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Label-free capacitance based aptasensor platform for the detection of HER2/ErbB2 cancer biomarker in serum

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ABSTRACT

In this study, a label-free capacitive aptasensor was developed based on capturing of human epidermal growth factor receptor 2 (HER2) protein by anti-HER2 ssDNA aptamers functionalized on interdigitated microelectrodes of capacitor as bio-recognition elements. The aptasensor response was measured by non-Faradaic Impedance Spectroscopy (nFIS) method. Capacitance signal specific to target HER2 protein is based on changes occurred due to charge distribution upon interaction of aptamer–protein molecules against to the applied AC frequency (50–350 MHz). HER2 protein in spiked human serum was successfully detected through concentration dependent changes in impedance/capacitance values as a result of the formation of aptamer–HER2 protein complex (0.2–2 ng mL^{-1} of HER2) on capacitor microelectrodes. The label-free capacitive aptasensing is a versatile and promising approach for early detection of cancer biomarkers in dilute human serum and the method potentially be extended to a wide variety of diseases biomarkers.

1. Introduction

Breast cancer is the most common cancer in women which is the second most common cause of cancer-related mortality [1]. HER2 has been shown overexpressed by ~20–30% in aggressive breast cancer cases [1]. The HER2 protein is a transmembrane tyrosine kinase receptor and a member of the epidermal growth factor receptors (EGFR or ErbB) family. The extracellular domain (ECD) of cleaved HER2 protein enters into the blood stream which serves as an indicator of increased expression of HER2. Increased level of HER2 in serum is associated with a high risk of cancer disease [1,2]. Recently, the clinical usefulness of serum HER2 levels determination is given more importance [3]. It is imperative to monitor HER2 levels because it is also overexpressed in other cancers including ovarian, lung, gastric, and oral cancers [4]. Breast cancer patients have elevated HER2 concentrations (15–75 ng mL^{-1}) in their blood compared to those observed for normal individuals (2–15 ng mL^{-1}) [5,6]. Currently, various HER2 detection techniques have been reported, including fluorescence in situ hybridization (FISH) assays for clinical testing of HER-2 gene amplification and immunohistochemical (IHC) assays for HER-2 overexpression [7]. However, these techniques are labor-intensive, time-consuming, require specially trained personnel and sophisticated instrumentations to carry out such procedures.

In the last decades, several classical biosensors were developed to detect the protein level that utilize recognition molecules often derived of living cells, such as enzymes, receptors, and antibodies have been reported [8–10]. The main disadvantage of using antibodies being their instability due to irreversible denaturation under external environmental perturbations. Therefore, alternative routes are ever demanding in order to develop stable biosensors with synthetic biorecognition elements. Novel synthetic molecules such as aptamers can fulfill these gaps associated with biomolecules derived of living cells. The aptamers are short, single stranded DNA or RNA oligonucleotides that can bind to their targets and offer specific properties, which favor them as new biorecognition elements for biosensing [11]. The different nature of these nucleic-acid recognition elements and their protein targets combined with the unique properties of aptamers has shown great potential for designing innovative sensing protocols including optical, electrochemical, and mass-sensitive approaches [12–15].

Recently, several electrochemical aptasensors based on Faradic approach have been proposed for the detection of cancer specific antigens such as cancer antigen CA15-3(MUC1) and Vascular Endothelial Growth Factor (VEGF) [16–18]. There are very few reports on electrochemical aptasensors based non-Faradaic

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approach for the detection of different protein diseases biomarkers [19] The Faradaic-based electrochemical aptasensors for the detection of HER2 have been proposed based on use of gold nanoparticle electrode [6,18] which heavily relied on active participation of electron transfer mediators in the medium for the analysis [20]. In some cases, diffusion of analyte through an unstirred layer limit greatly for its application in disease diagnosis.

