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Risk stratification of heart failure from one drop of blood using hand-held biosensor for BNP detection

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ABSTRACT

Continued risk assessment by evaluating cardiac biomarkers in healthy and unhealthy individuals can lower the mortality rate of cardiovascular diseases (CVDs). In this research, we have developed a hand-held biosensor system to rapidly screen for brain natriuretic peptide (BNP) from a single drop of whole blood. The sensor methodology is based on extended gate design of electrical double layer (EDL) field effect transistor (FET), that can directly detect BNP in whole blood, without extensive sample pre-treatments, thereby eliminating the limitations of charge screening in high ionic strength solutions. A simple sensor array chip is fabricated to integrate with the MOSFET sensor system. Sensing characteristics are elucidated using purified BNP samples in 1 × PBS (with 4% BSA), spiked BNP samples in whole blood and clinical whole blood samples. The blood cells can be gravitationally separated without the use of any external actuation. The sensor exhibits very high sensitivity over wide dynamic range of detection. The sensing characteristics are not adversely affected by the presence of background proteins or blood cells, even without gravitational blood cell separation. Thus, the biosensor system can allow users to perform rapid whole blood diagnostics with minimal user protocols, in 5 min. The features of high sensitivity, cost-effectiveness and convenience of usage empower this technology to revolutionize the mobile diagnostics and healthcare industry.

1. Introduction

Molecular biomarkers that provide physio-pathological information play an important role in the diagnosis and prognosis of diseases such as cardiovascular diseases (CVDs) and cancers (Mayeux, 2004; Liu et al., 2014). Timely diagnosis aids in the prevention of CVDs, which are the leading causes of death worldwide. Continuous monitoring of molecular biomarkers provides additional risk stratification for CVDs, beyond the traditional biomarkers (Gilstrap and Wang, 2012; Yin et al., 2014). High rates of morbidity and mortality could be prevented through regular assessment and management of cardiovascular risk, by providing cost-effective and convenient means of health monitoring systems available to all. Congestive heart failure (CHF) is essentially the inability of the heart to pump blood to all parts of the body, due to weakened heart muscles or defects (Taylor, 1996). Since heart failure can be presented with a variety of symptoms, physicians face challenges in definitive diagnosis and correct prognosis. Protein based biomarkers of heart failure such as brain natriuretic peptide (BNP) and N-terminal pro brain natriuretic peptide (NT-proBNP) are identified as the standard biomarkers for diagnosis and prognosis of heart failure (Lin et al., 2014; Natriuretic Peptides Studies, 2016). Clinical determination of the natriuretic peptides from blood plasma can be performed using conventional spectroscopic techniques such as radioimmunoassay and chemiluminescence (Cowie et al., 2003; Nishikimi et al., 2013). More recently surface plasmon resonance (SPR) based immunoassays and electrochemical enzyme immunoassays have also been developed to quantify BNP levels (Matsuura et al., 2005; Jang et al., 2014). However, these assays are quite complex, performed using bulky and costly equipment, often requiring trained laboratory staff to perform the assay.

Miniaturized electronic sensors present an opportunity to perform high sensitivity immunoassays at considerably low cost. The ease of integration of these miniaturized biosensors with the measurement system facilitates compact and portable sensor units that can be used for point-of-care assays. In this genre, field effect transistor (FET) based

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biosensors are highly desirable owing to its characteristic features such as high sensitivity, low cost, easy signal read-out and fast response times. However, FET based sensors suffer from charge screening effect in high ionic strength solutions such as blood due to the extremely short Debye length (Stern et al., 2007; Zheng and Lieber, 2011). As a result, extensive sample pre-treatment methods are adopted to facilitate biomolecule detection, such as dilution and desalting (Zheng et al., 2005; Zheng and Lieber, 2011; Stern et al., 2010). This severely impacts the reliability and practicality of FET based assays in clinical applications especially if a rapid, inexpensive in-vitro diagnostic platform is desired.

