



Surface plasmon resonance based immunosensor for the detection of the cancer biomarker carcinoembryonic antigen

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ABSTRACT

An immunoassay in optimised conditions with a highly sensitive surface plasmon resonance (SPR) based biosensor was developed for the detection of the cancer biomarker carcinoembryonic antigen (CEA). Different formats of the immunoassay were initially investigated on the surface of the gold sensor chip. A self-assembled monolayer (SAM) was formed on the gold chip using 11-mercaptopundecanoic acid (MUDA), before the immobilisation of the antibodies was conducted. The assay was then formed in a direct capture and a sandwich assay. In order to increase the sensor signal the CEA antigen was incubated with the detection/capture antibody before it was injected to the sensor chip surface and the results were recorded in real-time using the Biacore 3000 instrument. A detection limit of 3 ng ml^{-1} CEA was obtained with a dynamic detection range from 3 ng ml^{-1} to 400 ng ml^{-1} with correlation coefficients of 1.00 and 0.99 for the sandwich and rabbit anti-mouse (RAM) capture assay. Kinetic data analysis was performed for the standard capture test and subsequently for the developed assays and R_{max} showed an increase from 215 RU for the standard capture test to 428 RU for the RAM-capture assay and 734 RU for the sandwich assay, respectively. The developed SPR immunosensor using the sandwich assay format showed high sensitivity and reproducibility for CEA detection which makes it a promising procedure for cancer biomarker analysis.

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1. Introduction

Carcinoembryonic antigen (CEA) has been widely studied in clinical analysis as a tumour biomarker. CEA is a cell adhesion glycoprotein and is a member of the immunoglobulin super family [1]. The protein was first identified from human colon cancer tissue extracts in 1965 by Phil Gold and Samuel O. Freedman [2]. CEA is produced during foetal development and the production of it terminates before birth. In healthy individuals the normal level of CEA is between 3 and 5 ng ml^{-1} and this level may increase up to 10 ng ml^{-1} due to other benign diseases [3]. The protein scarcely exists in the blood of healthy people except cigarette-smokers. However, its concentration shows a significant increase in some conditions including lung cancer, colorectal carcinoma, pancreatic carcinoma and breast carcinoma [4]. Hence, it can be used as a biomarker for diagnosis and prognosis of cancer. CEA levels over 20 ng ml^{-1} are usually associated with patients with cancer in metastatic state [5].

The treatment of lung cancer is a long and difficult process and the survival scarcely reaches 5 years. The most crucial point for the

best result is to diagnose the disease at an early stage. To this aim, many methods are now available to diagnose the disease including chest X-ray, computerised tomography, magnetic resonance imaging, positron emission tomography, sputum cytology and biopsy. However, some of these methods are not suitable for all patients due to the other pathologies they may have [6]. Moreover, patients can often experience great pain and complications because of some diagnostic tools such as biopsy. Since the current diagnostic methods are also time consuming, a new sensitive and rapid method is necessary for both lung and colon cancers detection. CEA is one of the most investigated tumour markers in certain cancers [7], with several clinical and research-based applications [8]. However, due to the absence of both rapid and sensitive diagnostic tool, CEA related cancers cannot be detected at an early stage which is vital for successful treatment. Therefore, biosensor technologies can play a crucial role in achieving this aim [9,10]. Though enzyme-linked immunoassay (ELISA) has been generally used for both clinical and research field, the SPR-based biosensors will provide label-free and real-time detection system [11]. SPR based biosensors have also been used for other diseases which occur at very high incidence level using genetic [12] or protein markers [13] which exist in either tissue or body fluids or in both [14].

In the present paper we report on the development of a surface plasmon resonance (SPR) based biosensor platform for the

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detection of CEA, the most crucial tumour marker for lung and colon carcinomas. An immunoassay for CEA was developed and optimised on the SPR gold sensor surface to achieve high sensitivity for a real-time disease diagnosis. Different homogeneous assay formats were investigated including capture and sandwich immunoassay. By using this label-free real-time biosensor technology we were able to achieve a low detection limit for CEA which represents the critical CEA level in non-smoker individuals. This will help in the identification of possible cancer patient. The technique shows a promising future technology for the diagnosis of cancer at inchoate stage without the use of invasive surgical procedures.

