VEGF Cancer Biomarker Protein Detection in Real Human Serum Using Capacitive Label-Free Aptasensor

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Summary: Vascular Endothelial Growth Factor (VEGF) is a signalling protein known as a serum biomarker for a number of diseases including cancer. Therefore, it is necessary to develop a label-free, rapid and sensitive method for detecting VEGF in its native forms in human serum. In this work, we have demonstrated the proof-of-concept of a sensitive label-free biosensor based on capacitance changes induced by capturing of VEGF in human serum using anti-VEGF aptamers functionalized on interdigitated (IDE) capacitor arrays (aptasensors). Limit of detection of VEGF protein using this aptasensor was in the range 0.5–2 ng/mL in dilute human serum. The label-free capacitive aptasensing strategy provides a simple, reliable and effective means of biosensing serum VEGF levels for early cancer diagnosis.

Keywords: aptamer; biosensor; cancer; dielectric properties; protein

Introduction

Vascular endothelial growth factor (VEGF) is a signaling protein, also used as a serum biomarker for a number of human diseases, including cancer.[1] VEGF is an angiogenesis regulatory protein, which is implicated in tumour growth and invasion that represent a desirable therapeutic and diagnostic target. Cancer progression occurs when the balance in angiogenesis stimulators and inhibitors is disturbed, and it is understandable that the fluctuation in the levels of circulating angiogenesis regulatory proteins, such as VEGF has been evaluated as likely biomarker candidates in biological fluids (plasma, serum, and urine).[2,3] Thus, rapid, selective and sensitive detection of VEGF directly in the whole blood or serum is particularly important for disease diagnosis and subsequent therapy monitoring.[4]

Over the past few decades, several biosensors were developed to detect protein levels based on antibodies as a biorecognition element. Alternative emergence of a new class of synthetic biorecognition also known as aptamers can be employed for both therapeutic and biosensing applications.[5,6] Aptamers are single-stranded DNA, RNA or modified nucleic acids that have been designed through an in vitro selection process known as SELEX (systematic evolution of ligands by exponential enrichment), in which specific oligonucleotides are isolated from complex libraries of synthetic nucleic acids by repetitive binding of the oligonucleotides to target molecules.[5,6] Aptamers offer several advantages over antibodies, including the ease of synthesis, easy labeling and good stability. The unique properties of aptamers enable designing innovative sensing protocols through interfacing them with electrochemical, optical or mass-sensitive transducing approaches.[7–10] Recently, several aptamer-based biosensors (aptasensors or aptamer beacons)[11] have been used for probing...
binding-induced conformational changes in aptamers and monitor their interaction with targets by means of color changes\cite{12,13} or electron transfer.\cite{10,14} However, these methods are limited by sensor size, sensitivity or complexity of assays. For example, colorimetric or fluorescence quenching methods require collecting a large amount of particles to induce a discernable color change or fluorescent signals.

In this study, we developed an interdigitate electrode (IDE) based capacitor aptasensor for detection of VEGF in real human serum. An anti-VEGF ssDNA aptamer was employed as biorecognition element on capacitive transducer to develop a label-free aptasensor. The sensitive signal generated upon binding of aptamer specific to VEGF protein on sensor surface was measured using non-Faradaic electrochemical impedance spectroscopy (nFIS). This approach allows potential application of the developed sensor in real-time VEGF detection for cancer diagnosis and monitoring.

**Experimental Section**

**Reagents**

Human serum (male; blood type, AB) was purchased from PAN\textsuperscript{TM} Biotech, GmbH and reconstituted by appropriate dilution in phosphate buffered saline (PBS, pH 7.4) prior to use. 3-Mercaptopropionic acid (MPA), N-(3-dimethylaminopropyl)-N-ethyl carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), human VEGF165 and monoclonal anti-VEGF antibody were purchased from Sigma-Aldrich, Germany. Surface activated MBs (Dynabeads M-270 Carboxlic Acid) were purchased from Life Technologies Co., USA. All other reagents used in this study were of analytical grade. An anti-VEGF aptamer was developed in our laboratory using SELEX technology that had a following sequence 5’-GGG CCG TTC GAA CAC GAG CAT GGT GGG TGG TGG CCC TAG GAT GAC CTG AGT ACT GTC C-3’ (ssDNA) was employed as biorecognition element for the development of aptasensor.

**Fabrication of Capacitor Arrays**

Gold interdigitated electrode based capacitor sensor arrays were patterned on SiO\textsubscript{2} surface using image reversal technique. In this process, the metal layers were patterned using the dual tone photoresist AZ5214E. A 2 µm thick AZ5214E photoresist was patterned with the help of a mask for a lift-off process in pure acetone as a solvent. Following this step, a very thin tungsten layer of 50–60 nm size was layered to improve the adhesion of gold on the SiO\textsubscript{2} film by DC sputter deposition and about 200–210 nm thick gold layer was deposited. The dimension of each electrode was 800 µm in length, 40 µm in width with a distance between two electrodes of 40 µm. Each capacitor sensor contained 24-interdigitated gold electrodes within a total area of 3-mm\textsuperscript{2}.