In the present research, we have devised and characterized a hand-held biosensor system for the determination of BNP levels from a single drop of whole blood. The sensing methodology is based on extended gate electrical double layer (EDL) FET biosensor that can directly detect the target analyte in high ionic strength solution such as whole blood, offering bio sensing beyond the Debye length. EDL FET biosensors have demonstrated high sensitivity and selectivity in biomolecule detection (Chu et al., 2017). In the extended gate design, device to device variations are considerably reduced allowing easy sensor calibration for variety of clinical applications. Also, FET is protected from harsh chemical environments of the physiological fluids, providing enhanced stability and robustness. The use of single FET for multiple electrical measurements significantly lowers the assay cost. In here, we demonstrate the detection of BNP in 1 × PBS with 4% BSA and clinical whole blood samples obtained from different patients. The sensor exhibits very high sensitivity, selectivity and wide dynamic range of detection. Simple gravitational separation of blood cells from blood plasma is performed, without any external actuation to eliminate any interference of cell components of whole blood. A hand-held biosensor measurement unit is developed to integrate the sensor chip and the electrical test results are displayed in the LCD display. We demonstrate that our unique sensing methodology can facilitate rapid screening of protein biomarkers from a single drop of whole blood (< 10 μL) in 5 min, and the simplistic assay can be performed by consumers with minimal protocols, as per their convenience and requirements.

2. Experimental

2.1. Fabrication of sensor array chip

A simple sensor array chip is fabricated on an epoxy substrate, containing multiple gold electrode pairs. Epoxy substrate is fabricated by pouring thermo-curable epoxy resin in PDMS mold and curing at temperatures of 125 and 165 °C for 1 and 1.5 h respectively. Post curing, electron beam (e-beam) evaporator technique is used to deposit gold electrode pairs (Ti (200 Å) and Au (2000 Å)) on the epoxy substrate. Typically, an eight-electrode pair array is fabricated. The two electrodes forming the gold electrode pair are separated by a gap of 185 μm. The whole sensor array chip is passivated using photoresist and openings of 3600 μm^2 (600 μm x 600 μm) are made on each of the electrodes. When test sample is dropped on the sensor, the solution connects the two openings on the gold electrodes. Gate bias voltage is applied to one electrode and the other electrode is connected to the gate terminal of the MOSFET.

2.2. Sensor surface functionalization

Monoclonal antibody (mAb) is used to capture BNP samples from the test solution. BNP antibody is purchased from Hytest Inc (19c7). A mild reducing agent such as 2-mercaptoethylamine (2-MEA) is used to cleave the disulfide hinge region of IgG molecule, yielding free thiols to bind with gold. mAb and 2-MEA are mixed together in the molar ratio 10,000:1. The mixture is then incubated in reaction tube at 37 °C for 15 mins and on the sensor surface for 1.5 h in room temperature. The sensor is allowed to remain in 4 °C for 12 h following which the unbound antibodies are thoroughly washed away in 1 × PBS.

2.3. Purified BNP samples

Purified BNP purchased from Abcam, is diluted to target concentrations in 1 × PBS (pH 7.4, 150 mM NaCl, 10 mM PO_4^{3-}) containing 4% Bovine Albumin Serum (BSA). The target concentrations of purified BNP samples tested are 0, 100, 200, 500 and 1000 pg/mL. 4% BSA is added to the reaction buffer solution to simulate the real conditions of human blood serum.

2.4. Clinical BNP samples

The clinical whole blood samples are obtained from different patients admitted to the hospital as per the IRB No. 16MMHIS112. The whole blood samples are collected from different individuals at different times, in a vacutainer treated with anti-coagulant. The whole blood sample obtained from healthy individual with undetected BNP is also treated with anti-coagulant, as per the IRB no. 10610HE074.

2.5. Sensor regeneration

After each sample measurement, the sensor surface is thoroughly washed with 3 mL gentle protein elution buffer (Pierce gentle ag/ab elution buffer, pH 6.6) and 1 × PBS, alternatively for one hour, to remove target proteins and non-specifically bound proteins. Thus, the sensor surface is regenerated, and electrical baseline is restored.

2.6. Sensor measurement

A hand-held biosensor system is used to evaluate the sensor characteristics. The system houses a microcontroller unit, signal acquisition, LCD display, and read out circuitry. N-Channel Depletion-Mode DMOS FET (LND150) is used to carry out all electrical measurements and is connected to the hand-held device as shown in Fig. 1. The hand-held device is able to measure 8 sensors on one chip, which is inserted into a PCI socket in the hand-held device. The circuit in the device provides pulse Vd and Vg to each sensor electrode, by using switch component to lead the bias given to each sensor electrode. The device can be connected to laptop with USB wire or Bluetooth. A rechargeable Li-battery is embedded in the device. FET biasing, signal acquisition and display of measurement results are controlled with user interface software, which facilitates two distinct modes of operation: single and burst. In single mode, one measurement data is recorded and in burst mode, multiple data are recorded. Throughout this study, burst mode of operation has been used. A short duration gate pulse with a width of 100 μs and amplitude of 1 V is applied as the gate bias to one of the electrodes. A steady DC bias of 2 V is applied as the drain-source voltage during sensor operation. The detachable sensor chip containing gold electrode pairs is connected to hand-held system as shown in Fig. 1. More details about the test parameters, current gain calculations and typical drain current response are shown in Supplementary information.