2. Materials and methods

2.1. Materials and reagents

Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4), bovine serum albumin (BSA), N-hydroxysuccinimide (NHS), ethanolamine, Human CEA (cat no. C4835) and its monoclonal antibody (C2331) were purchased from Sigma Aldrich (Poole, UK). 1-ethyl-3-(3 dimethylaminopropyl)-carbodiimide (EDC) was purchased from Pierce-Thermo Scientific (Cramlington, UK). Mouse monoclonal antibody to carcinoembryonic antigen (CEA) (cat no. ab10037) and Mouse Monoclonal (1C11) to cardiac Troponin T: ab8295 was purchased from Abcam (Cambridge, UK), monoclonal PSA detection antibody (cat no: MCA2561) obtained from AbD Serotec (Kidlington, UK). Mouse IgG (cat no. 015-000-003) and rabbit anti-mouse IgG (RAM) was bought from Stratech Scientific Ltd./Jackson ImmunoResearch (Newmarket, UK). In the developed sandwich and RAM-capture assays, Sigma anti-CEA antibody (C2331) was used as the detection antibody to perform the assay. All other chemicals were of analytical grade.

2.2. Instrumentations

A fully automated SPR-based Biacore 3000 biosensor and the bare gold sensor chips were supplied by Biacore GE Healthcare (Uppsala, Sweden). The sensor possesses four sensing spots that provide four separate areas for different assay simultaneously. In the current study two sensing spots were employed for sandwich and indirect assay formats while the third spot provided the control surface. The operating temperature of the assays was 25 °C and the flow rate of the buffer was 10 $\mu\text{l min}^{-1}$ throughout the assay. The data presented in this work are the averages of 3 data points for the assays described unless otherwise stated.

2.3. Sensor chip cleaning and MUDA coating

Bare gold sensor chips were first cleaned using nitrogen plasma for one minute and then coated with self assembled monolayer (SAM) by immersing the sensors in 2 mM solution of 11-mercaptoundecanoic acid (MUDA) overnight followed by rinsing with ethanol and Milli-Q water and then dried under nitrogen. The SPR sensor chips were then stored at 4 °C until used.

2.4. Control surface selection

For the selection of the best control sensor surface, three different antibodies (mouse IgG, anti-PSA and anti-troponin produced in mouse) were examined. Since the samples were prepared using 5 $\mu\text{g ml}^{-1}$ BSA in all experiments, 300 ng ml^{-1} CEA was diluted in BSA and the non-specific binding of this solution to each control surface was measured. A high concentration of CEA antigen was

used in this confirmation study and the non-specific binding of the antigen to each control surface was recorded during the SPR assay.

2.5. Immobilisation of antibodies

The SAM coated sensor chip was first docked to the Biacore instrument and primed with running buffer (10 mM PBS, pH 7.4, 0.0027 M potassium chloride, 0.137 M sodium chloride) at a flow rate of 10 $\mu\text{l min}^{-1}$. Monoclonal mouse anti-CEA antibody was then immobilised via one flow path of the instrument for the sandwich assay whereas rabbit anti-mouse and mouse IgG antibodies (control antibody) were immobilized to the second and third sensor array of the chip, to conduct the capture assay and obtain control surface, respectively. The immobilisation stage of the immunoassay was obtained using conventional amine coupling chemistry. The running buffer in this stage was degassed phosphate buffered saline (PBS). During the immobilisation step firstly the sensor chip surfaces were activated with a mixture of 400 mM EDC and 100 mM NHS (1:1). Both reagents were prepared in deionised water and immediately mixed before use. EDC-NHS was injected onto the four sensor surfaces simultaneously for 3 min (30 μl) to activate the sensor chip surface. Then, 30 $\mu\text{g ml}^{-1}$ coating antibodies (anti-CEA antibodies, rabbit anti-mouse and mouse IgG) prepared in 10 mM sodium acetate buffer (pH 5.5) were immobilized to the sensor surfaces. After antibody immobilisation, the sensor surfaces were blocked with 30 $\mu\text{g ml}^{-1}$ BSA in PBS buffer for 3 min (30 μl). Finally, 1 M ethanolamine (pH 8.5) was used to cap the non-reacted NHS esters exist on the sensor surface for 3 min (30 μl). The RU changes were recorded 2 min after the protein injection was completed.

