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Direct detection of carcinoembryonic antigen autoantibodies in clinical human serum samples using a surface plasmon resonance sensor

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ABSTRACT

The ability to detect biomarkers in human serum is important for cancer diagnostics. The work presented focuses on the establishment of a surface plasmon resonance (SPR) biosensor as a means for detecting varying levels of autoantibody biomarkers in human serum samples. Carcinoembryonic antigen (CEA) is a biomarker that is present in human serum. It is thought that CEA levels become elevated in patients with colon and ovarian cancer, causing a corresponding increase in the autoantibody level in human serum. Detection of this CEA autoantibody increase could be used to diagnose cancer in patients. Using a SPR biosensor, human serum samples were screened directly for CEA antibody levels. Results using a sandwich assay with a SPR sensor demonstrated the same linear trend as seen from an established enzyme-linked immunosorbent assay (ELISA) method. Serum samples from five healthy individuals were used to establish a threshold value for differentiating a cancerous serum sample from a negative sample with a 95% confidence. Three serum samples from cancer patients with positive CEA antibody levels as evaluated by ELISA were used to test the criterion.

1. Introduction

Surface plasmon resonance (SPR) is a technique that allows for real-time, label-free, and quantitative detections of interactions between biomolecules. While a label-free, direct detection of analytes is possible using a SPR sensor, sandwich assays, similar to those used in enzyme-linked immunosorbent assay (ELISA), involving secondary antibodies for either verification or amplification of the sensor signal can also be performed. Recently, SPR sensors have been applied to clinical and medical diagnostics [1–3], with research focusing on assay development for antigen-specific detections in buffer. Few studies have been performed on clinically relevant diagnostics, where assays are performed in human fluids, such as urine, serum, etc. Detection of autoantibodies using a SPR sensor in clinically relevant matrices has not been previously reported.

Early cancer diagnosis is essential to effective treatment and monitoring of the disease. When cancer is diagnosed in its earliest stages, treatment for the disease is much more effective [4,5]. As the cancer progresses, treatment methods change and their efficacies decrease. Most cancers present a number of aberrant proteins or increased production of other proteins. These proteins and their *in vivo* concentrations can vary depending on the cancer progression and type [6,7]. Early diagnosis of cancer is difficult due to the low initial concentrations of these aberrant marker proteins in blood samples. One developing technique to aid in the early diagnosis of cancer focuses on the detection of an immune response to tumor antigens [8–12]. The detection of serum autoantibody responses to tumor antigens may be a more reliable method than detection of the antigen itself. Serum antibodies are more stable than the antigens [13,14] and may be more abundant and readily detectable, especially at early cancer stages [15] Recent studies have shown that serum antibodies to the HER-2/*neu* antigen in breast cancer patients were present at early stages of the disease [16,17].

Carcinoembryonic antigen (CEA) is a tumor-associated antigen that has been identified as a biomarker for colorectal, gastric and pancreatic cancer [18]. CEA is an oncofetal protein that is expressed in normal mucosal cells [19] and over-expressed in adenocarcinoma. Elevated levels of CEA, and corresponding elevated levels of autoantibody to CEA, can be used as a diagnostic criterion for early cancer detection, as well as diagnosis of disease progression. An elevated CEA level is found in fewer than 25% of patients with disease confined to the colon. Advancing tumor stage shows increased sensitivities up to 75% of the patients with distant metastasis [20].

In this work, we demonstrate an assay for differentiating anti-CEA levels in clinical ovarian and colorectal serum samples using a SPR sensor. The samples from cancer patients have been pre-

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viously tested by ELISA and are known to have varying amounts of CEA autoantibody. Direct detections of CEA autoantibody and secondary antibody detections from the SPR sensor were compared to ELISA results. Using SPR, samples from five healthy individuals were used to establish a criterion for direct detection of autoantibody levels by which other samples could be judged with 95% confidence. This criterion was then applied to three known CEA positive serum samples. Responses showed anti-CEA levels above the criterion value for all positive samples tested.