Biosensing using label-free capacitive aptasensors may prove a unique platform for cancer biomarker detection with the advantages of label-free nature, no use of electron mediators, high sensitivity, ease of use and low cost testing potency. Here, aptamers play a critical role as ligands that can be functionalized on microelectrodes of capacitors for specific capturing of cancer biomarkers on sensor surfaces. Capacitance can then be measured by the amount of charge that a capacitor can hold at a given AC electrical frequency. For instance, a charged molecule such as ssDNA aptamer or its target protein biomarker when introduced at the capacitor interface, capacitance value changes because of the differences in the nature of each interacting molecule on capacitor. The affinity with which the aptamer binds to its target on the metal surface also has a noticeable effect on the capacitance value [21]. The observed change in the capacitive signal can thus be used to quantify any reaction that take pace between the ligands immobilized and target captured on the sensor surface.

In this study, we fabricated interdigitated gold microelectrode (IDE) based capacitor arrays that were functionalized with anti-HER2 aptamers (H2) specifically bind to HER2 protein and detected HER2 in human serum (HER2 aptasensor). The aptamer employed here was previously selected and well characterized in our laboratory [22]. The nFIS signal was used to directly probe and quantify the aptamer–HER2 interactions on sensor surfaces. The developed aptasensor exhibited high sensitivity toward HER2, providing an easy means of HER2 detection in human serum for early diagnosis of breast cancers.

2. Experimental

2.1. Reagents

Human serum (male; blood type, AB) was purchased from PAN^{TM} Biotech, GmbH and reconstituted by appropriate dilution in phosphate buffered saline (PBS, pH 7.4) prior to use. Here, AB-type serum was used because of it is compatibility with other blood types and therefore represent more than one blood types. β-Mercaptopropionic acid (MPA), *N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich, Germany. All other reagents used in this study were of analytical grade.

2.2. Capacitor sensor fabrication

Gold IDE array based capacitors were patterned on a 525 ± 25 μm thick SiO_2 wafers (p-type, 0–100 Ω-cm resistivity, ⟨1 0 0⟩ orientation) using standard photolithography. The wafer surface was cleaned in a series of steps using isopropanol, acetone and distilled water, respectively and dried using N_2 gas. Image reversal was carried out using AZ5214E photoresist after layering it on SiO_2 wafers and baked at 120 °C for 5 min. Following this step, a 50–60 nm thin tungsten layer was deposited to facilitate improved adhesion of gold and a 200–210 nm thick gold layer was then deposited using DC sputter deposition. The deposition was carried out in argon atmosphere with power of 150 W for 3 min. The unpatterned gold was lifted off using acetone and the dimension of each patterned electrode was measured to be 800 μm in length and 40 μm in width with a distance between two electrodes of 40 μm. Each wafer contained an array of 45 independent capacitors each made of 24 IDEs within a total area of 3 mm^2 that served as individual sensors.

2.3. Immobilization and optimization of anti-HER2 aptamer (H2) on capacitor sensor

The sensor chips were incubated with 100 mM of MPA in ethanol for 12 h at room temperature, washed with ethanol and dried using N_2 gas. Free carboxyl groups of MPA on sensors were activated by adding a mixture of 200 mM of EDC and 100 mM of NHS in distilled water and incubated for 3 h. The activated sensors were functionalized with NH_2-modified H2 which was previously selected and characterized in our laboratory [22]. The efficient binding of H2 enabled employing this aptamer for capacitor sensor. This aptamer had a following sequence; 5′-NH_2-$(CH_2)_6$-GGG CCG TCG AAC ACG AGC ATG GTG CGT GGA CCT AGG ATG ACC TGA GTA CTG TCC-3′. Different concentrations of H2 aptamer (1, 2 and 4 μM) in 5 μL volumes was first subjected to thermal treatment by denaturation at 90 °C for 10 min, quickly cooled at 4 °C for 5 min and annealed at 25 °C in an air-tight humid chamber before immobilization on MPA activated sensor surfaces. The unprotected groups on aptamer functionalized sensors were capped first by incubating with 50 mM ethanolamine in PBS, pH 8 for 15 min and washed thrice with PBS (pH 7.4), dried and stored at 4 °C until use.