2.6. CEA detection

First assays were performed using direct assay approach without incubation. To increase the signal amplification the homogeneous assay was then applied as sandwich and capture methods with an incubation step added before the assay taking place in the instrument. Different incubation methods were examined, including water bath at 37 °C and with/without shaker at room temperature applied prior to the assay. The CEA and detection antibody were incubated in the 1.5 ml Eppendorf tube for each concentration of the antigen. The detection antibody concentration was chosen as always higher than CEA to prevent any free CEA in the solution that can interfere with the binding results. The incubation conditions were then optimized as time, temperature and detection/capture antibody concentration. The best results were achieved through applying incubation at room temperature for 2 h using a shaker. PBS buffer was used as the running buffer during the CEA marker detection and 5 $\mu\text{g ml}^{-1}$ BSA in PBS was used to prepare the CEA samples. For the sandwich assay, two different mouse anti-CEA antibodies (a coating and detection antibodies) were used while rabbit anti-mouse (RAM) was preferred as coating antibody for the capture assay. RAM-capture assay is an indirect assay here in which RAM was used to capture either mouse anti-CEA antibody or CEA bound mouse anti-CEA antibody. The sensor signal difference due to the mass difference of free or antigen (CEA) bound anti-CEA antibody was investigated to obtain the results. The anti-CEA captured on RAM causes an SPR signal, however the SPR signal is higher (due to higher mass) when antigen bound anti-CEA antibody is captured on RAM immobilised surface. By subtracting the two responses the affect of antigen to the assay can be calculated. Before samples injection, 5 $\mu\text{g ml}^{-1}$ BSA and anti-CEA detection antibody were injected to all sensor surfaces as negative controls in the experiments. Each CEA sample and negative controls were injected onto the sensor surface for 3 min and RU changes were recorded. After each binding step the sensor chip surface was regenerated by injecting 100 mM HCl (1 min, 10 μl) and additional

20 mM NaOH (1 min, 10 μ l) where these were found to give the best sensor surface regeneration without hindering the affinity of the immobilised antibody. All the data points presented are the averages of the triplet measurements unless otherwise stated. The limit of detection (LOD) was calculated as the signal obtained from the CEA concentration that is equivalent to the 3 times the standard deviation of the signals obtained from the blank standards.

3. Results and discussions

In this study an SPR based assay for the detection of human CEA tumour marker was developed and optimised using different immunoassay formats constructed on the surface of a Biacore bare gold sensor chip including a standard capture, rabbit anti-mouse (RAM) capture and sandwich assays.

3.1. Assay optimisation

Bare gold SPR sensor chips were employed in this work as the sensor platform for the CEA detection. Each chip consists of four sensing arrays. The modification of the chips using self-assembled monolayer's (SAM) was carried out on the sensor surface. The SAM coated sensor chip was first docked to the Biacore instrument and primed with running buffer using a flow rate of 10 μ l min⁻¹. To eliminate non-specific binding to the control sensor array surface, control surface selection study was conducted. Three different antibodies (mouse IgG, anti-PSA and anti-troponin) were investigated and used in this study. The antibodies were immobilized to the three different sensor arrays on the Biacore chip using different flow channels of the sensor, respectively with conventional EDC-NHS chemistry [15]. A 3 min injection of the antibodies was sufficient

to achieve the signal with concentration of 30 μ g ml⁻¹ antibody saturation. A 300 ng ml⁻¹ CEA solution in PBS buffer containing 5 μ g ml⁻¹ BSA was then injected to all immobilised control surfaces on the sensor array. In addition to the CEA, a 5 μ g ml⁻¹ BSA solution was also examined in a separate experiment in order to measure the non-specific binding caused by this solution alone. The recorded RU change for non-specific BSA binding was 1 ± 1 RU for anti-PSA and anti-troponin immobilized surface while it was 1 ± 0.5 for mouse IgG. Moreover, non-specific binding of the CEA antigen against each surface was observed at zero level and therefore mouse IgG was selected as the control surface for further experiments (Fig. 1A).