3. Results and discussion

The schematic representation of the extended gate design of EDL gated FET biosensor is shown in Fig. 1(a). The real image of the sensor mounted on the handheld biosensor system is shown in Fig. 1(b). The sensor structure is uniquely different from traditional FET based biosensors. In the extended gate design, the gate metal is connected to a pair of gold electrodes which are separated by a short gap. The gold electrode pair simulate a two-plate capacitor, with the test solution (containing the target protein) as the dielectric. One of the gold electrodes of the pair is functionalized with the receptor and the other is connected to the FET gate metal. The test solution is dropped on the gold electrode pair, forming a dielectric between the two gold surfaces. In this way, the FET does not come in contact with the bio sample

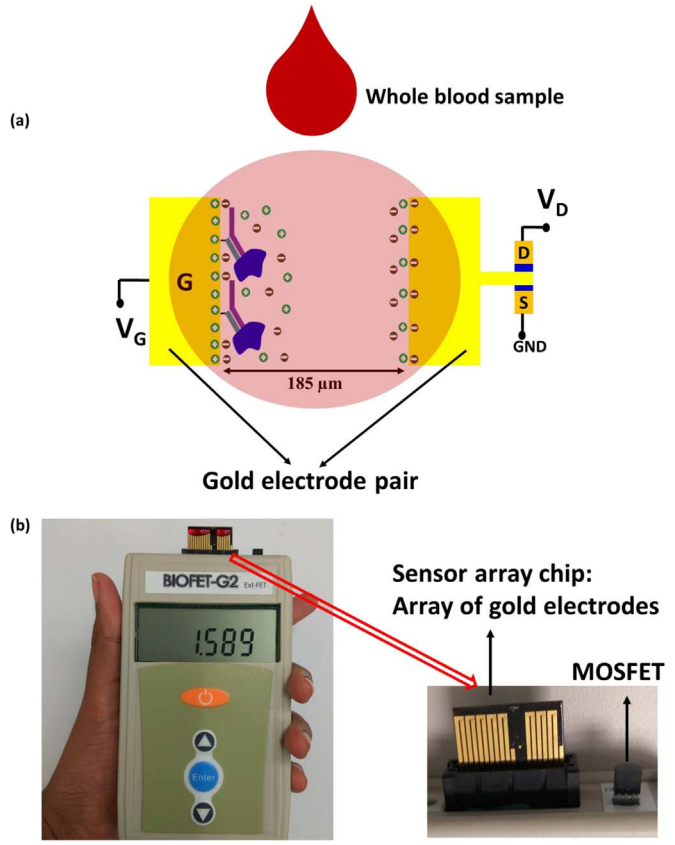

Fig. 1. (a) Schematic illustration of extended gate EDL FET biosensor (b) Real image of the hand-held EDL FET biosensor system integrating the sensor array chip for electrical measurement.

containing solution, which considerably reduces device to device variations and electrical drift (van der spiegel et al., 1983; Toshiya et al., 2005; Kaisti et al., 2016). The extended gate design also reduces the cost of the sensor as a single FET can be used repeated electrical testing. A high-density sensor array consisting of simple gold electrodes can easily be realized with enhanced sensing performance compared to traditional FET based biosensors. This sensor array chip can easily be integrated with portable or mobile diagnostic systems allowing rapid biomolecule sensing, at the convenience of the user, at homes or hospitals. The sensing methodology described in this research can be applied to all types of FETs including novel 2-dimensional materials based FET sensors. Since FET is not subjected to harsh chemical environments, considerable time and efforts are saved in stabilizing FET performance in aqueous environments.

In our sensor structure, each sensor chip contains an array of gold electrodes. The gold electrodes are designed as a pair that is separated by a gap of 185 µm. The entire chip is passivated in photoresist and openings of 600 μm^2 are made on the gold electrode pair, such that the test solution comes in contact solely with the electrode open areas. When a pulsed gate voltage, Vg is applied, the mobile ions in the test solution instantly polarize and form electrical double layer (EDL) on each of the electrode open area-solution interface. Thus, a solution capacitance Cs is formed, which modulates the potential drop on the transistor oxide dielectric (Vox). The changes in Vox control the channel conductivity. Thus, the applied potential can be envisioned as the summation of potential drops across the test solution and the transistor dielectric, according to Eq. (1).

$$V_g = \Delta V_s + \Delta V_{ox} \tag{1}$$

Where V_g is the applied gate bias and V_s and V_{ox} are the potential drop across the solution and the MOSFET gate oxide, respectively. Considering the capacitances are in series, the potential drop across the dielectric can be modeled as in Eq. (2).

$$\Delta V_{ox} = \frac{\frac{1}{j\omega C_{ox}}}{\frac{1}{j\omega C_{ox}} + \frac{1}{j\omega C_s}} \times V_g = \frac{C_s}{C_{ox} + C_s} \times V_g \tag{2}$$

Where C_s and C_{ox} are the solution capacitance and the oxide capacitance, respectively which are connected in series circuit, and j is complex function and ω is angular frequency.