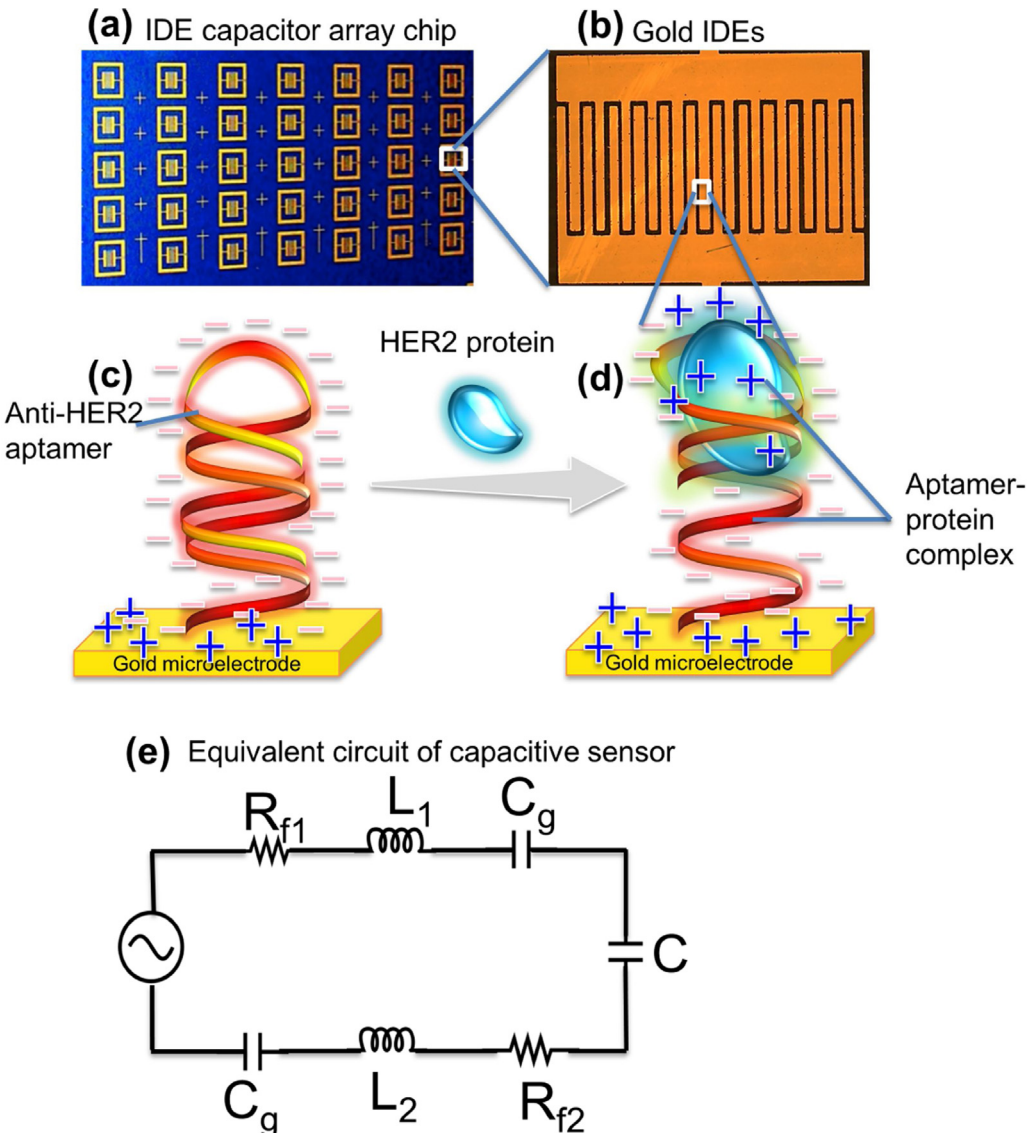
The sensors were subjected to thermal treatment as described above just before the start of the experiment and blocked the sensor surface by immersing chips in a solution of 5% BSA in PBS pH 7.4 for 1 h followed by thorough washing with PBS and finally dried. The optimized concentration of anti-HER2 aptamer immobilized on sensor surface was later used for the determination of HER2 protein in spiked human serum. The immobilization of anti-HER2 aptamer on capacitor chip was confirmed by Fourier transform infrared spectroscopy (FTIR, Nicolet^{TM} iS^{TM}10).