2. Materials and methods

2.1. Reagents

Oligo (ethylene glycol) (OEG) alkanethiol ($HS–(CH_2)_{10}–(OCH_2)_4–OH$) and COOH-terminated oligo (ethylene glycol) (COOH–OEG) alkanethiol ($HS–(CH_2)_{10}–(OCH_2)_6–COOH$) were purchased from Prochimia (Gdansk, Poland). Bovine serum albumin (BSA), phosphate-buffered saline (PBS) (0.01 M phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.4), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), trifluoroacetic acid, ammonium hydroxide (NH_4OH), Trizma–HCl (Tris), pH 7.0, sodium chloride (NaCl) and polyoxyethylenesorbitan monolaurate (Tween-20) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$) and hydrochloric acid (HCl) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Glass coverslips (25 mm × 25 mm) were purchased from VWR (West Chester, PA, USA). CEA was obtained from Protein Sciences, Corp. (Meriden, CT, USA). Clinical serum samples from five healthy individuals and four patients with colorectal or ovarian cancer were obtained from the Tumor Vaccine Group at the University of Washington (Seattle, WA, USA). TMB development reagent for ELISA was obtained from Kirkegaard and Perry Laboratories (Gaithersberg, MD, USA). CEA and polyclonal goat anti-CEA were purchased from BioDesign International (Saco, ME, USA) and used for surface verification. Goat anti-human IgG-HRP Conjugate (γ chain) used for secondary antibody binding was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Surface plasmon resonance sensor

In this study we use a SPR biosensor developed at the Institute of Radio Engineering and Electronics (Prague, Czech Republic). The biosensor is based on wavelength modulation and the Kretschmann geometry of the attenuated total reflection method, and has been previously been described in detail [21,22]. Briefly, a collimated, polychromatic light beam passes through an optical prism and contacts a thin metal layer at a defined angle of incidence. The reflected light from the metal surface is then collected by four independent spectrophotometers, thus creating an instrument with four independently measured channels. The refractive index close to the metal surface creates a characteristic dip in the detected light spectrum at a specific wavelength. Changes in refractive index cause this characteristic dip to shift. By tracking these changes in dip position, binding events occurring on the surface can be quantified. Changes could result from a bulk refractive index change, a temperature change or a binding event occurring on the sensor surface. Temperature stabilization is used to maintain a constant temperature during the experiments, thus eliminating effects from temperature changes. Because the data of interest was the net shift in SPR wavelength, bulk refractive index changes were not important. Washing the sensor surface with the same buffer solution both before and after exposure to the sample of interest eliminates the bulk effect from this data.

2.3. Preparation of self-assembled monolayer

A mixture of OEG and COOH–OEG was dissolved in absolute ethanol. The COOH–OEG is used as the specific binding element of the self-assembled monolayer (SAM) and the OEG creates a non-fouling background. The mixture had a total thiol concentration of 100 μM and consisted of a 3:7 ratio of COOH–OEG:OEG.

SPR sensor chips, BK7 glass chips 32 mm × 18 mm × 2 mm, were coated with 2 nm of Cr and 55 nm of Au by electron beam evaporation. These chips were exhaustively rinsed with absolute ethanol and blown dry with compressed, filtered air. The chips were then cleaned by UV-ozone for 20 min. Following UV cleaning, the chips were exhaustively rinsed with 18.2 MΩ cm DI water followed by absolute ethanol. The cleaned chips were immersed in the COOH–OEG:OEG solution overnight. After incubation overnight, chips are removed from the SAM-forming solution, rinsed with absolute ethanol and blown dry with compressed, filtered air.

2.4. Protein immobilization

2.4.1. CEA immobilization

Proteins were immobilized to the sensor surface using EDC/NHS chemistry, which chemically links an amine group to a carboxylic acid group. SAM-functionalized chips were immersed in an aqueous mixture of 0.2 M EDC and 0.05 M NHS for 10 min. The chips were then rinsed with 18.2 MΩ cm DI water and blown dry with compressed, filtered air. CEA (5 μl) in PBS at a concentration of 250 μg/ml was then placed on each chip surface. A glass coverslip was placed on top of each chip, allowing the droplet to cover the surface. The chips were then placed in a plastic Petri dish along with a piece of filter paper saturated with water. This Petri dish was sealed with parafilm and placed in the refrigerator overnight. Upon removal from the Petri dish, chips with coverslips were immersed in 18.2 MΩ cm DI water. The coverslips were removed and the chips were washed exhaustively with 18.2 MΩ cm DI water. Chips were then blown dry with compressed, filtered air.