Thus, changes in Cs can modulate Vox and hence, the transistor drain current. The changes in Cs can be due to different ionic strength of the solution, surface functionalization of sensor electrodes and target protein binding on to the receptor. When target protein binds to the receptor i.e., when BNP binds to the antibody, the charge distribution in the local region of the EDL changes, which effectively changes Cs and hence, the transistor drain current. Thus, the drain current changes in a BNP concentration dependent manner. The high sensitivity obtained in our method can be attributed to the EDL gating mechanism, similar to the working of high k dielectrics (Chu et al., 2017). Throughout our study, we rely on current gain or simply gain as sensor signal rather than absolute drain current. Current gain is defined as the change in drain current before and after applying Vg. This index is more stable compared to absolute drain current, which may be prone to thermal drift and impedance loading. The baseline drift is further minimized by adopting short duration (100 µs) pulsed gate bias rather than continuous DC bias, which results in heating induced drain current shifts. Also, test solution does not touch the MOSFET, which enables the use of sensor in harsh conditions of biological samples, without suffering from baseline drifting.

3.1. Electrical response in whole blood

Our primary goal is to perform protein detection directly in whole blood, without extensive pre-treatments. However, previous studies based on electronic and electrical sensors have serious non-specific signals from the high concentration of proteins, metabolites and cellular components in whole blood. In a previous study, Stern et al. describes the fabrication of a microfluidic purification chip to capture antigens from whole blood, wash repeatedly and dilute the ionic strength several orders of magnitude lower than physiological salt concentration, to facilitate protein detection using FET biosensor (Stern et al., 2010). This technique involves extensive sample pre-processing and hence is not suitable for a low-cost, in-vitro diagnostic assay for biomarker detection. In a different platform based on DNA functionalized electrochemical sensor, target protein detection in whole blood was demonstrated (Mahshid et al., 2015). However, this technique requires additional labeling reagents and is only applicable to aptamer based immunoassays. In our study, we demonstrate the detection of BNP from whole blood, without any additional steps of labeling or washing, in 5 min with a single drop of blood.

Firstly, we investigated the effects of whole blood incubation on the sensor chip. The electrical results are depicted in Fig. 2. The whole blood samples are collected in a vacutainer coated with anti-coagulant. After dropping the sample of whole blood on the sensor chip, it is turned downwards such that the blood cells are separated from the plasma, gravitationally, without requiring any external actuation (as shown in Fig. 2(a)). The device is held in the upturned position during the incubation period of 5 min, followed by electrical testing. In contrast, whole blood sample is dropped on the sensor chip and allowed to incubate for 5 min, with the chip facing the upward direction, as shown in Fig. 2(b). In this configuration, the blood cells sediment to the bottom, and are separated from the plasma. The electrical test results for both device configurations (chip facing up and down) are shown in Fig. 2(c). The current gain response of the sensor is similar in both the device configurations. This means that the sedimentation of blood cells on the sensor surface does not cause a significant change in sensor