2.4. Spiking HER2 protein in dilute human serum

Human serum (male; blood type, AB; PAN^{TM} Biotech, GmbH) was filtered using 0.22 μm syringe-filter and the intrinsic HER2 from the filtered serum was removed by mixing the serum with anti-HER2 antibody functionalized magnetic beads (Invitrogen) followed by magnetic separation. HER2-free serum thus obtained was spiked with known concentration of pure and carrier-free HER2 protein (Cat. No. 1129-ER-050, R&D systems) with a molecular mass of ∼220 kDa in dimeric/non-reduced form. The spiked serum samples were appropriately diluted with phosphate buffered saline (PBS, pH 7.4) to obtain 10 times diluted human serum containing a series of HER2 concentrations ranging from 0.2 to 5 ng mL^{-1} corresponding to 0.9–22 pM, respectively and stored at −20 °C until use. The unspiked 10 times diluted HER2-free serum was used as blank control. Non-specificity tests were conducted using varying concentrations of Vascular Endothelial Growth Factor (VEGF-165, Cat. No. V7259, Sigma–Aldrich) and human Epidermal Growth Factor Receptor (EGFR, Cat. No. E2645, Sigma–Aldrich) spiked in 10 times diluted serum. These samples were incubated on aptasensors under identical conditions as described for HER2 protein.

2.5. Dielectric measurements

A series of HER2 protein concentrations ranging 0.2–5 ng mL^{-1} in 5 μL volumes of 10 times diluted human serum were incubated for 2 h on aptasensors at room temperature. The samples covered an area of 3 mm^2 IDEs on each sensor. Changes in dielectric parameters (impedance/capacitance) in between the gold interdigitated microelectrodes of capacitors before and after the incubation of



Scheme 1. (a) Photographic image of a real capacitor array chip, (b) microscopic image of a representative capacitor, (c) schematic illustration of a gold IDE functionalized with ssDNA (anti-HER2 aptamer) whose phosphodiester backbone contributes abundant negatively charged species on capacitors, (d) formation of aptamer–target protein (HER2) complex when HER2 interacted with ssDNAs that induce charge distribution and thus influence specific changes in capacitance that can be measured and (e) equivalent circuit of the interdigitated capacitor sensor.

samples on the sensor surfaces was measured against the applied frequency range from 50 to 350 MHz using a Network Analyzer (Karl Süss PM-5 RF Probe Station and Agilent-8720ES), which was pre-calibrated using SOLT (short-open-load-through) method. The impedance of capacitors was calculated which is defined by the following Eq. (1),

$$Z_C = \frac{1}{j\omega C} \tag{1}$$

where ω is the frequency and C is the capacitance. The impedance values were exported to Matlab® software for the normalization and analysis of the change in capacitance values at an effective frequency (f) range between 50 and 350 MHz. The results were normalized with respect to blank controls and the final response values of independent replicates ($n = 3$) were averaged, plotted and respective standard deviations were used errors in plots. Percent relative standard deviations (%RSD) was calculated from the replicate experiments that were calculated to be less than ~12%.

3. Results and discussion

In this study, we fabricated a capacitance based aptasensor for specific detection of HER2 protein in human serum. The specificity of the aptasensor was derived from the anti-HER2 aptamer that specifically recognizes HER2 protein from dilute human serum. The gold IDEs patterned as capacitor arrays on SiO_2 wafers served as transducing sensors onto which anti-HER2 aptamers were immobilized. Capturing of anti-HER2 aptamer with HER2 protein was determined by the specific changes in the capacitive signal. Use of anti-HER2 aptamers provided more resistance to thermal or physical degradation, unlike normally seen with antibodies. The inherent abundant negatively charges of ssDNA aptamer enabled sensitive capacitive signal generation was translated upon target-induced surface interactions into a measurable capacitive signal. This type of target-induced capturing altered the surface charges that constituted a "signal-on" type of sensor unlike other methods that required mediating chemical, competitive binding and target-induced displacements [18]. Therefore, we employed ssDNA aptamers that were previously trained in vitro to specifically bind