2.4.2. BSA immobilization for referencing

Chips functionalized with the COOH–OEG/OEG SAM described above were mounted on the SPR sensor. Phosphate buffer (10 mM phosphate, pH 7.0) (PB) was flowed for 10 min at 50 μl/min to establish a baseline. An aqueous mixture of EDC and NHS at 0.2 and 0.05 M, respectively, was flowed for 10 min at 50 μl/min, followed by a 10 min PB rinse at a flow rate of 50 μl/min. BSA at a concentration of 1 mg/ml in PB was flowed for 10 min at 10 μl/min. This was followed by a PB rinse for 10 min at 10 μl/min.

2.5. CEA antibody detection in serum samples

Tris was flowed for 10 min at 50 μl/min to convert activated, but unreacted, sites on the sensor surface to hydroxyl groups. BSA at a concentration of 10 mg/ml in PBS (PBSA) was flowed for 10 min at 50 μl/min to establish a baseline. Serum samples were diluted in PBSA to final concentrations of 1%, 0.5% and 0.25% (v/v). These samples were flowed at 4 μl/min for 45 min, followed by a 30 min PBSA wash at 4 μl/min. Finally, a 0.1% Tween-20 solution was flowed for 10 min at 50 μl/min followed by a 10 min PBSA wash at 50 μl/min to remove any loosely bound proteins from the surface.

2.6. Secondary antibody binding

Following the binding of anti-CEA to the surface from the serum samples, goat anti-human IgG-HRP conjugate at a 1:50 dilution was flowed for 10 min at 10 μl/min, followed by a PBSA wash for 15 min at 10 μl/min.

2.7. Enzyme-linked immunosorbent assay

CEA was diluted to 125 ng/ml in a carbonate buffer (1.6 g Na_2CO_3/l, 2.94 g $NaHCO_3$/l, pH 9.6). Specified wells of the 96-well plate were coated with 50 μl/well of the diluted CEA solution. The plate was sealed with saran wrap and incubated at 4 °C overnight. The plate was removed and allowed to come to room temperature. Wells were blocked with 100 μl/well of the PBSA solution for 1 h on a rocker at room temperature. This step creates some wells coated with only BSA, which will be used as references. The plate was washed four times with a 0.1% (v/v) Tween-20 in PBS solution. Serum samples at 1%, 0.5% and 0.25% (v/v) in PBSA were then added to the BSA-coated and CEA-coated wells. The plate was covered with saran wrap and incubated for 1 h on a rocker at room temperature. The plate was then washed four times with the PBS/0.1% Tween-20 solution. 50 μl/well of goat anti-human IgG-HRP conjugate was added to the wells at a 1:50,000 dilution and incubated for 45 min at room temperature on a rocker. The plate was washed four times with the PBS/0.1% Tween-20 solution. TMB substrate was added at 75 μl/well and allowed to incubate for 45 min. Seventy-five microliters per well of 1N HCl was added to the wells to quench the reaction, and the plate was read at 450 nm.

3. Results and discussion

3.1. ELISA and SPR comparison

ELISA is a method that has previously been used to detect serum antibodies that recognize tumor-specific antigens [23–27]. This detection method relies on autoantibody binding to the antigen of interest and a labeled secondary antibody to produce a quantifiable change in adsorbance. SPR is a technique that allows for real-time, label-free, and quantitative detections. The capability of SPR sensors to directly detect biomolecules of interest eliminates the need for modified secondary proteins to produce a signal.

To establish the direct detection of autoantibodies by SPR as a valid assay, SPR responses were compared to those from an established ELISA method. Fig. 1 shows a comparison for three serum dilutions from a single positive sample detected using both SPR and ELISA. ELISA results are obtained by subtracting the plate reading for a BSA-coated well from a CEA-coated well. This subtracts out the non-specific adsorptions and gives a reading of only the specific autoantibody binding. SPR sensor responses are converted into units of ng/cm^2 using previously described correlations [22]. ELISA shows a relatively linear decrease in response as the serum percentage decreases, as expected. A similar trend is seen for the secondary antibody binding from SPR experiments using an assay similar to the ELISA. The direct analyte response is higher than the observed amplification response. This is likely due to interactions of serum proteins with the CEA autoantibody, blocking secondary antibody-binding sites and limiting the effectiveness of amplification.