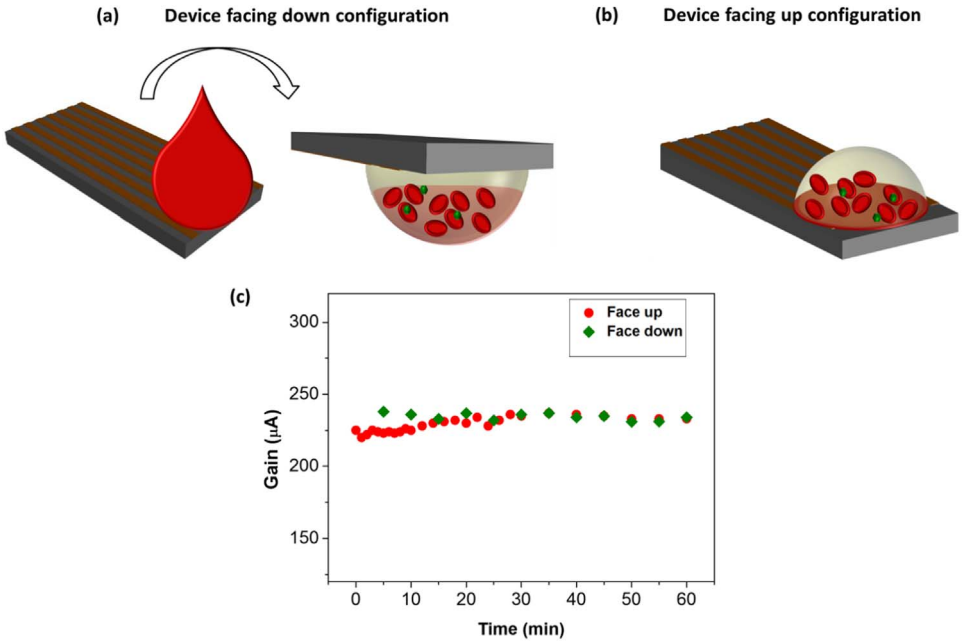

Fig. 2. (a) and (b) Schematic representation of gravitational separation of blood cells in device facing down and up configurations, respectively (c) Electrical characteristics of extended gate EDL FET in whole blood.

response. This behavior characterized by the electrical response of the sensor can be explained by considering the microscale vicinity of the blood cells and the sensor surface. Previous literature on electrical impedance sensors designed to study cell to substrate interactions suggest that when the cellular adhesion to the substratum increases, impedance increases (Giaever and Keese, 1991; Lo et al., 1995). This is due to the decreased cell to substrate space and tighter intercellular junctions which impede the electric field, thereby increasing the electrical impedance. Since the whole blood samples used in this study is anti-coagulated, clotting is prevented and hence, blood cells do not form tight intercellular regions. The cells do sediment on to the surface of the sensor but stay afloat and do not form strong adhesion on the sensor surface. A previous study shows that when cell is not strongly bound to the sensor surface, the electrical response is not significantly affected (Pulikkathodi et al., 2018a, 2018b). This mechanism of cell proximity can explain the electrical response shown in Fig. 2(c). In another research, the charge displacement upon cellular adhesion on sensor surface is characterized (Svetličić et al., 2001). In our sensor, since the blood cells are afloat on the sensor surface, the charge displacement in the local region of the EDL is minimal and hence, Cs and effectively current gain of sensor is not significantly changed. This result shows that our sensing methodology is not significantly affected by the presence of blood cells, which facilitates easy and speedy detection of target analytes from whole blood.

3.2. Detection of purified BNP in 1 × PBS with 4% BSA

Fig. 3 demonstrates the sensing characteristics of extended gate EDL FET biosensor in high ionic strength buffer. Purified BNP samples are prepared in 1 × PBS with 4% BSA. The addition of albumin is carried out to simulate the real conditions of blood serum. In order to reduce the drop to drop variations in buffer and person to person variations in clinical samples, we evaluate the current gain response of functionalized and bare gold electrode pair. The sensor response is denoted as the current gain of functionalized gold electrode pair minus the current gain of bare gold electrode pair. This differential current gain can