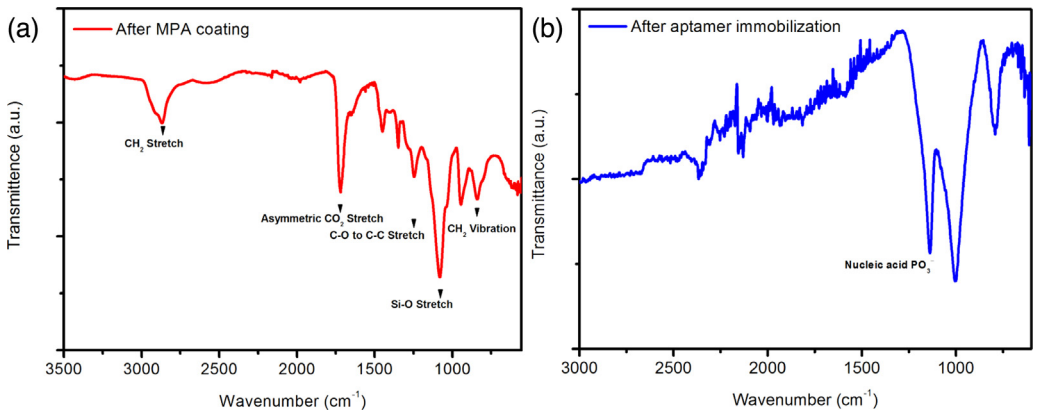


Fig. 1. Changes in FTIR spectra after capacitor chips subjected to; (a) self-assembled monolayer formation with MPA and (b) covalent coupling of anti-HER2 ssDNA aptamers.

HER2 protein for aptasensor development. A series of steps as illustrated in Scheme 1a–d were employed for sensitive label-free detection of HER2 protein in human serum and the details of results are presented in the following sections.

The equivalent circuit of the interdigitated capacitor used in this study is shown in Scheme 1e. In this circuit, R_{f1} and R_{f2} represent ohmic resistances in between the electrode fingers. Magnetic coupling between the fingers are represented by self-inductances, L_1 and L_2. The capacitance C represents the path between the insulated electrodes through the SiO_2 layer and Si substrate, which is also the fringing capacitance of the electrode into the air. The capacitance C_g is associated with the insulating layers on the electrodes. The aptamer probe immobilization and target binding on sensor surface influence change in the thickness of the surface film on sensor electrodes. This enables change in surface conductivity and capacitive responses of sensors.

3.1. FTIR analysis of aptasensor surface

The surface activation and immobilization of anti-HER2 aptamer on sensor surface was characterized by FTIR analysis. The FTIR spectra of MPA activated IDE capacitor chips showed CH_2 symmetric and asymmetric stretch modes at 2855 and 2950 cm^{-1} (Fig. 1a). The spectra also revealed an out of plane mode rocking vibration of CH_2 bond at 740 cm^{-1} and the stretch at 1150 cm^{-1} corresponding to anti-symmetrical coupling between C—O and C—C bonds. The presence of these characteristic stretching modes confirmed the SAM formation with MPA on capacitor chips. The FTIR spectra of NH_2-modified anti-HER2 aptamer immobilized on IDE capacitor chip is shown in Fig. 1b. The presence of characteristic stretching mode for nucleic acid phosphate (PO_2^-) at 1225 cm^{-1} and 1084 cm^{-1} indicated the successful immobilization of ssDNA aptamers on capacitor chips.

3.2. Optimization of anti-HER2 aptamer concentration on sensor surface

To optimize the concentration of anti-HER2 aptamer, three different concentrations, such as 1, 2 and 4 μM of anti-HER2 aptamer was immobilized on IDEs and capacitance responses of aptasensors were measured as a function of scanned AC electrical frequency ranging 50–300 MHz. The capacitance response was first observed for bare capacitor chips and compared the responses with those immobilized with different concentrations of anti-HER2 aptamers. It was found that blank capacitor surface was weakly charged, which then transformed it into a significantly enhanced charged surface after aptamer immobilization (Fig. 2). Distinct response of capacitor chip was observed with different concentrations of aptamer (1–4 μM) immobilized on capacitor sensor surface. The relative capacitance response tended to increase with different aptamer concentrations and the maximum change in capacitance was observed with chips immobilized with 2 μM concentration of anti-HER2 ssDNA aptamer (Fig. 2 and inset). There was no further increase in capacitance values with higher ssDNA concentrations (4 μM), indicating that the IDEs of a capacitor sensor surface area of 3 mm^2 saturated at 2 μM concentration of anti-HER2 ssDNA aptamer (Fig. 2 and inset). The observed reduction of capacitance signal after saturation can be attributed to the tight competition between the free and immobilized forms of aptamers on sensors, which probably have influenced the high gradient induced displacement of bound aptamer molecules. Based on the above result, 2 μM concentration of anti-HER2 ssDNAs was considered as the optimized concentration on a given area of each IDE for HER2 protein detection. Cumulative negative charges resulting from 2 μM of anti-HER2 aptamer on an area of 3 mm^2 appeared to be maximum capacity of each fabricated capacitor.