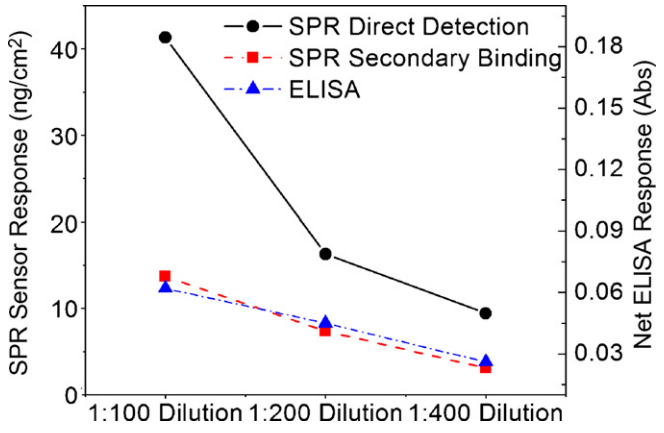

Fig. 1. Comparison of responses from ELISA and SPR using the same sample. Trends show that responses from the ELISA and from the SPR amplification antibody are similar, indicating that the two assays are producing similar data. The trends between SPR amplification antibody and the direct autoantibody response are also similar, indicating that direct detection of the autoantibody is a valid assay. SPR response in nm was converted to ng/cm^2 by multiplying by a factor of 17 [22].

ELISA and SPR sensors, though based on different sensing principles, follow similar procedures to produce a sensor signal. While ELISA relies on a secondary antibody, SPR is not reliant on sandwich assay, but can utilize a secondary antibody to provide both validation of immobilized analyte and to increase the observed sensor response. The response from the ELISA used in this work is calculated by subtracting the non-specific signal that results from the serum sample binding to a BSA-coated surface. The response from the 1% serum case on the BSA-coated surface was ~0.09, approximately half of the response seen on the CEA-coated surface. This indicates that a significant amount of non-specific adsorption occurs between the serum and the surface in the ELISA experiments. A similar experiment was performed using the SPR sensor, where a 1% serum sample was flowed across a surface coated with BSA. The response from this sample dilution, the highest dilution of serum used in this work, was negligible. This eliminates the necessity for a BSA reference channel, similar to the BSA referencing well used in the ELISA experiments.

There are two main differences in the ELISA and SPR assays for these studies that can account for the difference in non-specific binding: surface chemistry and sample delivery. The most significant difference between the detection methods involves the surface chemistry and protein immobilization. The ELISA performed in this work mimics many other ELISA procedures, where the molecular recognition element, in this case CEA or BSA, is immobilized on the surface through physisorption to a polystyrene 96-well plate. This method of immobilization does not offer a nonfouling background and there is a possibility that the recognition element (in this work CEA or BSA) can desorb from the surface. The SPR sensor assay uses a covalent linkage through the EDC/NHS chemistry to ensure the recognition element remains on the surface throughout the assay and also employs a surface with a nonfouling background, consisting of OEG groups, which resists non-specific protein adsorptions. Sample delivery to the surface also differs between the two assays. ELISA was carried out under static conditions, while assays using the SPR sensor were carried out under flow. Under flow conditions, the transport of analytes to the surface will be enhanced and loosely bound proteins can be washed away to eliminate some background signal. These differences in surface chemistry and sample delivery enable SPR sensors to perform detections without the BSA reference channel used in ELISA.

3.2. CEA surface characterization

The CEA-functionalized surfaces used for detection in this work were created outside of the SPR sensor. Therefore, the amount of CEA immobilized on the surface and its viability need to be determined. To establish that a sufficient amount of CEA is bound to the surface, and that it remains active, an online immobilization was performed using the CEA purchased from BioDesign International. As seen in Fig. 2a, CEA binds to an EDC/NHS activated SAM surface with coverage of ~300 ng/cm^2. This corresponds to approximately one monolayer of protein on the surface. To test the activity of the CEA, polyclonal anti-CEA was flowed across the surface and showed an average binding of ~48 ng/cm^2, Fig. 2b. A chip functionalized outside of the SPR sensor was also tested