A standard direct assay format in which the coating anti-CEA antibody was immobilized onto the active sensor surface and mouse IgG immobilised to the control surface was then developed. CEA antigen was then injected on the sensor surface in the concentration range of 100–400 ng ml⁻¹. Though a clear difference was observed between the active sensor surface and the control surface, the obtained results were low despite the high concentration of CEA used in the test (Fig. 1B). The recorded response changes were 258 ± 19 RU using the standard direct assay for the binding of 300 ng ml⁻¹ CEA. These preliminary tests with high concentrations of CEA showed that the direct detection of CEA biomarker using the SPR sensor may not be suitable for the measurement of low CEA concentrations. This was confirmed when the optimised direct assay conditions were then applied for the detection of lower CEA concentrations (down to 100 ng ml⁻¹) achieving a low and irreproducible signal. Kinetic data analysis was performed for this assay results and the data was fitted to 1:1 Langmuir binding model to determine the binding association and dissociation rates [16]. With this binding model, K_A , K_D , R_{max} values were calculated as 1.13×10^8 M⁻¹ s⁻¹, 8.8×10^{-9} M and 215 RU for the concentration

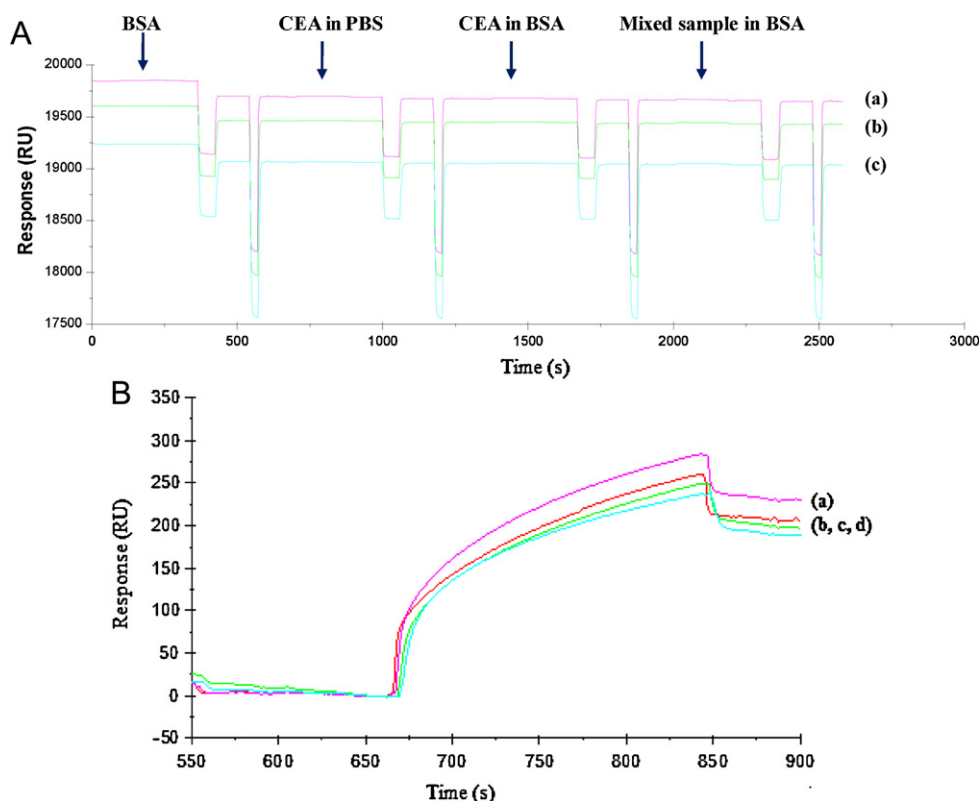


Fig. 1. (A) Confirmative assay for the control surface selection with PBS buffer (10 mM, pH 7.4, 0.0027 M KCl, 0.137 M NaCl). The non-specific binding of (5 μ g ml⁻¹) BSA to each antibody surface (30 μ g ml⁻¹) (first binding), the non-specific binding of CEA antigen in PBS (300 ng ml⁻¹) or BSA solution (second and third bindings), the non-specific binding of the mixed sample included anti-CEA detection antibody and CEA antigen (last binding). The immobilized surfaces: anti-PSA (a), anti-troponin (b) and mouse IgG (c). (B) Direct assay sensorgram with a 300 ng ml⁻¹ concentration of CEA biomarker using the SPR sensor. CEA antigen binding on Abcam's anti-CEA immobilized (a) and Sigma's anti-CEA immobilized (b–d) sensor surfaces.

