Flexible low-cost cardiovascular risk marker biosensor for point-of-care applications

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The detection and quantification of protein on a laser written flexible substrate for point-of-care applications are described. A unique way of etching gold on polyethylene terephthalate (PET) substrate is demonstrated by reducing the damage that may be caused on PET sheets otherwise. On the basis of the quantity of the C-reactive protein (CRP) present in the sample, the risk of cardiac disease can be assessed. This hsCRP test is incorporated to detect the presence of CRP on a PET laser patterned biosensor. Concentrations of 1, 2, and 10 mg/l were chosen to assess the risk of cardiac diseases as per the limits set by the American Heart Association.

Introduction: Diagnostics become more important in third world countries as the people have limited access to medical care systems and have less awareness of healthy lifestyles [1, 2]. There is certainly a need for on-site detection in the life science fields; and for point-of-care diagnostics in rural areas of underdeveloped countries so that even an unskilled person can use the device to determine the presence of disease-causing markers. Currently, diagnostics commonly employ long assay time, trained personnel, sophisticated instruments, and require financial support. A good approach to overcome this current situation would be the use of flexible and paper-based point-of-care devices to detect specific biomarkers [3]. Biomarkers provide insight into normal biological processes, pathogenic processes, and pharmacological therapeutic interventions. Hence, the development of more compatible, reliable, convenient, simple, easy-to-use systems would be of great use to a person less skilled in medical diagnostic procedures. To achieve this, we have demonstrated a simple device that can detect the presence of proteins in a few minutes. This can load to a reduction of healthcare costs compared with traditional strategies performed at centralised laboratories. In our case, the transducer was a gold IDEs array coated with 350 nm of Parylene-C fabricated on polyethylene terephthalate (PET) flexible sheets.

Immunoassays are vital and versatile analytical techniques that have numerous applications in biomedical diagnosis [4]. The determination of C-reactive protein (CRP) assists in detecting disease risks earlier and can be detected via the immunoassay technique. Efforts have been made to improve on agglutination assays for their potential in detecting biomarkers. The agglutination process occurs when anti-CRP antibodies interact with CRP protein (antigen). In general, agglutination takes place when an antigen is in a mixture with its corresponding antibody called isoagglutinin. The degree of agglutination depends on the concentration of antigen added to a specific concentration of antibody and vice versa. After coating with Parylene-C, the devices were ready to be used as sensors for which immobilisation of biomolecules were necessary.

The biosensor was surface treated to immobilise hsCRP antibodies. First, all the flexible sensors were subjected to plasma cleaning. It was then immersed in an ethanolic solution of 3-mercaptopropioninc acid (MPA) for 24 h because it acts as a cocatalyst and its unique reducing properties make it an ideal candidate for a wide variety of chemical reactions including addition, elimination, or cyclisation reactions. The flexible sensors were then thoroughly rinsed with distilled water and dried over with pure nitrogen gas. The surfaces were now abundant of free carboxyl groups of MPA and incubating the sensor surfaces with a mixture of 0.05 M of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.03 M of *N*-hydroxysuccinimide (NHS) in distilled water for 5 h activated these surfaces. EDC is the carboxyl-activating agent for the coupling of primary amines to yield amide bonds in preparation of immunoconjugates. NHS was used to provide stability and activity characteristics of the reagents.

The self-assembled monolayers that were formed on the sensor surfaces were now ready to be seeded with anti-CRP antibodies. ASI Arlington CRP test kits were used throughout the experiments. The kit consisted of latex beads coated with anti-CRP antibodies, and these were introduced on the surface-treated flexible sensors to attach antibodies onto the surface. To have more open sites of the antibody on the surface of the sensor, we used a new surface modification method by exposing the antibodies to ultraviolet (UV) light [5] and during immobilisation of antibodies onto the sensor. The detailed procedure for the immobilisation of antibodies is depicted in Fig. 1. The sensors were then washed with DI water for 2 min to remove any unattached antibodies. After immobilising antibodies, it was then introduced with series of antigen concentrations (1, 2, and 10 mg/l) and incubated for 60 min. The CRP complex so formed due to antibody and antigen interaction was then measured at 1 MHz and 1 V using an LCR meter.