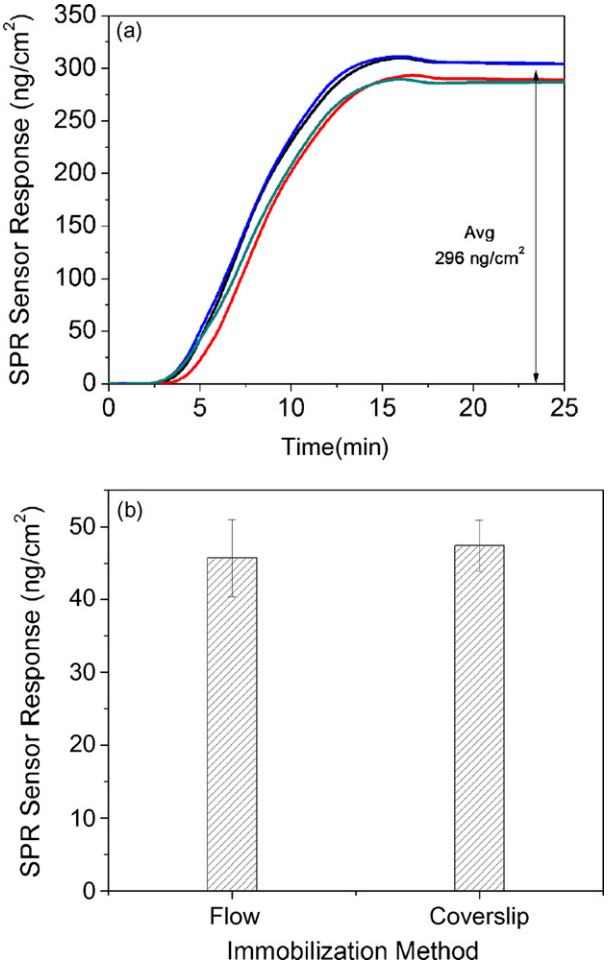

Fig. 2. Characterization of the CEA surface being used in the autoantibody assay. (a) CEA binding to a COOH–OEG/OEG surface that has been activated with EDC/NHS chemistry. The four curves shown are the SPR sensor response to CEA from four independent flow channels run simultaneously. (b) Comparison of anti-CEA binding to a CEA surface functionalized online and a CEA surface functionalized offline using the "coverslip method.".

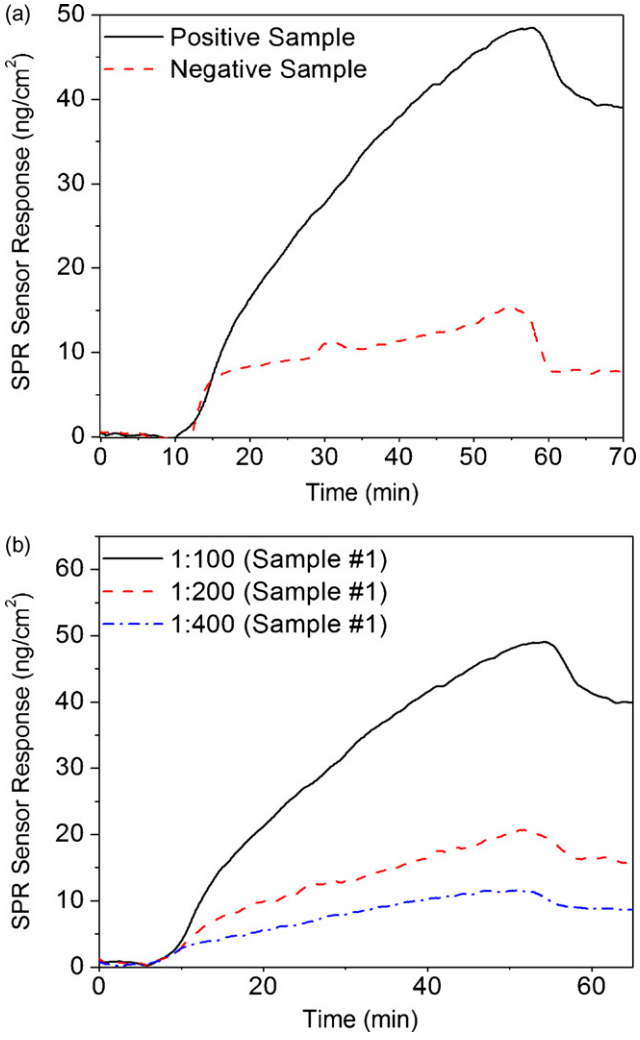

Fig. 3. Sensorgrams comparing (a) the response of a positive serum sample to a negative serum sample, both at a 1% dilution (v/v) in 10 mg/ml BSA, and (b) three different dilutions of the same positive serum sample diluted in 10 mg/ml BSA.

using polyclonal anti-CEA and showed an average binding of ~46 ng/cm^2. The consistency in antibody response indicates that chips functionalized outside of the sensor have similar levels of active CEA immobilized on the surface as those functionalized in the sensor.