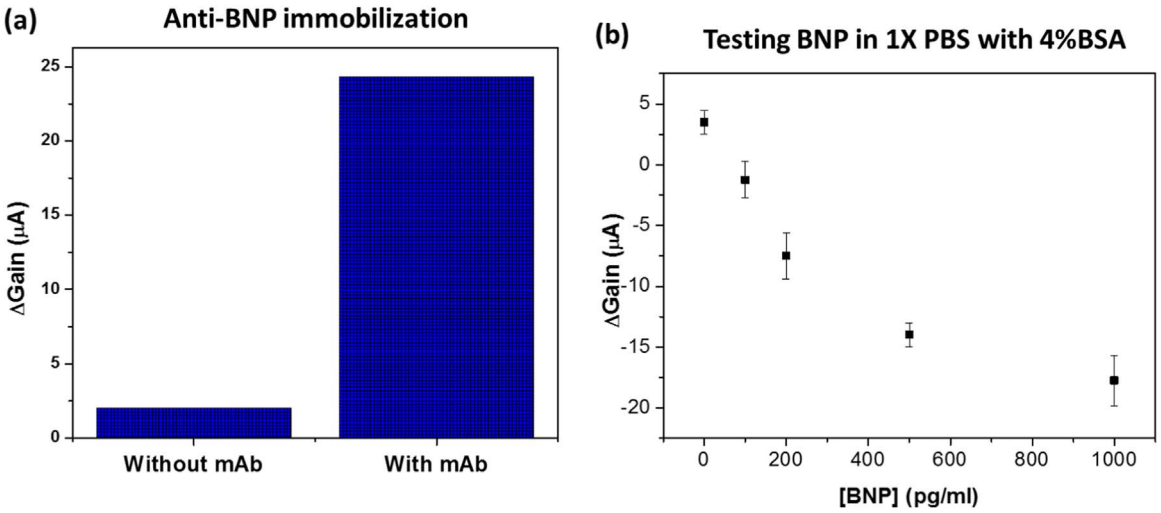

Fig. 3. (a) Sensor response after surface functionalization (b) BNP sensing characteristics in 1 × PBS with 4% BSA (n = 4).

reduce the variations arising from external sources such as temperature and humidity changes, fluid pressure and surface energy of droplet. This method of using differential gain can also eliminate the inconsistencies observed in clinical samples obtained from different patients as well. Fig. 3(a) depicts the sensor response before and after antibody immobilization. The change in current gain can be used to monitor effective surface functionalization. Fig. 3(b) demonstrates the sensor response curve in 1 × PBS with 4% BSA. As seen from Fig. 3(b), the current gain decreases as the concentration of BNP increases. The BNP concentrations are 0, 100, 200, 500 and 1000 pg/mL. The sensor exhibits very high sensitivity over a wide dynamic range of detection. BNP which is a long- term marker of congestive heart failure has an average clinical cut off value of 450 pg/mL for patients < 50 years of age and 900 pg/mL for patients > 70 years of age. The results in Fig. 3 demonstrate that our sensor is suitable for clinical determination of BNP as a cardiac biomarker. Also, our sensing methodology can facilitate direct protein detection in high ionic strength solutions (1 × PBS), thereby overcoming the limitations of traditional FET sensors set by Debye screening. This is indeed protein detection beyond Debye length.

3.3. Detection of purified BNP in whole blood

As a means of positive control, we tested BNP samples spiked in whole blood. The samples are prepared in whole blood (containing BNP less than detection limit of laboratory standard instrument) in the concentrations 100, 200, 500 and 1000 pg/mL. After dropping the sample on the sensor, the device is held in downward facing position during the incubation period (5 min) and electrical measurement is carried out. The sensing characteristics are shown in Fig. 4. The current gain of the sensor decreases as the BNP concentration increases, similar to Fig. 3. The sensor displays high sensitivity from 0 to 1000 pg/mL. More importantly, the sensor has a target concentration dependent signal change even in the presence of myriad of background proteins in whole blood. This response elucidates the high selectivity of the sensor towards the target protein. The whole blood samples contain variety of proteins and other biomolecules, in significantly high concentrations. But our sensor can yield a BNP concentration dependent sensor response amidst such noisy background. This is an important characteristic of the sensor, which can be attributed to its sensing mechanism. During the incubation period of 5 min, the binding kinetics of antibody and BNP (specific interaction) dominates over non-specific binding. The strong electrostatic interaction in the local region of antibody-antigen moiety in the EDL modulates Cs to a considerably larger extent than the non-specific interactions in the bulk of the solution. This results in high selectivity towards the target protein. Results in Fig. 4 demonstrate that our sensor methodology can be used to directly detect target proteins in whole blood without extensive sample processing methods.

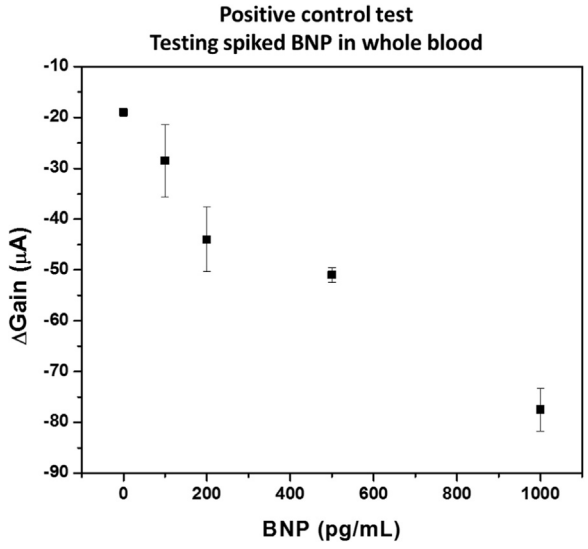

Fig. 4. Sensing characteristics of BNP samples spiked in whole blood (n = 3). The BNP concentrations are 0 (undetected BNP), 100, 200, 500, 1000 pg/mL.

