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A new microfluidics system with a hand-operated, on-chip actuator for immunosensor applications

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ABSTRACT

This paper presents the realization of a portable, point-of-care and multi-target immunosensing lab-on-a-chip (LOC). The chip utilizes a novel on-chip actuating mechanism which uses a negative pressure to create thrust inside the channels to facilitate and control the fluid flow. No external instruments and/or sources were used to drive or control the liquid throughout the experimentation. LOC demonstrates a highly sensitive detection and quantification of a cancer marker, human-epidermal growth factor receptor (hEGFR) and a cardiac marker, interleukin 6 (IL-6) by impedimetric measurement approach. Without the use of any signal amplification methods (chemical), a dynamic detection limit of 3–8 ng/ml of hEGFR and 0.1–5 ng/ml of IL-6 in human serum was obtained. The extracted dissociation constants were calculated as 5.86 ng/ml (hEGFR) and 2.45 ng/ml (IL-6) by kinetic analysis.

1. Introduction

The advent of micro-fabrication technologies has played a significant role for the development of fluid-based devices at microscopic length scales. The use of microfluidics in chemical and biological sensing has been previously investigated [1]. Since then, it has provoked many researchers for the development of microfluidic technologies in application areas of chemistry and life sciences. It is possible to combine several complex laboratory functions on a single chip, such as pre-treatment, analyte sample separation, bio-chemical reaction, label/label-free detection, and fluidics. This combination of functions on a chip is referred to as lab-on-a-chip (LOC) [2,3]. The dexterity of LOC delivers numerous advantages such as low consumption of reagents, integration to multiple processes, lower analysis time, higher sensitivity and the reliability. Thus, LOC technologies are widely used in the applications of drug discovery [4–6], controled drug delivery [7,8], single/multiple cell analysis [9–11], genome analysis [12–14], proteomics [15,16] and portable point-of-care systems [17–19]. Among the various applications of LOC, the immunoanalysis for the detection of disease markers using LOC technology with on-chip actuated mechanism is not explored to the extent. There is a bottleneck in the development of all next generation immunosensing LOC systems, which has insufficient stability and reproducibility of their interface properties in the different environments of their practical applications [20].

In addition to the cost, complexity and reliability, the microfluidics technology for immunosensing applications involves several key functional aspects, namely the immobilization method with ensured stability, the development of efficient signal transducing strategies, the adaptable fabrication schemes, and the design of microfluidic network. There have been considerable interests to immobilize a biomolecule via adsorption, entrapment, site-specific binding and avidin-biotin technology [21]. However, the immobilization/functionalization method used for immunoanalysis varies according to the signal transducing material and the specific detection methodology. The effective signal transducing mechanism strongly depends on the type of immunoassays (IAs). The affinity-based IAs are rapidly progressing at the conjunction of chemistry, biochemistry, material science and physics. Affinity-based IAs can be divided into two main categories as label-free (quartz crystal microbalance (QCM) [22], surface plasma resonance (SPR) [23]) and labeled techniques (enzyme-linked immunosorbent assay (ELISA) [24], radio immunosorbent assay (RIA) [25] and fluorescence immunosorbent assay (FIA) [26]). Labeled affinity biosensors are derived from the immunoassay technology, where signal detection is facilitated by the use of enzyme/fluorophore labels [27]. These methods have several limitations including the requirement for complex reagents, qualifying the presence of the target molecules/analytes only, generation of radioactive residues in RIA, fluorescent-labeling as in sandwich-type arrays when applied to biological fluids limit the sensitivity due to background noise, and

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the necessity for heavy/expensive instrumentation [28]. Therefore, there is a clear need for a clean and rapid method to quantify the levels of multiple proteins in serum to determine potential disease candidates that may benefit from therapeutic interventions in the early stages of diseases. The label-free techniques are well suited for portable applications in which the expected variations of impedimetric/dielectric properties when the target is bound in instantaneously measurable format [29].

Rapid prototyping with different fabrication methodologies, such as hot embossing [30–32], injection molding [33,34] and lamination techniques [35,36] developed some interest for the low cost microfluidics devices. However, these processes require high temperatures that may limit the device use in biological applications. Moreover, the precise and low cost fluid control is hard to achieve without the integration of on-chip fluidic components to the off-chip control systems. Thus, until recently, the precise fluid control can only be achieved by the expensive and complex methods such as externally controled electro-kinetic flows and active components (micropumps and micro-valves). Therefore, on-chip actuating sources/hand-operated micropumps are more suitable and cost effective for portable point-of-care applications where the function is not limited by specialized personnel or additional needs of power sources/instrumentation [37]. These limitations can be surpassed with single-use strip tests (immunochromatographic assays) with simple yes/no results [38–40]. However, reliable output can only be ensured by quantitative analysis.