**Immobilization of Anti-VEGF Aptamer on IDEs of Capacitor Chips (Aptasensors)**

The IDE capacitor chips were first immersed in a solution of 100 mM of β-mercapto propionic acid (MPA) in ethanol for 12 h at room temperature. Self-assembled monolayer (SAM) of MPA containing free carboxyl groups 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 sensor chip made of arrays each capacitors with an area of 3-mm\textsuperscript{2} were incubated with 2 µM of 5’-NH\textsubscript{2}(CH\textsubscript{2})\textsubscript{6}-modified anti-VEGF aptamer (ssDNAs) in 5 µL volumes for 3 h and washed with PBS, pH 7.4 and pre-incubated at 37°C for 1 h in an air-tight humid chamber.

**Capacitive Electrochemical Detection of VEGF Protein in Dilute Human Serum**

The aptamer immobilized sensors were subjected to pre-treatment for unfolding the binding site and this was carried out by placing the chips in a pre-heated airtight moist chamber at 90°C for 10 min, quickly cooled at 4°C and finally incubated at
25°C for 10 min. This facilitated stable ssDNA confirmation which was required for its binding to VEGF protein. A series of VEGF protein concentrations in 5 µL 0.1X human serum ranging 5 pg/mL to 5 ng/mL were incubated on aptasensors, each covering an area of 3 mm² IDEs and incubated for 2 h at room temperature. For negative controls, bovine serum albumin (BSA) at different concentrations were spiked in human serum and used as negative controls under standard assay conditions.

**Dielectric Measurements**
Changes in dielectric parameters (impedance/capacitance) in between the gold interdigitated microelectrodes of capacitors before and after incubation of samples on the sensor surfaces was measured in the frequency range 50 MHz to 300 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.

**Results and Discussion**
The capacitive response signal with the aptasensor was gradually increased with minimum 5 pg/mL VEGF protein concentrations in human serum (Figure 1a). The concentration response of the sensor was observed up to its saturation at maximum of 2 ng/mL. The sensor response trend was dramatically lost at further higher VEGF concentration (5 ng/mL) (Figure 1a). It was observed that the response of aptasensor chip with different concentrations of VEGF protein was also dependent on applied frequency. However, the capacitive signal with the aptasensor was less-dependent on concentration of VEGF protein at frequency >250 MHz (Figure 1a). Low concentrations of VEGF protein showed relatively small capacitance change at lower frequencies. Therefore, selecting an appropriate applied frequency for extrapolating the effective capacitance change with respect to the target concentration was critical. It was found that 212 MHz yielded a better resolution of the capacitive signal under standard conditions described in experimental methods, which was also concentration-dependent with VEGF protein incubated on the chips (Figure 1b). A linear increase in relative capacitance change was noticed with dynamic concentrations from 5 pg/mL to 2 ng/mL of VEGF protein (Figure 2a). Non-specific protein BSA (negative control) however, as expected failed to show any response and therefore it was confirmed that no non-specific binding occurred on the sensor surface (Figure 2b).

The underlying mechanism by which the capacitance changes occurred with
aptasensor was postulated to occur due to the charges present on aptamers and/or VEGF protein. The capacitance response was generated as a result of change in total surface charges on IDEs. Capacitor IDEs immobilized with VEGF aptamer transform 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 VEGF protein combined with negatively charged backbone of ssDNAs (aptamer) probably contributed to the enhanced surface capacitance. The charges on the surface, such as by increasing the VEGF-protein concentration directly affect the charge distribution on the capacitor surface, enabling effective concentration dependent change in capacitance. However, it was found that the values of capacitance changes for the tested biomarker proteins varied probably because of the size and charge on their surface combined with area and geometry of gold interdigitated electrodes that greatly influenced the dielectric properties. The sensor response with a protein biomarker under the applied frequency is strongly dependent on the nature of protein as well as the geometry of metal electrodes. Here, the developed aptasensor showed detection of a broad dynamic detection range (0.5–2 pg/mL), however with a low relative change in signal. Therefore, we are carrying out the extension of this work to conduct new assay design to have better sensitivity toward detection of VEGF protein using aptasensor chip, which is through signal enhancement strategy.

**Conclusion**

In this study, a non-faradiac electrochemical capacitive aptasensor was developed for rapid detection of VEGF cancer biomarker in human serum. The change in capacitive signal was brought by target-induced binding in conformational change of the aptamer probe through change in charge distribution. The developed aptasensor was successfully utilized for a label-free capacitive biosensing of VEGF protein that showed a dynamic detection range of 0.5-2 ng/mL. This method did not require any additional chemical reagents enabling detection of VEGF protein in its native forms in human serum which is most desired in clinical diagnosis.

**Acknowledgements:** This work was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) 1001 grant no.
110E287. We thank Divya Mahalingam for her assistance in chip fabrication.