3.3.1. Detection of BNP in clinical whole blood

Fig. 5 describes the sensing characteristics of extended gate EDL FET biosensor in clinical whole blood samples. The samples are obtained from different patients at different times. The electrical testing of whole blood samples has been performed in two device configurations: sensor chip facing downwards and upwards, during sample incubation period (5 min). Fig. 5(a) and (b) depict the sensor response with device facing down and up, respectively. The BNP concentrations in the clinical samples are 140, 644, 1570, 2980, 3900 pg/mL. As seen from the figures, when the BNP concentration increases, the current gain decreases. The trend of sensor signal agrees with that shown in Figs. 3 and 4. The sensor not only depicts high sensitivity but also high selectivity towards BNP. The sensor can also offer wide dynamic range of detection. The clinical samples obtained from different patients may contain variations in the protein and metabolite consistency. However, since we use bare gold electrodes to monitor the background response of the whole blood sample under test, the person-to-person variations can be minimized considerably. The sensor calibration curves depict good concentration dependency, even though the blood samples are collected from different patients. This demonstrates that there is very little effect from non-specific binding on the sensor signal. Moreover, in our previously published work (Chu et al., 2017), we have proved that in this sensor methodology, there is no significant non-specific binding even in untreated clinical samples.

The electrical response depicted in Fig. 5 for face up and down configurations may not have exactly same electrical response owing to chip to chip variations, but both show good sensitivity over wide dynamic range. This result further confirms our findings described in Fig. 2, about non-interference from blood cells. Even though blood cells sediment on to the sensor surface in device facing up configuration, the electrical response of the cell does not change significantly. This sensor response is observed in cases where cells are not tightly bound to the sensor surface. However, while testing real clinical samples, cellular adhesion to substrate may not be consistent across different patient samples. Hence, gravitational separation of blood cells may eventually be performed, to ensure optimal sensor performance and to avoid any interference from cells. Since no external energy/actuation is required to perform the gravitational separation of blood cells, the assay simplicity, overall cost and sensor robustness are not compromised.

After testing each whole blood sample, the sensor is washed in mild elution buffer to elute the specific protein and non-specific biomolecules away from the sensor surface. In this way, the sensor surface is regenerated for further testing and sensor baseline is restored. This allows us to strictly control the electrical response of the background test solution. During each sample measurement, which includes the sample incubation period of 5 min, the background of the test medium is controlled to be the same, as all the specific and non-specific binding entities are washed away from the sensor surface. This method is adopted to allow the same test background for all sample measurements and hence enables target detection, without washing process. This reduces the assay complexity while providing stability and reliability. The sensor baseline can be monitored electrically to verify regeneration, as shown in Fig. 6. The baseline of sensor in device facing down and up configurations is shown in Fig. 6(a) and (b), respectively. These results demonstrate that we can wash off the whole blood components from the sensor surface using the protein elution process and therefore, the change in sensor signal in Fig. 5 is indeed responsive to the BNP concentrations. Even when the blood cells are allowed to sediment on to the surface of sensor in device facing up configuration, the sensor