Recently, alternate strategies have been extensively studied to develop on-chip actuating mechanisms with no external instrumentation. For example, Park et al. have developed an electrochemical immunosensing disposable LOC integrated with latch mechanism for hand operation for the detection of glucose [41]. Hydraulic pressure (pressing with the finger) is applied on the inlet section (reservoir cum actuator) to sprout liquid into the reaction chamber. The methodology has severe drawbacks to be modified for point-of-care and hand-held applications. Firstly, the complexity in multilayered fabrication and the bonding between the glass and first PDMS layer is not permanent; hence the liquid leak during the latch operation is inevitable. Moreover, the design is limited for label-free single target detection. Secondly, the signal transducing method, the faradic measurement where same working electrode on the LOC was used without considering the unavoidable electrode fouling effects. The setup to measure the transducer signal and use of micro syringes for loading analytes hinders its real time application. In another work, Moorthy et al. have performed a calorimetric, disposable botolinium toxin enzyme-linked immunosorbent assay (ELISA) with an integrated micro-fluidic device [42]. However, the approach to sprout liquid from multiple reservoirs using multiple integrated micro-pumps and lack of proper air-vent to avoid the backward flows impedes its objective in hand-held applications.

This study presents a portable PDMS-based microfluidic system integrated with hand-operating on-chip actuation mechanism for specific multi-target immunosensing applications. The analyte was transferred without any external source by the release of micropump actuated by hydraulic pressure (by pressing with a finger). An interdigitated transducer (IDT)-based affinity-type sensor was employed for the detection and quantification of target molecules in complex mixtures by affinity-based interactions. A single integrated micro-pump is operated to perform all necessary washing and surface modification procedures. The function of the on-chip actuated fluidic system as a LOC was tested with two target biomarker protein models, such as human epidermal growth factor (hEGFR) and interleukin (IL-6) antibodies. In a healthy individual, the normal level for hEGFR is lower than 8 ng/ml and for IL-6 it is lower than 100 pg/ml. Any level higher than the specified would drastically increase the risk of cancer and cardiac diseases [43–45].

2. Materials and methods

2.1. Materials and reagents

Slygard 184 (polydimethylsiloxane, PDMS) was purchased from Dow Corning, USA. Epidermal growth factor receptor (anti-hEGFR) antibodies and human epidermal growth factor receptor (hEGFR) protein antigen were purchased from Sigma–Aldrich (USA). Monoclonal antibody for IL-6 and purified IL-6 antigen were purchased from Fitzgerald (Concord, MA, USA). Bovine serum albumin (BSA) was purchased from AbD Serotech, Germany. Phosphate buffer solution (pH 7.4), potassium iodide, iodine, acetone, isopropyl alcohol, ethyl alcohol (EtOH) and 10 mercaptoundecanoic acid (MUDA) were purchased from Sigma–Aldrich, Germany. HPR 504 and the developer OPD 4280 were purchased from OCG microelectronic material, Belgium. All other chemicals used in the experiments were prepared with double distilled water (dH_2O), and during the fabrication deionized water was used.

2.2. Design of microfluidics

The design of LOC consisted two stacked PDMS layers on glass layer. The middle PDMS layer was used for channel microstructures and waste reservoirs. Microfluidics were driven by application of hydraulic pressure (simple pressing with a finger). Each LOC was designed to have eight IDTs, three inlets and an air vent. Additionally, three integrated microfluidic channels each with 100 μm width were connected to the inlets and the actuating chamber passes over the IDT surface. The top PDMS layer was designed to deform over the wastage reservoirs of middle layer. The essential components of immunosensing LOC were an actuation chamber and fluid channels. The actuation chamber is vented to the ambient through an air vent/venting aperture. Fig. 1a shows the schematic of the hand-operated LOC. The transfer of liquids was accomplished by the relaxing the elastic deformation caused by the release of hydraulic pressure, which induces a suction (negative pressure inside the channel). Thus, the driving force utilized in this design does not require an external power source as in the case of an off-chip actuated micro-fluidic systems.

In static assay immunoanalysis technique, the incubation period is essential to allow the reaction to occur on the transducer surface. Thus, the proposed LOC with a series of coordinated steps allowing incubation, as well as performing complex experimental lab procedures, such as loading of analytes, washing (cycle-1) and draining (cycle-2) processes as shown in Fig. 1. During the cycle-1, an analyte sample is loaded into the inlet ports. The sample flows inside the molded wells due to the surface tension and capillary action to finally stop at the well boundary. The analytes run and fall onto the IDT surfaces by pumping the actuator. The channels and the IDT surfaces were washed before and after repeating cycle-1 with wash buffer (1× PBS). After the completion of cycle-1 process, the unbound sample analyte or washing solution was drained to collect it in the waste reservoir. During cycle-2, the inlet ports were closed and the reservoir is emptied through the air vent. Thus, the two processes (cycle-1 and cycle-2) facilitated all lab procedures for immunosensing applications.

The reversible van der Waals contact of PDMS–PDMS was used for closing and opening of all ports (inlet and air vent) with an additional PDMS cover layer that controls the flow of fluids. Following steps were involved in the performance of the microfluidics. First, the analyte sample is loaded into an inlet well connected to a channel. After opening the air vent, a hydraulic pressure is created by pressing the actuator on the waste reservoir chamber. Next, by closing of air vent using a removable PDMS cover layer releases hydraulic pressure, which creates a negative pressure inside the closed channel which pulls the analyte sample into the channel