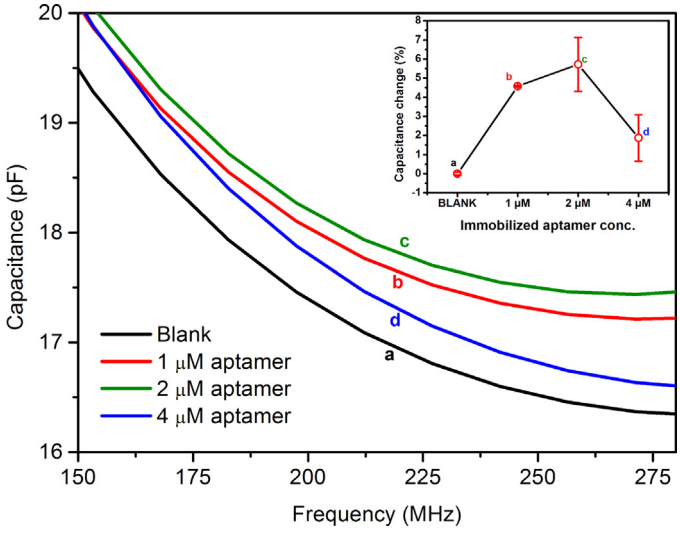


Fig. 2. Capacitance change occurred as a function of applied AC frequency with (a) bare IDEs of capacitor sensor and IDEs immobilized with (b) 1 μM, (c) 2 μM and (d) 4 μM anti-HER2 aptamer The line plots shown in the figure were plotted using the average values of independent replicates ($n = 3$), respectively. The inset figure shows relative percent change in capacitance responses occurred with different concentrations of anti-HER2 aptamers at a specific frequency (242 MHz). The colored alphabets matching the colored lines represent the concentration of aptamer used to immobilize on IDEs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

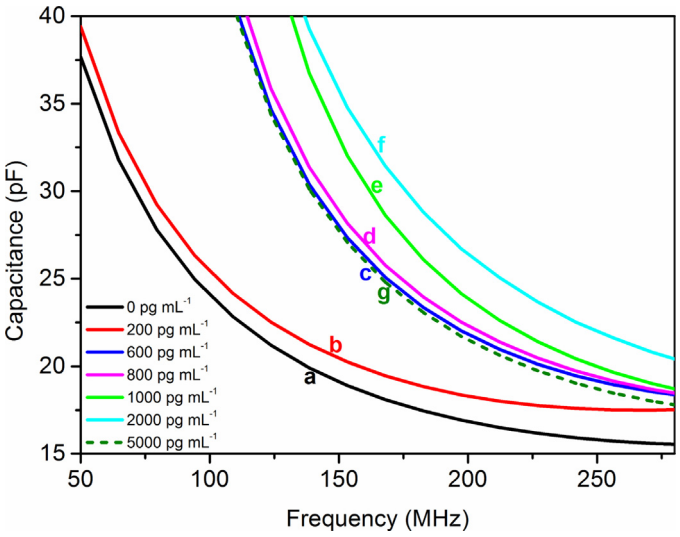


Fig. 3. Capacitance response profiles showing HER2 protein dependent changes against frequency sweep from 50 to 300 MHz. The data lines are derived from average capacitance values of independent replicates ($n = 3$) at different frequencies for varying concentrations of HER2 protein. The colored alphabets (a–g) represent HER2 protein concentrations indicated in the legend matching to their respective line colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

