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An aptamer based competition assay for protein detection using CNT activated gold-interdigitated capacitor arrays

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ABSTRACT

An aptamer can specifically bind to its target molecule, or hybridize with its complementary strand. A target bound aptamer complex has difficulty to hybridize with its complementary strand. It is possible to determine the concentration of target based on affinity separation system for the protein detection. Here, we exploited this property using C-reactive protein (CRP) specific RNA aptamers as probes that were immobilized by physical adsorption on carbon nanotubes (CNTs) activated gold interdigitated electrodes of capacitors. The selective binding ability of RNA aptamer with its target molecule was determined by change in capacitance after allowing competitive binding with CRP and complementary RNA (cRNA) strands in pure form and co-mixtures (CRP:cRNA = 0:1, 1:0, 1:1, 1:2 and 2:1). The sensor showed significant capacitance change with pure forms of CRP/cRNA while responses reduced considerably in presence of CRP:cRNA in co-mixtures (1:1 and 1:2) because of the binding competition. At a critical CRP:cRNA ratio of 2:1, the capacitance response was dramatically lost because of the dissociation of adsorbed aptamers from the sensor surface to bind when excess CRP. Binding assays showed that the immobilized aptamers had strong affinity for cRNA (K_d = 1.98 μM) and CRP molecules (K_d = 2.4 μM) in pure forms, but low affinity for CRP:cRNA ratio of 2:1 (K_d = 8.58 μM). The dynamic detection range for CRP was determined to be 1–8 μM (0.58–4.6 μg/capacitor). The approach described in this study is a sensitive label-free method to detect proteins based on affinity separation of target molecules that can potentially be used for probing molecular interactions.

1. Introduction

Identification and characterization of molecular interactions is important in designing novel assays for biosensing. A number of methodologies have been established in recent years to probe molecular interactions (Berezovski and Sergey 2003; Foulds and Etzkorn, 1998; Pagano et al., 2011; Royer and Scarlata, 2008; Ryder et al., 2008; Wan and Le, 2000; Wong and Lohman, 1993). These interactions occur at the molecular interfaces and they are mainly electrostatic in nature. Various methods were employed to monitor formation of protein/nucleic acid complexes. These include the optical method, such as Surface Plasmon Resonance methods, fluorescent approaches, and others such as, electrophoretic mobility shift assays, affinity capillary electrophoresis and nitrocellulose-filter binding assays (Berezovski and Sergey, 2003; Foulds and Etzkorn, 1998; Karlsson, 2004; Pagano et al., 2011; Ryder et al., 2008; Singhal and Otim, 2000; Wan and Le, 2000). In addition, label-free methods, such as non-Faradaic type can also be employed by immobilizing biological receptors on the surface of a suitable electronic transducer, which converts the molecular interaction signal into a quantifiable electronic signal.

Until now, the different nature of nucleic acid recognition elements and their protein targets indicated great promise for designing innovative sensing protocols (Strehlitz et al., 2008). Synthetic oligonucleotides, called aptamers, have attracted much of attention because of their binding abilities similar to antibodies, as well as their relative ease of isolation, modification, tailored binding affinity, chemical synthesis and resistance against denaturation. These are short, single stranded DNA or RNA oligonucleotides that can bind to their targets and offer specific properties, which favor them for developing protein arrays, drug delivery and as new biorecognition elements for biosensing of disease-related proteins (Bagalkot et al., 2006; Hansen et al., 2006; Mukhopadhyay, 2005; Strehlitz et al., 2008). Aptamers are typically generated by an iterative screening process of complex synthetic nucleic acid libraries against specific target molecules, making them more specific to target molecules. The targets can be ions, small organic molecules, proteins or even whole cells. The molecular interactions derived from their interaction with target molecules generally follow natural rules of biomolecular interactions.

Most of the aptamer based assays were designed to detect specific proteins as disease markers and rely on standard sandwich

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type bio-affinity assays in connection to common enzyme (Xu et al., 2009), fluorophore (Wang et al., 2008) or nanoparticle tracers (Chiu and Huang, 2009). Until now, electrochemical aptamer-based biosensors, such as redox-mediating faradaic type or label-free direct measurement of charge distribution/capacitance by non-faradaic type have been reported on different platforms to detect specific disease biomarkers and cells using aptamers (Lei et al., 2009; Sassolas et al., 2009; Velasco-Garcia and Missailidis, 2009; Xu et al., 2009) or by using antibodies (de Vasconcelos et al., 2009; Laczka et al., 2008; Varshney et al., 2007).