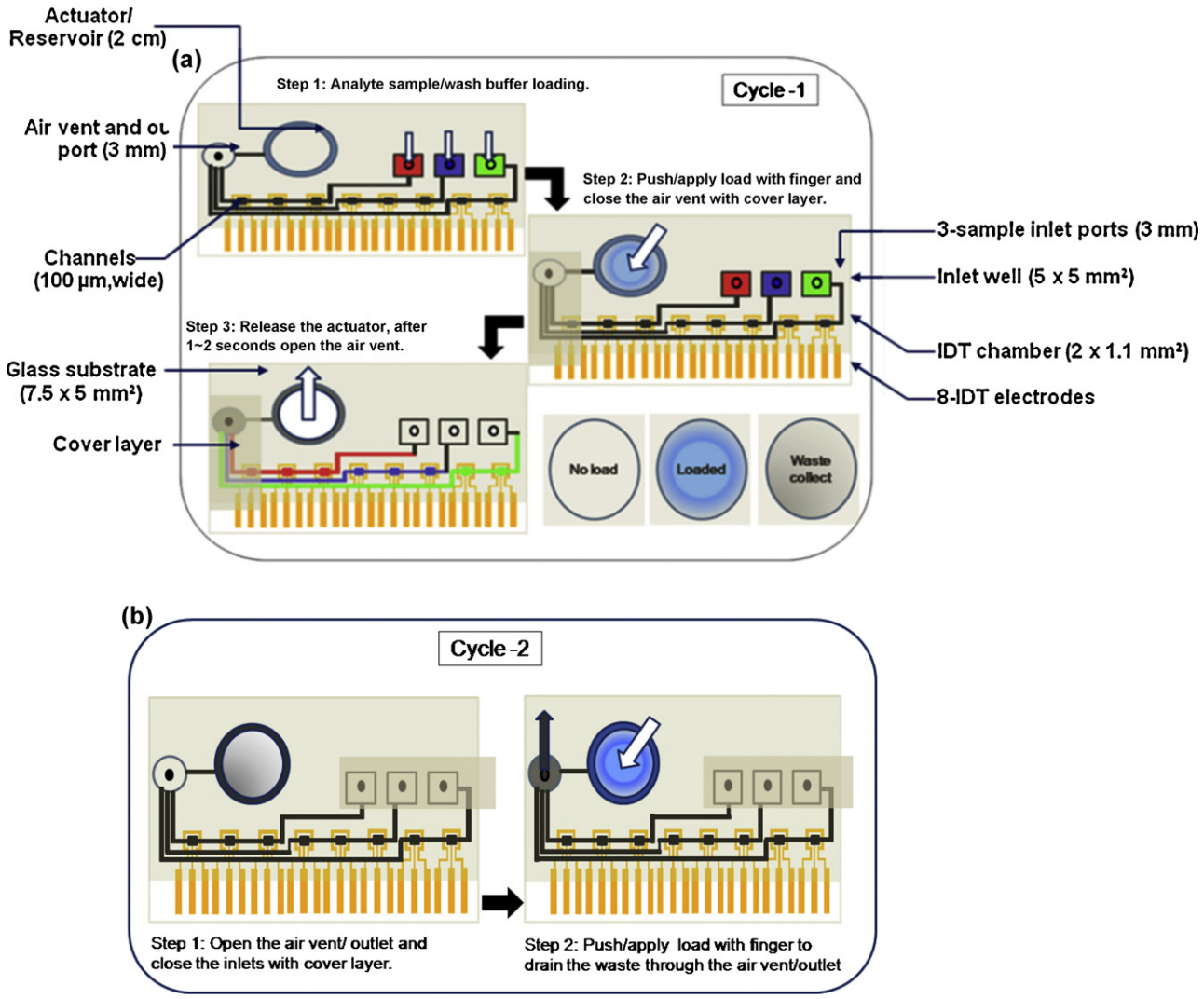

Fig. 1. Schematic representation and the operating mechanism of the immunosensing LOC. (a) Step 1: the analyte sample/wash buffer (20 µl) is dropped to fill the three inlets; Step 2: without closing the air vent with cover layer, a hydraulic load is applied by pressing the actuator with finger; and Step 3: the actuator is released with load after closing the air vent with a cover layer and after few seconds, the cover layer is removed to stop the fluid flow action for incubation. (b) Step 1: open the air vent and close the inlets with same inlet cover layer sequentially; and Step 2: a hydraulic load is applied on the actuator to drain the collected waste through the air.

to dispense onto the surface of IDTs. The same process was also applied for washing the channel and sensor surface (Fig. 1a and b).

The liquid flow in the channel is propeled by the relaxation of the released actuator. Hence, the accurate flow control and its characteristics can be addressed with the volume and flow rate during the pulling action of the actuator. The actuator was modeled as a circular diaphragm with clamped edges under a uniform mechanical pressure in the lateral direction [46]. To initiate the flow in the channel, it is considered that the volume change during the application of hydraulic pressure is larger than the total channel and inlet port volume. Therefore, the negative pressure created after the release of hydraulic load decreases the fluidic resistance in the microchannel running over the IDTs. This enables the sample to flow through the channel from inlet ports. Finally, in the proposed hand-operating LOC design, the direct application of a static immunoassay from very small sample volume of a real serum sample could be realized without any external power sources or technical skills.

