in capacitance/dielectric properties which was corresponding to the antigen. The detection limit in formats I and II was in the range 25 pg/ml to 25 ng/ml. The highly sensitive method demonstrated in this study can potentially be applied to detection of multiple biomarkers in real serum.

The levels of one or more biomarkers in an unknown sample can be determined by chips immobilized with multiple antibodies. However, the unknown sample if contained normal level of one biomarker and elevated level of the other. In such cases, the chips once tested with unknown sample can be tested again but this time using known standard biomarker proteins. This eventually allow determining which biomarker level was elevated. Under optimized conditions, label-free detection of multiple protein biomarkers using GID-capacitor arrays on SiO_2 background offers advantages of speed, simplicity, sensitivity, less expensive and has a potential for miniaturization to a hand-held device for point-of-care diagnosis. However, there are few challenges to address such as the consistency of the chip responses as a result of batch-to-batch variations due to the geometrical errors during fabrication of GID-capacitors, variations in the size of nano-particles during sputter deposition of GID arrays combined with stability of antibodies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.03.018.

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