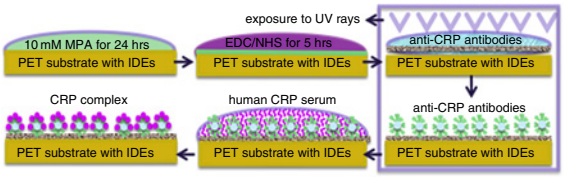

Fig. 1 *Procedure of antibodies immobilisation*

Results and discussion: We take advantage of the subtractive fabrication process to fabricate PET flexible sensors which involved three major steps, as depicted in Fig. 2. The PET sheets' surfaces were cleaned thoroughly with DI water. It was then sputtered by gold via DC sputtering to a thickness of 600 nm at a deposition rate of 59 nm/min using Quorum 300T D. Selective maskless laser etching was employed to fabricate IDEs, which allows rapid prototyping in patterning the features. A Universal laser engraving system was used for patterning the structures with a power of 70% and speed of 10%. The selection of these parameters was based on a trial and error procedure, which attempted to avoid a short circuit between the electrodes and undesirable metal peel off, which was going to shrink the overall capacitance. The fabricated structure had a 100 µm gap and a finger width in a cell of 36 mm^2. The final step, to preserve the capacitors' quality factor, was to coat the structures with a Parylene-C (after patterning gold electrodes) dielectric layer at a thickness of 350 nm using the chemical vapour deposition (SCS PDS 2010 LABCOTER 2) system. The dimensions and flexibility of the fabricated flexible low-cost biosensors are represented in Figs. 2*e* and *f*. Selective etching using a laser greatly reduced the time for the fabrication of the sensors when compared with other label-free detection sensors fabricated using MEMS techniques [6]. A laser ablation method was used, whereby a wavelength of 1.06 µm was chosen, to selectively etch a thin gold film, being a photon absorbing layer and being transparent to the PET substrate film. This allows very thin polymer substrates to be used, without being damaged, as would be the case with a 10.6 µm CO_2 laser.

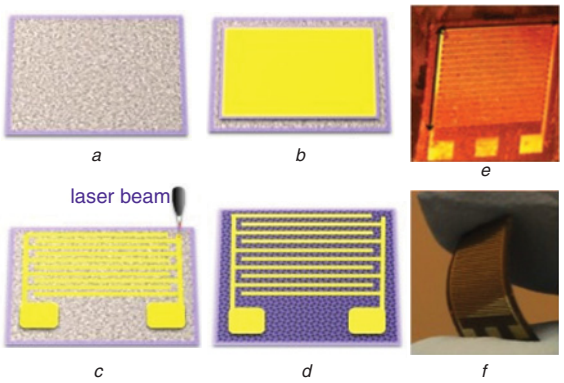

Fig. 2 *Fabrication process of flexible transducer: bare PET sheet (Fig. 2a); Au sputtering (Fig. 2b); laser engraving (Fig. 2c); deposit Parylene-C (Fig. 2d). Dimensions (Fig. 2e) and flexibility (Fig. 2f) of fabricated biosensors*

We used a low-cost setup, without the use of any clean room facilities. This is a maskless fabrication methodology, where the design is done on CorelDraw software that saves time and the need for complicated software to draw images. In reference to sources from [7], the absorbance spectra of gold shows that it absorbs the laser at 950–1050 nm. Moreover, the transmittance spectra of PET with reference to [8] show that PET transmits the laser at ~300–1500 nm. An ytterbium-doped fibre laser with a wavelength of 1060 nm, to be transparent to polymers such as PET, PMMA, and also glass, was used. This laser selectively absorbs the gold thin film, which is sputtered on the surface and passes through the polymer without damaging the PET. These key features allow us to etch selectively only the metal and that makes it unique from other laser writing techniques.

Short pulses (2 s of three pulses) of UV light were exposed during immobilisation. The antibody absorbs one UV photon, and the disulphide bridge is opened, thereby forming thiol groups. Their interaction with the gold surface leads to an oriented Fab region so that the upside down position is hampered and the antigen binding is more efficient.

Immobilisation of antibodies using UV light paved the way for more open sites for antigens to be attached. Efforts have been made to immobilise antibodies alone on capacitive sensors [9] but no efforts have been made to immobilise beads coated with antibodies, which could give numerous open sites for antigens to attach. The beads coated with antibodies also enable using less sample, which is an essential feature in diagnostics. To determine the specificity of CRPs we performed two sets of experiments. One used CRP proteins and the other used bovine serum albumin (BSA) proteins as antigens. On agglutination with antibodies, the capacitance increases by 50 pF when compared with the non-agglutinated process. The change in capacitance, when CRP proteins and BSA were added to anti-CRP antibody immobilised sensors, is shown in Fig. 3.