Table 1
Results of kinetic calculations for CEA marker detection with standard and optimised assay formats.

Parameters/ assay type	Standard assays	Optimised RAM-capture assay	Optimised sandwich assay
k_a (1/M s)	8.17×10^4	1×10^3	6.88×10^5
k_d (1/s)	7.29×10^{-4}	1.46×10^{-6}	2.09×10^{-5}
R_{max}	215 RU	428 RU	734 RU
K_D (M)	8.8×10^{-9}	1.46×10^{-9}	3.04×10^{-11}
K_A (1/M)	1.13×10^8	9.97×10^4	3.29×10^{10}

of 300 ng ml⁻¹ CEA (using Abcam antibodies in a direct affinity assay) (Table 1). Due to the weak responses with the direct assay, other assay formats were then investigated.

A sandwich and RAM-capture assays were then developed under optimised conditions that gave much higher response when compared to the standard capture assay. Langmuir binding model was also performed for the optimised assays in the linear dynamic range of 3–400 ng ml⁻¹ of CEA and the results are reported in Table 1. The developed assays provided higher responses than the standard direct assay format using Rabbit anti-mouse and Abcam anti-CEA antibody as the surface capture antibodies and in both assays the anti-CEA antibody (Sigma) was employed as the detection antibody.

To enhance the sensor signal and improve the sensitivity of the assay further an incubation step was introduced where the detection anti-CEA antibody (from Sigma) was incubated first with CEA antigen in buffer before the sample was applied to the sensor surface. To optimise this step various incubation procedures were examined including temperature (37 °C, or 22 °C and with/without shaking conditions). Optimal results were achieved when a 22 °C with a shaker incubator was used. The principle of the applied homogenous assays (RAM-capture and sandwich assays) is shown in Fig. 2.

The concentration of the anti-CEA detection antibody used in the assay was also optimised. Various concentrations of detection antibody in the range of 1–5 µg ml⁻¹ were examined using CEA sample concentration range of 50–400 ng ml⁻¹. Optimal results were achieved when 5 µg ml⁻¹ detection antibody was used. Higher concentrations of anti-CEA detection antibody were also tested but did not give higher responses. The time of incubation between the detection antibody and the CEA before injecting on the sensor surface was then optimised under these conditions to achieve maximum sensitivity. The RU responses were measured throughout 5 h and the highest RU changes were recorded in the

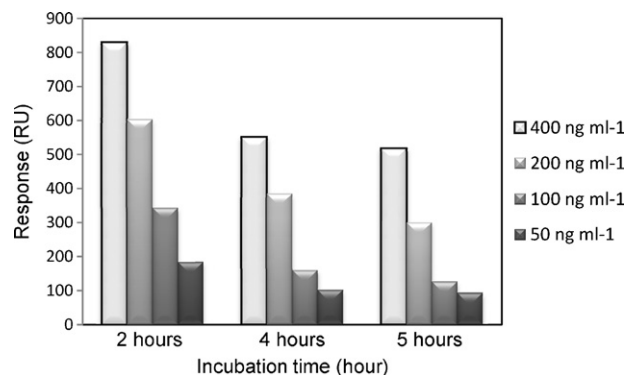


Fig. 3. Optimisation of the incubation time for the CEA antigen with the anti-CEA detection antibody. A coating anti-CEA antibody was used on the sensor surface and the detection anti-CEA (5 µg ml⁻¹) was incubation with the sample CEA (50–400 ng ml⁻¹) for different incubation time before injection on the sensor chip.

first 2 h of incubation; however, the obtained RU changes for each CEA concentration showed gradual decrease after 2 h as depicted in Fig. 3. However, it must be noted that these samples did not contain preservatives or protein stabilisers. After obtaining these results the assays were performed using 1 or 2-h incubation to observe the difference; however, the recorded RU changes were similar in both incubation periods (data not shown for 1 h). Therefore, 1 h incubation was preferred to perform the assay at ambient temperature on a shaker in order to minimise the total assay time. This incubation step was performed prior to the measurement of CEA binding on the Biacore 3000 biosensor.