2.3. Fabrication of LOC for immunosensing applications

Firstly, glass substrates were cleaned by dipping in piranha solution, a mixture of sulfuric acid (H_2SO_4), and hydrogen peroxide (H_2O_2) in volumetric ratio of 5:1 at 135 °C for 10 min. A very thin tungsten layer of 30 nm and gold layer of 170 nm thick were then sputter deposited on the glass surface, followed by 1.8 µm-thick positive photo resist (HPR 504) spinning over the glass surface. The glass substrate was then exposed to UV with a dose of 100 mJ/cm^2 and developed using positive developer (OPD 4280). Finally, the electrodes were patterned by etching the gold, using a mixture of potassium iodide (KI) and iodine (I_2) solution in 4:1 ratio, followed by etching titanium layer using nitric acid (HNO_3).

The schematic of fabrication process flow of the IDTs is shown in Fig. 2a. The microfluidic section of the LOC was realized in polydimethylsiloxane (PDMS) by soft lithography. The channels and actuators were microfabricated through coordinated casting, curing and bonding processes of PDMS–PDMS and PDMS–glass. The microfluidic channels used to transfer the analyte samples and incubation wells over the IDT area were formed of PDMS. A 3 mm thick PDMS layer was dispensed onto a pre-patterned SU8 master supported on a silicon wafer and degassed for 2 hrs. After curing the elastomer for 1 h at 80 °C, the replica layer was peeled off from the masters and the hole for main actuator was punched out. Each of the incubation well has an enclosure cavity of 0.1344 mm^3 in volume. The channel lines were rectangular

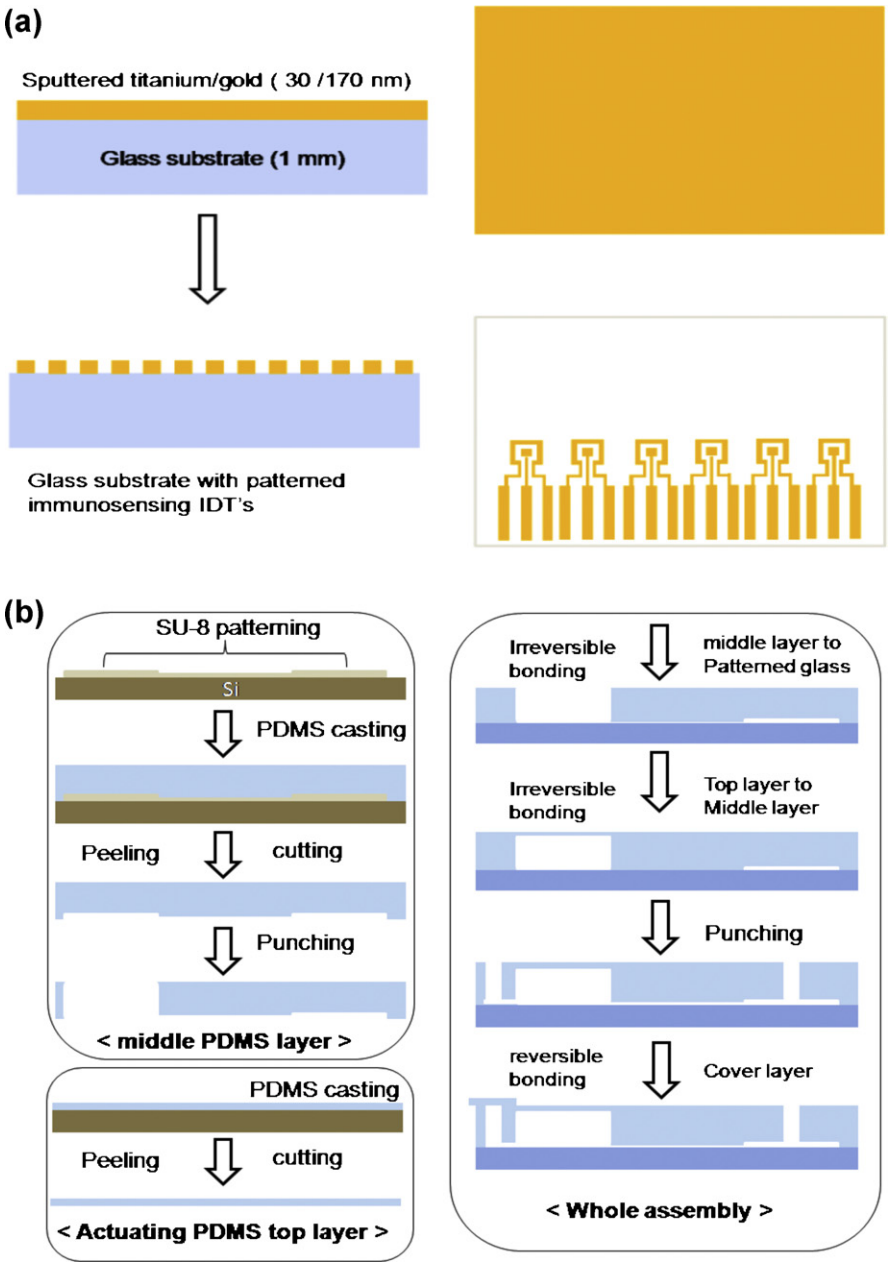

Fig. 2. (a) Fabrication process flow of inter-digitated transducers on glass substrate. (b) Fabrication process flow for the PDMS-based immunosensing LOC with pre-patterned glass as substrate.