3.3. Negative sample analysis

In order to distinguish between two different sample populations, a criterion needs to be established. In the case of SPR sensing, that criterion is a threshold sensor response above which a sample could be considered as part of a separate population. Serum samples from healthy individuals were diluted in PBSA and run on the SPR sensor at dilutions of 1:100, 1:200 and 1:400. Dilutions to lower serum concentrations were not incorporated into this assay development. CEA autoantibody typically exists in patient serum at concentrations around 1 μg/ml, resulting in concentrations as low as ~2.5 ng/ml after dilution.

Responses from five different healthy samples were averaged and statistically analyzed. By assuming that the sensor response from negative samples has a Gaussian distribution, we can establish an upper value for the negative samples using the standard deviation of the population. By setting the threshold value one standard deviation (σ) above the negative average, 85% of the negative samples will be below the threshold. A threshold value two standard deviations above the negative average will result in 97.5% of the negative sample population being below the threshold. A threshold of 2σ above the negative average for each dilution was therefore chosen as the basis to distinguish the positive sample population from the negative population. A sample could be judged positive if one of the serum dilution responses was above the corresponding threshold value. By requiring three different serum dilutions to be above the corresponding 2σ threshold values, we raise the stringency of the test, and increase confidence in a positive result.

3.4. Positive sample testing

Using the criterion established from the negative sample population, serum samples from patients with ovarian and colorectal cancer were screened to validate the established criterion. As seen in Fig. 3a, the amount of specific binding from a positive sample is much higher than what was seen from a negative sample at the same dilution. The sensor responses from three serial dilutions, seen in Fig. 3b, exhibit a linear trend. This is typical of sensor responses to solutions with low analyte concentration. To determine if direct detection of CEA autoantibody could be potentially

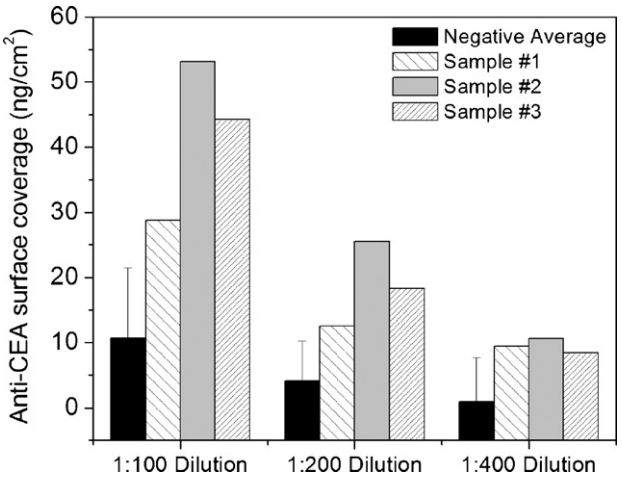

Fig. 4. Evaluation of four positive serum samples using the developed threshold value. The negative average value was obtained from five separate negative samples. The error bar represents two standard deviations of the negative sample population.

useful as a means of differentiating serum from cancer patients from that of healthy individuals, three serum samples from cancer patients were run and compared to the criterion values established for the healthy samples. CEA autoantibody levels in three serial dilutions, 1%, 0.5% and 0.25% serum, were interrogated to improve the reliability of the assay. Because CEA autoantibody levels are elevated in individuals with colorectal or ovarian cancer, a higher sensor response is expected for positive samples. As seen in Fig. 4, the sensor responses from positive serum samples are all above the threshold values established through analysis of the negative samples. Sensor responses from different serum samples vary, which is to be expected, as autoantibody levels will vary among individuals. A common trend within individual serum samples is the approximately linear decrease in sensor response that is observed in increasing dilution. With a small set of samples analyzed, we are able to demonstrate the feasibility of using direct detection on a SPR sensor to differentiate positive samples from negative samples based on the comparison of specific autoantibody levels.