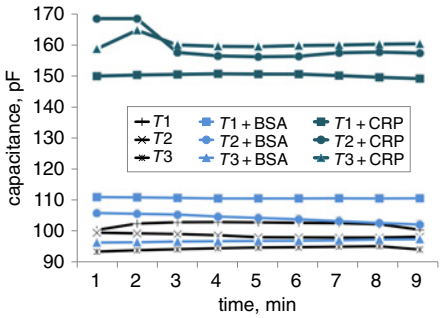

Fig. 3 *Detection of CRP in presence of CRP and non-specific protein (BSA) (T1, T2, and T3-capacitance readout for three trials after immobilisation of hsCRP antibodies)*

When compared, the results clearly show that there was a substantial increase in capacitance when CRP antigens were used because antigens were attached to immobilised antibodies. In contrast, as for the BSA protein, the capacitance was slightly increased because of the biological remnants, which proved that there was not a specific reaction. This inferred that the biosensors were specific in detection to a particular protein. To further test the sensitivity and reliability of these flexible sensors, we used various concentrations of CRP proteins and quantified them as enumerated in Fig. 4.

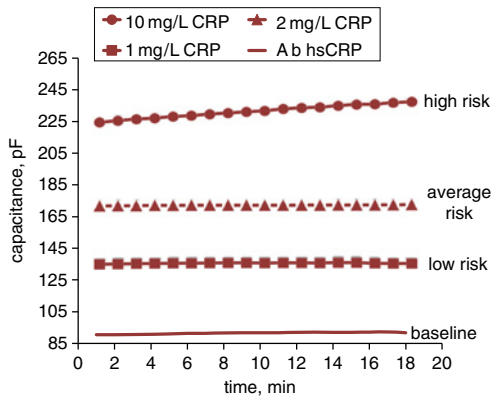

Fig. 4 *Capacitance increasing with concentration shows quantification of CRP in sample as per limits set by AHA [10]*

The increases in the percentage of the capacitance (C_{CRP} – baseline/baseline) for each sample after agglutination are plotted in Fig. 5*a*; they signal that the sensors can quantify any given sample. However, to ensure that the sensors could be a useful tool to quantify a given sample, we calculated the ratio (C_{CRP}/baseline) of each sample after agglutination of the samples and the result is plotted in Fig. 5*b*. The ratio gives an insight into how capacitance varied with consecutive concentrations. This also gave a measure of quantification between consecutive higher concentrations. The linearly increasing trend in Fig. 5*b* was a result of the increase in concentration and indicated that the sample was quantified. All the quantification data were compared with reference to baseline capacitance.

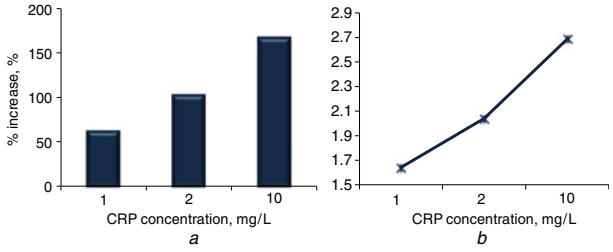

Fig. 5 *Percentage increase of capacitance (Fig. 5a), and ratio of increase in capacitance with concentration (Fig. 5b)*

The concentrations were chosen based on the risk a patient can be prone to cardiac disease. According to the American Heart Association (AHA) and the United States Center for Disease Control, a CRP concentration below 1 mg/l represents low risk; a range from 1 to 3 mg/l represents medium (average) risk; a measurement over 3 mg/l represents high risk [10]. Thus, the concentrations of 1, 2, and 10 mg/L were selected as these assess risk according to the aforementioned limits. An Agilent LCR meter was used to measure capacitance at a frequency of 1 MHz, as in the previous experiment, and the read out value gave an indication of the analyte quantification, which is plotted in Fig. 4. The capacitance measurements are frequency dependent. The water (used for diluting) molecules on an addition of CRPs can be more polarised at lower frequencies. For this reason, the capacitance readout at higher frequencies is more reliable without significant fluctuations and, thus, a frequency of 1 MHz was selected.

Conclusion: We have demonstrated a low-cost, fast method for developing flexible capacitive sensors using laser etching. This UV modification method is applicable to all antibodies to have more attaching sites and provides better agglutination of the sample. The device would be more available and less expensive and can identify or anticipate disease in a pre-symptomatic stage. The sensor is capable of detecting and quantifying the CRP protein associated with cardiovascular diseases.

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One or more of the Figures in this Letter are available in colour online.

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