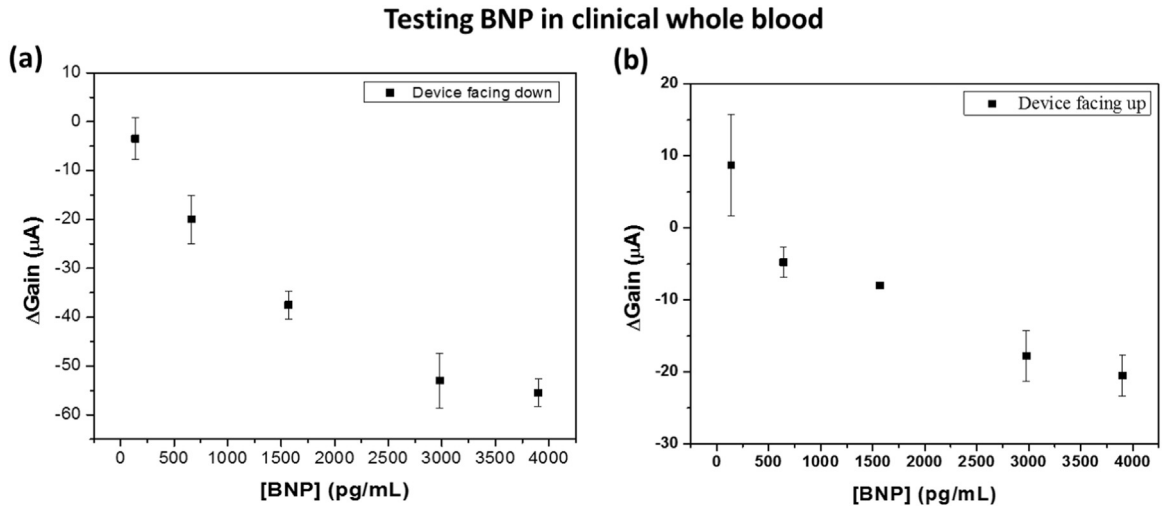

Fig. 5. BNP sensing characteristics in clinical whole blood samples (a) in device facing down configuration (b) in device facing up configuration (n = 3). The BNP concentrations are 140, 644, 1570, 2980, 3900 pg/mL.

baseline can be restored and controlled efficiently.

4. Conclusion

In this research, we have devised a hand-held diagnostic system based on extended gate EDL FET biosensor for the detection of BNP as a biomarker for congestive heart failure. We have developed FET sensor methodology to directly detect protein biomarkers in whole blood, without extensive sample pre-treatments. This methodology is based on the high field generated across the test solution which modulates the FET channel conductivity, enabling detection beyond Debye length in high ionic strength solution. We have demonstrated the detection of BNP prepared in 1 × PBS with 4% BSA and in real clinical whole blood samples obtained from different patients. The blood cells can be gravitationally separated from the blood plasma, without any external actuation or additional machinery. This significantly reduces the complexity of the assay and maintains the biosample integrity, preserving the proteome consistency. The sensor exhibits high sensitivity over a wide dynamic range of detection, in buffer and clinical whole blood samples. The selectivity of the sensor is demonstrated by testing spiked BNP concentrations in whole blood (with undetected BNP), as a means of positive control. Thus, we have overcome the drawbacks of traditional FET based biosensors and facilitated the opportunity of FET based biosensors to explore clinical applications. Since extensive sample pre-treatments or additional reagents are not required, our simplistic assay is ideal for in-vitro diagnostics intended for rapid screening of biomarkers of diseases. The sensor array chip consisting of gold electrodes may be used with a single FET embedded in the PCB in a disposable or 'use and discard' format. Therefore, the end user does not need to perform any additional assay protocols, making it highly convenient and accessible to all.

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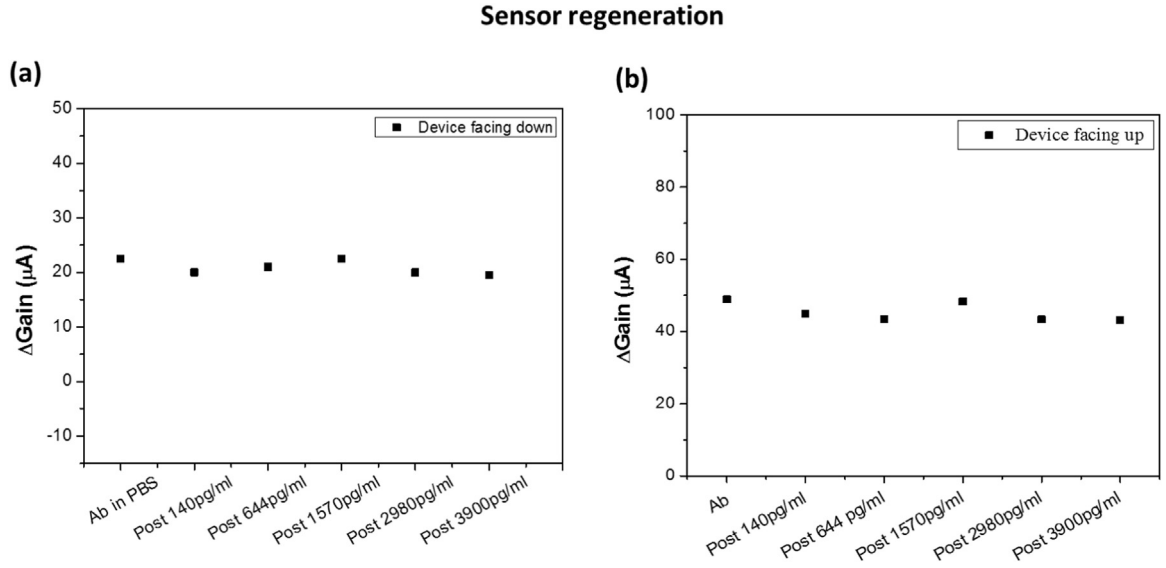

Fig. 6. Sensor baseline characteristics after protein elution (a) in device facing down configuration (b) in device facing up configuration.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2018.02.036.

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