3.5. Assay specificity

The response from a SPR sensor is a convolution of all surface-binding events from a sample. Therefore, minimizing the amount of non-specific interactions with the sensor surface is essential for accurate SPR biosensing. It has been well established that OEG can provide a surface that is relatively nonfouling to proteins [28–30] and has been previously used as the background of mixed SAMs for sensor detections [21,22,31,32]. Thus, for this work, OEG-terminated thiol chains are used to create a nonfouling background. The COOH-terminated thiol chains used as reactive groups have an OEG spacer, to facilitate their incorporation into the OEG nonfouling background. To test the nonfouling properties of the CEA-functionalized surfaces, BSA at 1 mg/ml and a monoclonal anti-human chorionic gonadotropin at 16 μg/ml were separately flowed across surface. No binding was seen from either the BSA or the monoclonal antibody, Fig. 5a and b, respectively. This antibody concentration is in the range of anti-CEA that would be found in a 1% clinical serum sample. Dilution of the serum samples in a solution of 10 mg/ml BSA in PBS decreases the non-specific interactions with the surface compared to similar dilutions without BSA (data not shown). BSA is known to prevent the adsorption of other proteins onto a surface and is often used as a blocking agent in sensor applications.

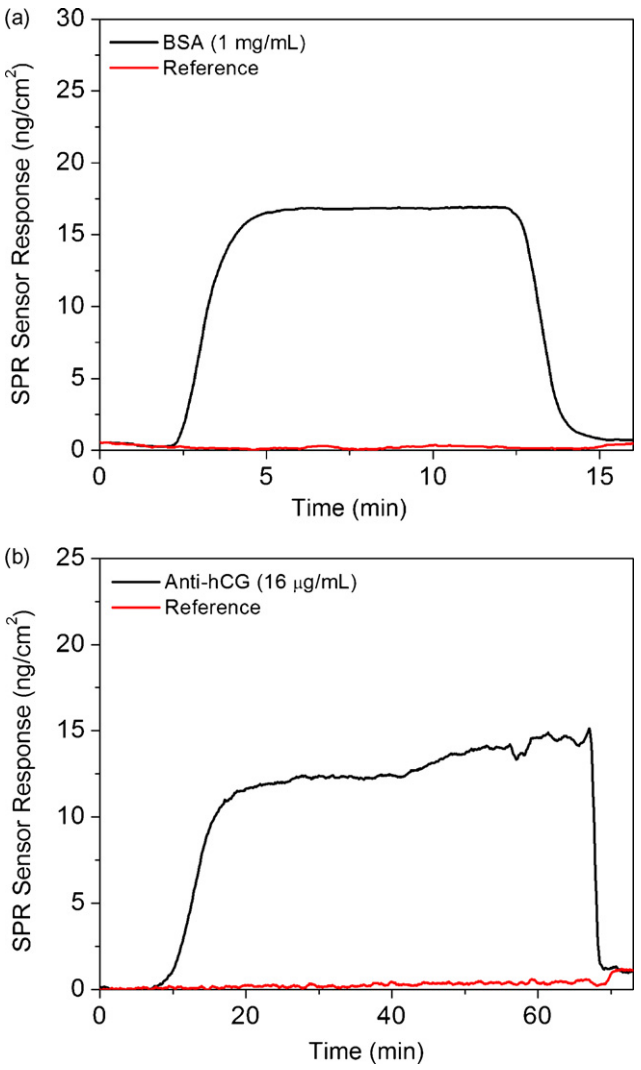

Fig. 5. Specificity of the assay was validated using BSA and anti-hCG. (a) Sensorgram showing no BSA (1 mg/ml) adsorption on a CEA-functionalized surface. (b) Anti-hCG at a concentration of 16 μg/ml does not bind to a CEA-functionalized surface. Therefore, the binding seen from the positive serum samples is attributed to a specific interaction between CEA on the surface and anti-CEA in the serum sample.

4. Conclusions

In this work, an assay for differentiating CEA autoantibodies levels in serum samples using a custom-built SPR sensor is demonstrated. Trends from an ELISA correlated well with secondary antibody response and direct detection of CEA autoantibody from samples using the SPR sensor. Furthermore, through analysis of a small set of clinical samples, we were able to show that serum samples with overexpressed CEA autoantibody were distinguishable from healthy samples using a criterion based on direct detection. Dilution with BSA along with nonfouling surface chemistries helped to minimize the amount of non-specific adsorption to the sensor surface, removing the necessity of a secondary antibody and allowing for direct detection of the CEA autoantibody.

The work presented here focuses on the development of an assay using a SPR biosensor as a means for discriminating varying levels of autoantibody in human serum samples. Recent developments in SPR instrumentation have focused on expanding sensors from a few channels [33,34] to many channels using imaging capabilities [35,36]. These SPR imaging sensors have the ability to screen for tens to thousands of biomarker autoantibodies simultaneously,

making them an intriguing technology for cancer screening and biomarker discovery.

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