3.2. Sandwich and RAM-capture assays characterisation

In the development of the immunoassay on the sensor chip, three different antibodies were used and these included; monoclonal mouse anti-CEA antibody (from Abcam), rabbit anti-mouse and mouse IgG. The antibodies were immobilised through the separate flow paths of the three arrays on the sensor platform. Anti-CEA monoclonal antibodies and rabbit anti-mouse antibodies were used as the coating antibody for the sandwich and RAM-capture assays, respectively, whereas mouse IgG provided the control surface.

The immobilization signal of each antibody was measured during a 3 min duration and the evaluated RU changes for the immobilization reaction were recorded as 3500 ± 95 for anti-CEA (Abcam), 3000 ± 120 for rabbit anti-mouse and 2800 ± 37.6 for

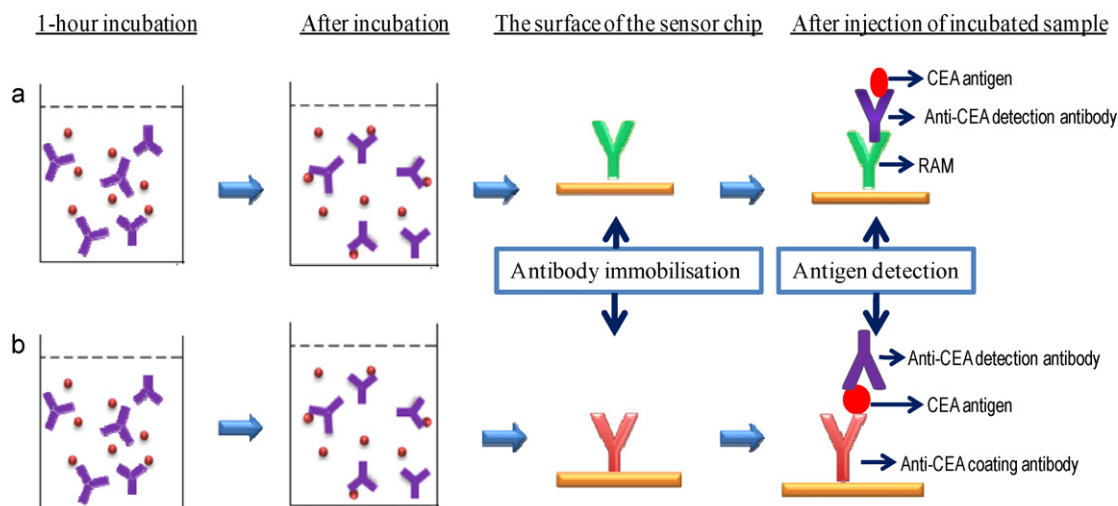


Fig. 2. Schematic representation of homogenous RAM-capture (a) and sandwich assay (b).