in cross-section with area of 100 μm wide by 60 μm deep. A second layer of PDMS was again dispensed onto the blank wafer to act as an actuator and the same casting process was used while the thickness of this layer was ~1.5 mm. The microfluidic channel was then sealed by bonding the punched PDMS layer to a pre-patterned glass substrate with a 30 s oxygen plasma treatment to activate both surfaces prior to the bonding process. The process was repeated to seal the second PDMS layer over the punched-out PDMS layer. The whole assembly was cured for 1 h at 80 °C. Finally, after punching out the inlet and air vent ports, the openings were covered with a PDMS layer (~1.5 mm), which enabled reversible bonding. Fig. 2b shows the fabrication process of the immunosensing LOC. In the design of a static assay on LOC, many fabrication constraints have been considered. For instance, during the application of load and irreversible bonding were useful for preventing leaks as well as controlling the environment over the IDT surface area.

2.4. Experimental characterization and process setup

The liquid flow rate was analyzed for the release of applied hydraulic load. A high-speed, digital CCD camera (Phantom V311) with a maximum 500,000 frames per second speed was used to capture the images. The experimental setup to analyze the flow-rate is shown in Fig. 3. High-speed micro-PIV measurements of transient flow were performed [47]. The majority of the images were recorded at 1000 fps and the lens used was a macro lens with a field of view up to 2.8 mm at a focal length of 15 cm.

Karl Suss MA 56 mask aligner was used for all soft lithographic processes. PDC-32G-Harrick scientific oxygen plasma generator was used during bonding. The flow characteristics were determined using Phantom V311 high-speed camera. Impedimetric electrochemical measurements were performed using a portable network analyzer, Via echo 2500 [48]. The network analyzer was

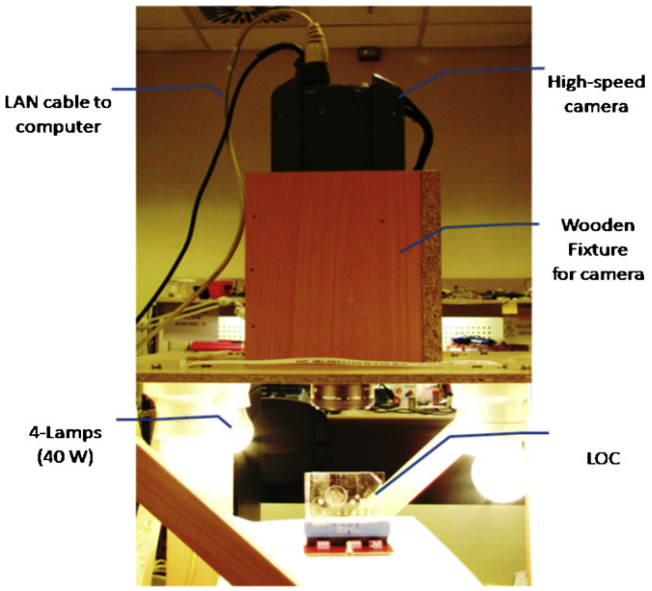

Fig. 3. Photographic representation of the experimental setup used for the flow characterization.

calibrated (50–300 MHz range) with the SOLT (short-open-load-through) method and scattering parameter (S-parameter) data of the IDT was measured. The IDTs were utilized for the non-Faradic signal transduction. Each planar structured IDT constitutes an array of 24 gold interdigitated transducing electrodes (IDT) within an area of 1.1 mm × 1.8 mm, Fig. 4b. A simple assembly was built for taking measurements and the experimental setup for non-Faradic measurements is as shown in Fig. 4c.

2.5. Preparation of sensor chips: SAM formation and Ab-immobilization

The signal transducing IDTs in the LOC were washed with sterile dH_2O. The blank measurements with bare IDTs were taken prior to any application on the surface. The surface of the sensor was then coated with self-assembled monolayer (SAM) with 10 mM solution of mercaptoundecanoic acid (MUDA) for overnight at room temperature followed by washing with EtOH and dH_2O (cycle-1 process) to remove traces of unbound MUDA.

The IDTs were coated with SAM (MUDA) to obtain a uniform layer of the linker molecule and the maximum sample surface coverage. The surface was then activated using a mixture of 0.1 M EDC and 0.05 M NHS (1:1) which enables binding of protein antibodies. Both reagents were prepared in sterile deionised water and immediately mixed before use. Activation of each IDT was performed through microfluidic channels of the system by cycle-1 process. After 4 h incubation at ambient temperature, the sensor system was then washed with PBS buffer and antibody immobilization was performed. Triplicate IDTs were coated with 30 μg/ml concentrations of anti-hEGFR and IL-6 antibodies in buffer. After the antibody immobilization process, the sensing platform was washed with PBS and subjected to antigen binding along with appropriate controls.

2.6. Multiple protein marker detection

In this study, hEGFR and IL-6 markers were investigated for simultaneous detection of cancer and cardiovascular system disease. hEGFR (a cancer marker) and IL-6 (one of the cardiac markers) were studied in the concentration range of 0.1–10 ng/ml spiked in human serum. 10 ng/ml of each marker was prepared in 100% human serum and lower concentrations were then diluted from these stocks. BSA protein (50 ng/ml in serum) was used as negative control on the antibody immobilized surface to validate the specificity of the binding between target antibody–antigen pairs. Each concentration of the markers was tested with three independent LOC and triplet measurements were taken with the help of Network analyzer. The antigens were incubated for 2 h at room temperature in binding step and the microfluidics system was then carefully washed with PBS followed by quickly washing with dH_2O prior to taking the measurements. The functionalization of the IDT surface is schematically shown in Fig. 4a.