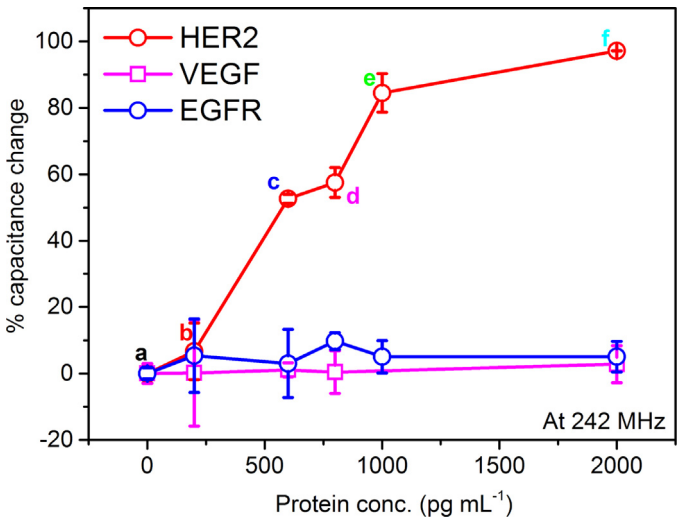


Fig. 4. Percent change in capacitance responses against increasing concentration of target HER2 protein at a constant 242 MHz frequency. The sensor responses were also compared with non-specific VEGF and EGFR proteins under identical conditions showing no significant change in the signal. Capacitance responses with different concentrations of HER2 protein (a–f) are color coded to match the colored plots shown in Fig. 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. HER2 protein detection using capacitive aptasensor

Anti-HER2 ssDNA aptamer activated sensors (aptasensor) were employed for detection of HER2 protein in spiked human serum. A series of HER2 protein concentrations ranging 0.2–5 ng mL^{-1} were prepared in 10-times diluted human serum were incubated on aptasensors for 2 h as described in Section 2. The capacitive responses of aptasensor chips were measured as a function of applied AC frequency and responses are shown in Fig. 3. Change in capacitance signal with the aptasensor began with a minimum of 200 pg mL^{-1} of HER2 protein in 10 times diluted human serum. The level of capacitance was dose-dependent with HER2 concentration from 200 pg mL^{-1} to 2 ng mL^{-1} and the signal was dramatically lost with higher concentration of 5 ng mL^{-1} of HER 2 protein (Fig. 3). It was observed that the response of aptasensor with different concentrations of HER2 protein was also dependent on applied frequency. However, we observed change in capacitance was more stable at higher frequency above 240 MHz (Fig. 3). Selecting an appropriate applied frequency for extrapolating the effective capacitance change with respect to the target concentration was critical. In this study for HER2 protein, it was found that 242 MHz yielded a better resolution of the capacitive signal under standard conditions described in experimental methods. A linear increase in relative capacitance change was obtained with dynamic concentrations from 200 pg mL^{-1} to 2 ng mL^{-1} of HER2 protein at around 242 MHz (Figs. 3 and 4).

The aptasensor had a limitation in sensitivity from 0.2 to 2 ng mL^{-1} HER2 protein probably due to the limited availability of HER2 protein molecules on a given sensor area (3 mm^2). At highest concentration (5 ng mL^{-1} HER2 protein), the sensor signal dramatically declined. We speculate that this could be because of the strict competition between the abundant or high gradient target molecules (HER2 protein) and limited local aptamer binding sites. This type of competition likely to alter the local/proximal physicochemical environment and therefore displacement or dissociation of previously captured target molecule may have occurred. However, the detection range can also be fine-tuned by optimizing the sensor area and geometry of the electrodes for obtaining a desired detection range.