CRP is one of the acute-phase plasma proteins under cardiovascular disease (CVD) conditions and serves as a target for early diagnosis and prevention of CVD. CRP has been regarded as low risk at a concentration below 1.0 mg/l, moderate for 1.0–3.0 mg/l, and high risk for concentrations over 3.0 mg/l (Pearson et al., 2003). It can rise as high as 1000-fold because of inflammation induced by infection/injury that often lead to CVD (Casas et al., 2008). Recently, a label-free capacitive biosensor for CRP detection has been reported from our laboratory that utilized antibodies (Quershi et al., 2009), which are prone to lose their activity over time and the advantages in relation to previous works is described in Supporting Information (SI) section 1. Therefore, synthetic aptamers made of ssDNA/RNA were employed as affinity ligands on capacitor arrays that are stable and specifically bind CRP. Studies pertinent to the binding affinity interactions of aptamers with target molecules by direct charge distribution are scarce. Therefore, here, a competitive assay was designed by utilizing RNA aptamers immobilized on capacitors and competitively assayed with different molar ratios of CRP (as a model protein) and cRNA. This study demonstrates that it is possible to determine the target concentration by affinity separation, which is facilitated by bound/free aptamer in presence of its complementary strand and the target by capacitive responses. The interaction events were captured based on charge distribution under the applied frequency using capacitors made of two electrodes for ground and signal in the absence of redox mediators. This method is effective for probing biomolecular binding events such as ligand–target interactions. The measuring principle of these sensors is based on simple changes in dielectric properties or charge distribution and conductivity when a ligand–target complex formed on the surface of an electrode without redox mediators (Gautier et al., 2007; Quershi et al., 2009; Tlili et al., 2005). The determinants of quantitative parameters of binding specificity, affinity and stoichiometry were measured.

2. Materials and methods

2.1. Materials and chemicals

Silicon wafers of 4″ size, <1 0 0> oriented, p-type with the resistivity of 9–12 Ω cm and thicknesses of 500 ± 25 μm and 1 μm thick SiO_2 layer on top were obtained from University Wafers, USA. Carboxy-functionlized multiwalled CNTs (carboxy-CNTs) were obtained from Arry®, Germany. Cysteamine, dimethyl sulfoxide (DMSO, 99.9%), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich, Germany. All RNA oligonucleotides were custom synthesized by Bioresearch Tech. Inc., USA, and a random ssDNA oligonucleotide was purchased from SynGen, Inc., USA.

2.2. Patterning gold interdigitated electrode (GID) array for fabrication of capacitor arrays

GID arrays were patterned on SiO_2 surface by image reversal photolithography. 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, the SiO_2 film was covered by a layer of 50–60 nm of tungsten (W) to improve the adhesion of gold, and then, a 200–210 nm layer of gold was deposited on W by DC sputtering method. The dimension of each electrode was 800 μm in length, 40 μm in width with a distance between two electrodes of 40 μm. Each sensor contained 24-interdigitated electrodes with a total area of 3 mm^2.

2.3. Immobilization of CNTs and RNA on sensor arrays

Steps involved in CNTs and RNA immobilization are schematically shown in Scheme S1 of the SI section 2. The sensor chip was first plasma cleaned and immersed in 1 mM cysteamine (prepared in 95% ethanol) for 24 h and was washed with ethanol and dried using N_2 gas. The self-assembled monolayer (SAM) of cysteamine formed on gold surface contained free NH_2 groups that were utilized for covalently attachment of carboxy-CNTs. For this, 100 μl of 1 mg/ml carboxy-CNTs suspension in DMSO was mixed with equal volume of 1:1 mixture of 200 mM of EDC and 100 mM NHS. The suspension was sonicated with alternative cycles of 10 s pulse with an interval of 10 s for total 5 min using an ultrasonicator probe (Vibra cell 75043). The suspension was then incubated for 4 h at room temperature. About 5 μl of this suspension was dropped on each sensor covering an area of 3 mm^2 that was previously activated with cysteamine SAM. The sensors were then incubated in an airtight humid chamber for 10 h for covalent attachment of carboxy-CNTs and rinsed with sterile distilled water. The free carboxyl groups on sensors were blocked by adding 5 μl of 50 mM ethanolamine and finally washed with 50% DMSO in water followed by acetone to remove traces of unbound CNTs and dried over N_2 gas. A sensor array without CNT immobilization was used as a control for comparison.

A modified 44-mer RNA aptamer that specifically bind CRP (Bini et al., 2008) was custom synthesized after modifying with alkane thiol-linker at the 5′ end. The ability of modified aptamer to bind CRP has been recently reported (Qureshi et al., 2010). The resulting modified RNA aptamer had the following sequence; 5′-HS-(CH2)6-GCCUGUAAGGUGGUCGGUGUGGCGAGUGUGUUAGGAGAGAUUGC-3′ and its complementary RNA strand (cRNA) used had the following sequence; 5′-GCAAUCUCUCCUAACACACUCGCCACA-CCGACCACCUUACAGGC-3′.

In order to determine the influence of the coverage on the overall response of the aptamers on the sensor surface, we initially conducted a preliminary test experiment with thiol-modified RNA aptamers directly immobilized on GID region of the sensor arrays by self-assembled monolayer (SAM) formation (Qureshi et al., 2010). For this, different concentrations of thiol-modified RNA aptamers (2 μl of 4, 6, and 10 μM) were incubating in phosphate buffered saline (PBS), pH 7.2 for 2 h at 25 °C, under sterile conditions. The immobilized RNA aptamer concentration (10 μM) that gave a significant change in dielectric responses (impedance/capacitance) was subsequently used to immobilize on all CNT activated sensor arrays in this study. The CNT activated GID regions of sensor arrays were immobilized with 10 μM modified RNA aptamer by physical adsorption in PBS for 2 h at 25 °C under sterile conditions (see SI section). After the adsorption of RNA aptamers, the sensors were thoroughly washed thrice with sterile distilled water and dried using N_2 gas and stored at 4 °C until use.

2.4. Surface characterization

The surface characterization was performed using Atomic Force Microscopy (AFM, Nanoscope) with the tapping mode. The surface topology of sensor surface was carried out with bare GID