Triplicate measurements were taken for each IDT of the LOC in the frequency range of 50–300 MHz and inter-assay analysis was performed. The S11 parameters during surface functionalization and the immobilization were recorded. For the analysis, capacitance was deduced from the S11 parameters. The deduced capacitance after antigen binding was normalized with the values obtained from the respective antibody immobilization and the results were analyzed as the normalized capacitance change ($|\Delta C|$).

$$\%|\Delta C| = \frac{C - C_O}{C_O} \times 100 \quad (1)$$

where C and C_O represent the capacitance after target binding and antibody immobilization.

3. Results and discussion

In this study, the functionality of the LOC with a dimension of 60 mm × 45 mm × 5.5 mm was tested with hEGFR-2 and IL-6, cancer and cardiac biomarkers, respectively, as model analytes for the detection of multiple disease markers.

3.1. Flow characteristics of the immunosensing LOC

For the determination of fluid velocity through the channels, a flow rate characterization unit (Fig. 4) was setup. It was observed that, the flow behavior depends only on the internal thrust created inside the channel due to the relaxing of the actuator. The working fluid (a methylene blue dye to visually inspect) was driven from the inlet liquid reservoir using internal actuators pulling action. The flow rate of the on-chip actuated LOC was measured after releasing the applied load. It was observed that the working fluid flows into the wastage reservoir through the channel because the released hydraulic load exponentially increases the fluidic resistance of the channel over the ambient pressure. When the actuator was released by removing the down pressed finger, and the measured average flow rate in the channel was recorded as 300 nl/s, which is lower than the reported previous experimental value (15.4 μl/s) [41].

3.2. Surface modification of transducer and immunoanalysis LOC performance

A stable immobilization method was employed during the immunoanalysis using the LOC. The antibody immobilized IDTs were then subjected to incubation with the target antigens. The specificity of the sensor to hEGFR and IL-6 antigens was derived from the specific binding between the anti-hEGFR-hEGFR, and anti-IL-6-IL-6 antigens while no binding was observed with a negative control (BSA). In Fig. 5a and b, we observed a significant change in $\%|\Delta C|$ for the hEGFR antigens (scan range: 230–280 MHz) and IL-6 antigens (scan range: 140–200 MHz) concentrations. From the above data, it is clear that the antigens were detected in the concentration range of 0.1–8 ng/ml. Here, the target antigens interacted to bind to the specific antibody by which they achieve certain active and vibrating three dimensional (3-D) conformations [49,50]. Thus, the vibrations lead to the transfer of charges in the 3-D structure which predominantly contribute to the