The underlying mechanism by which the capacitance changes with aptasensor occurred was postulated to occur due to the charges present on aptamers and/or HER2 protein at a specific frequency (242 MHz). The capacitance response was generated as a result of change in total surface charges on capacitor sensor surface [23]. Capacitor IDEs functionalized with anti-HER2 ssDNA aptamers transformed them into abundant negatively charged surface because of the phosphodiester backbone of ssDNAs and thus reduced surface capacitance. The charges distributed on the outer surface of HER2 protein combined with negatively charged backbone of H2 ssDNAs probably have contributed to the enhanced surface capacitance. The capacitance of typical two parallel electrodes of area 'A' (3 mm^2), separated by a distance 'd' is given by $C = A\varepsilon\varepsilon_0/d$, where '$\varepsilon$' is the relative static permittivity (dielectric constant) of the material between the plates and 'ε_0' is the permittivity of free space. The main factors that affected the capacitance are the distance between the electrodes and the area of the electrodes. Capacitive responses of the presented sensors are derived from the aptamer–protein interactions. Such interactions may induce change in conductivity of the insulating layers on the electrodes, charge redistribution and recombination within these layers. Capacitance changes over low frequency are related to double-layer effects on sensor surface while changes at high frequencies could be derived from the adsorption of molecules on electrode surface [24]. Therefore, capacitance responses over low or high frequency can be attributed to the formation of a double-layer or adsorption of captured target protein molecules, respectively by the aptamers on the electrode surfaces. The difference in dielectric constant calculated using capacitance equation at 241 MHz with and without protein capturing on sensors was in the range ~30–40. This result indicated the formation of organic molecule layers on the sensor surface. However, this is an indirect method to calculate the dielectric constant of the immobilized molecules on the sensor surfaces. Since, the large overall dielectric constant is almost entirely due to the charged protein side chains, located at the protein surface that have flexibility [25].

Use of multiple capacitor system has an advantage of increased capacitance value which would ultimately help in sensing any difference in capacitance value with more accuracy [26]. Therefore, immobilization of anti-HER2 ssDNA aptamer and formation of anti-HER2 ssDNA aptamer and HER2 protein complexes eventually contributed to the charge distribution on capacitor chips. Any molecular interactions on the surface, such as by increasing the HER2-protein concentration directly affected the charge distribution on the capacitor surface. Therefore, effective concentration dependent change in capacitance was occurred that appears to be governed by the maximum target concentration gradient (here 2 ng mL^{-1}) and the number of binding sites (2 μM immobilized aptamer concentration) on sensors. The HER2, EGFR and VEGF proteins currently represent the validated targets for cancer therapy and that HER2 and EGFR belong to the same family of human epidermal growth factor receptors [27]. Therefore, it is imperative to test the specificity of the aptasensor with VEGF and EGFR as non-specific proteins that were also spiked in dilute serum under identical conditions. Our result revealed that the aptasensor did not exhibit significant change in capacitance signal against varying concentrations of either EGFR or VEGF proteins suggesting that the aptasensor was specific to HER2 protein (Figs. 3 and 4).

4. Conclusions

This study reports on a simple and sensitive approach for detecting HER2 protein which is based on label-free capacitive aptasensor platform. The aptasensor utilizes anti-HER2 aptamers (ssDNAs) to capture serum HER2 protein and the specific change in capacitance response corresponded to the target concentration at an applied frequency of 242 MHz. The change in capacitance signal was based on several critical factors, such as (a) changes in the charge distribution upon binding of target to aptamer probe, (b) concentration of the target protein, (c) applied AC electrical frequency and (d) geometry and design of microelectrode structures of capacitor. The developed aptasensor was successfully utilized for a label-free capacitive biosensing of HER2 protein that showed a dynamic range between 0.2 and 2 ng mL^{-1} without requiring any additional reagent or redox-chemical. The developed aptasensor for detecting HER2 offers speed, simplicity and less-expensiveness because it required no labeling of biomolecules, unlike in other conventional methods, such fluorescence in situ hybridization (FISH) or enzyme linked immunosorbant assays (ELISA) that also relies on pathologists or trained laboratory personnel. This study also brings some of the key improvements to different transduction techniques applied previously for the analysis of HER2 in terms of detection limit and for the use of real serum samples [28–30]. However, the performance of the developed aptasensor platform can be further improved by fine-tuning the above factors (a–d) through fabrication of nanostructured capacitors and minimizing the batch-to-batch variations as well as the assay conditions. The developed bio sensing approach can provide a convenient and sensitive means to recognize target molecules in its native forms that is most desirable in clinical diagnosis.

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