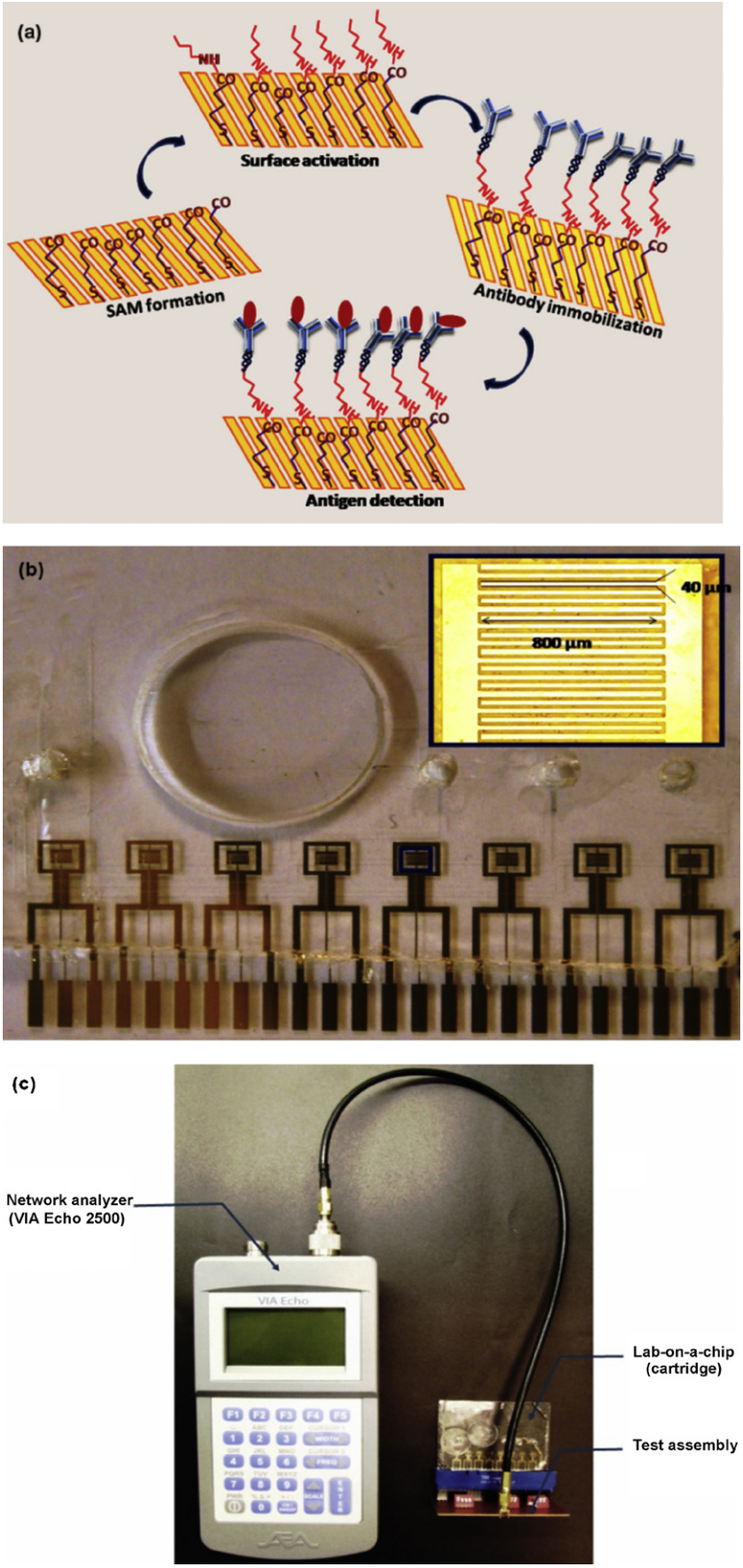

Fig. 4. (a) Schematic diagram of the bio-functionalization of the IDT surface. (b) Photographic image of the LOC system. (c) Hand-held format of measurement system.

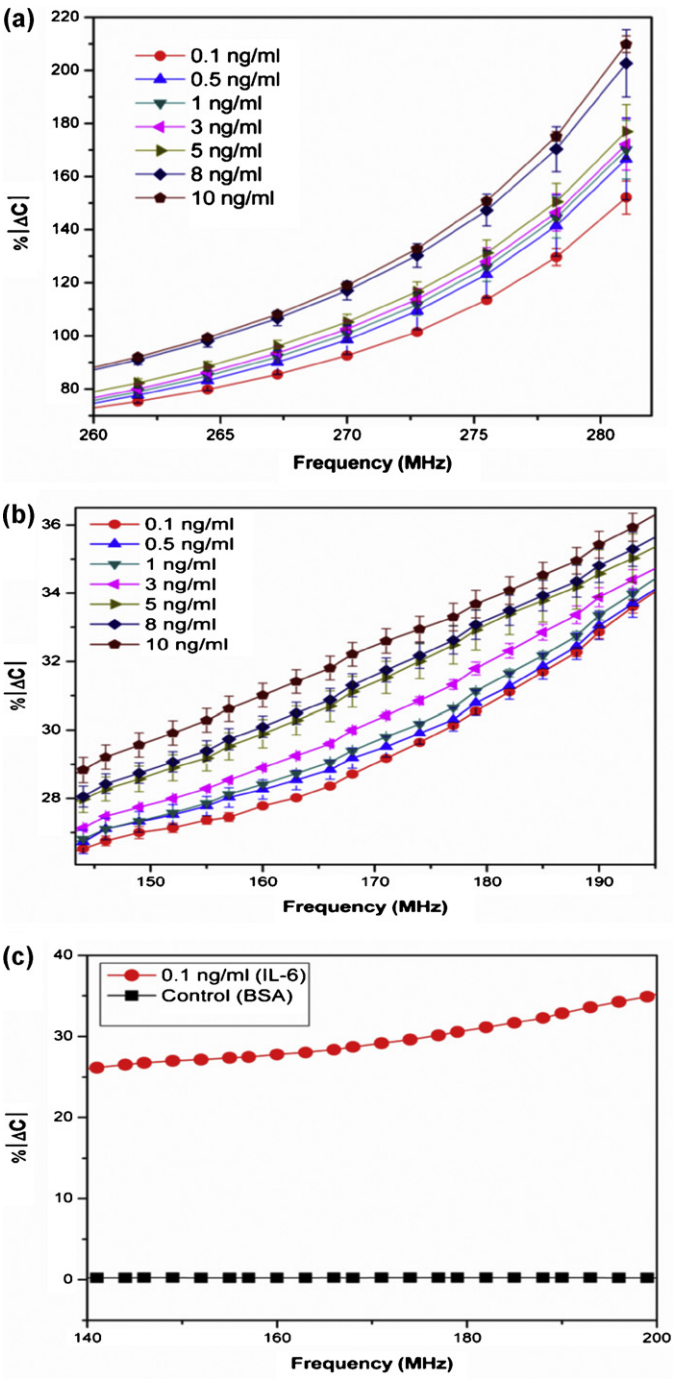

Fig. 5. (a) Relative change in capacitance obtained after anti-hEGFR immobilized IDTs were incubated with various concentrations of hEGFR antigens in serum in frequency range of 260–280 MHz. (b) Relative change in capacitance obtained after anti-IL-6 immobilized IDTs were incubated with various concentrations of IL-6 antigens in serum in frequency range of 140–200 MHz. (c) Comparison of relative change in capacitance obtained after anti-IL-6 immobilized IDTs when incubated with 0.1 ng/ml concentration of IL-6 antigens in serum in frequency range of 140–200 MHz and 50 ng/ml control (BSA).

frequency and consequently to the observed function. Hence, the frequency of conformational vibration strongly depends on size of the 3-D structure and surface charges that vary from one protein to another. For instance, the hEGFR (324 amino acids) [51,52] is larger in size than IL-6 (184 amino acids) [53]. Thus, the response of IDTs treated with hEGFR protein was consistent in the range of 230–280 MHz, whereas other IDTs treated with IL-6 were consistent in the range of 140–200 MHz. Furthermore, with standard

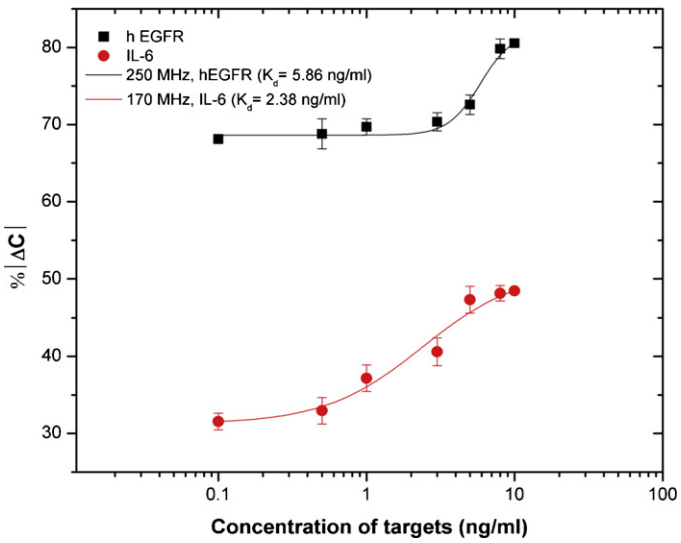

Fig. 6. Dose-dependent change in relative capacitance occurred after the Ab–Ag complex formation on IDT surface against varying concentrations of hEGFR at 270 MHz and IL-6 target in serum (0.1–10 ng/ml) at 170 MHz frequency.

experimental conditions (geometry of the IDTs and immobilization procedure), the $|\Delta C|$ levels of IDTs with hEGFR complex are higher when compared to the responses of IDTs with IL-6. Therefore, the binding of an analyte to an immobilized molecule can be detected without labeling or secondary reactions using IDT measurements. Also the response with BSA (control) shown in Fig. 5c verifies the specificity of the assay by giving rise to negligible response which was lower than 0.1 ng/ml of antigen(s) tested.

3.3. Kinetic analysis of hEGFR and IL-6 proteins

The affinity of the sensor surface immobilized with anti-hEGFR and anti-IL-6 binding to their respective targets was determined by non-linear regression fit analysis and extracted dissociation constants. It was noted that the K_d values were lower when tested with the concentrations of targets from 0 to 10 ng/ml. The sensitivity of the IDT was dependent on specified amount of immobilized antibodies within 1.4 mm × 1.8 mm sensor surface area with a defined geometry under standard conditions as described in Section 2. The system response reaches to a saturation level above a concentration of 8 ng/ml (hEGFR) and 5 ng/ml (IL-6) (Fig. 6). The binding kinetics of the interaction of antigens and antibodies are dependent on avidity, that is, the synergistic binding affinity of multiple interactions. The binding affinity can be significantly increased when multiple protein binding sites are available for binding. The lower the K_d value the stronger the binding according to the Langmuir adsorption isotherm [54]. Here, IL-6 showed strong binding compared to hEGFR. Thus, the density of the available binding sites greatly impacted the avidity effect, resulting in an apparent affinity between the protein and target when binding sites are clustered in a close proximity. Thus, higher concentrations of target did not increase the sensor response because of saturation of the binding sites. Hence, we observed saturation at target concentrations of 8 ng/ml and 5 ng/ml for hEGFR and IL-6, respectively (Fig. 6).

4. Conclusions

In this study, we developed a sensitive, real time and label-free immunoassay using a hand-operated LOC for the first time, for the detection of a cancer biomarker (hEGFR) and a cardiac biomarker (IL-6) on a single platform. Further, with proper design rules and a simple fabrication method, we have established the

technology to fabricate a novel LOC, which does not require external power source/additional instrument for fluid transfers. This micropump system can deliver sample solutions at a flow rate of 300 nl/s. Very low sample volume consumption (~60 μl) required for assaying biological samples is an added advantage. Moreover, the flexibility of the design to stop the liquid flow at any instant of time can be used for incubation purposes in immunosensing LOC applications, which is rarely seen in the literature. Thus, using non-Faradic electrochemical-based immunosensing methodology, we have developed a LOC platform for the sensitive detection of hEGFR and IL-6 markers.

The multi-target detection with dose dependent analysis was achieved with a sensitive detection limit of 3–8 ng/ml for hEGFR and 0.1–5 ng/ml for IL-6. The hand-operated pumping system employed in this study can be easily integrated to the systems where there is no availability of external fluid driving mechanisms or power sources. We expect that our novel approach will play important role in the delivering sensitive samples for portable and point-of-care applications. In the near future, multiple markers representing same disease will be tested for its applicability.

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