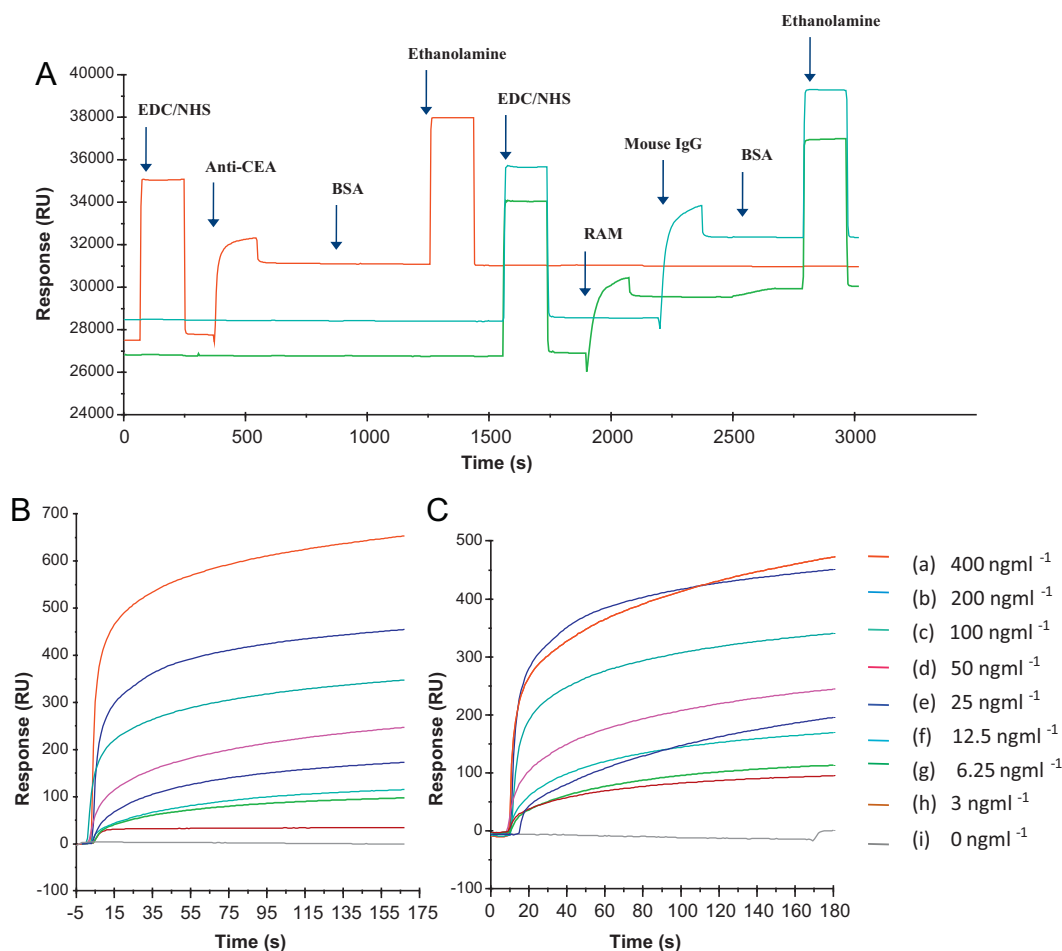


Fig. 4. Immobilisation of anti-CEA coating antibody (red), rabbit anti-mouse (green) and mouse IgG (blue) antibodies on the sensor chip surface (A). Sensorgram of the CEA using sandwich assay (B), and RAM-capture assay (C) methods in the concentration range of 3–400 ng ml⁻¹. The lowest line represents the control in each assay and the RU change gradually increased from the bottom to the top according to the increased CEA concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

mouse IgG antibodies, respectively (Fig. 4A). A 3 min injection of antibodies was sufficient for the signal to reach equilibrium; therefore, the immobilisation time was kept at 3 min for the assay. Although the RU changes for the immobilized antibodies showed similarity to each other they were different antibodies produced by different companies.

In the sandwich assay method, the CEA antigen in the sample was first incubated with the anti-CEA detection antibody (Sigma, 5 μ g ml⁻¹) for 1 h at 22 °C and then was injected on the anti-CEA coated sensor surface. Whereas for the indirect capture assay the anti-CEA detection antibody coupled with CEA antigen (Ab–Ag complex) was injected on the Rabbit anti Mouse (RAM) and mouse IgG coated sensor arrays for the RAM-capture and control assays, respectively (Fig. 2a). Each incubated sample was prepared in 5 μ g ml⁻¹ BSA and the non-specific binding of both 5 μ g ml⁻¹ anti-CEA detection antibody and 5 μ g ml⁻¹ BSA were recorded before each experiment. The non-specific binding of 5 μ g ml⁻¹ anti-CEA detection on the anti-CEA coating antibodies was recorded as 5.1 ± 5.2 while non-specific binding of the 5 μ g ml⁻¹ BSA on all surface caused only 3 ± 2 RU change (data not shown).

The selected concentration range of CEA samples for the detection was 3–400 ng ml⁻¹ and this concentration range was studied through two different assay types. The recorded RU changes were from 30 to 802 RU in the concentration range of 3–400 ng ml⁻¹ CEA and 5 μ g ml⁻¹ detection antibody control caused only 3.5 ± 2.7 RU change in the sandwich assay. On the other hand, the obtained

results were between 13 and 430 RU change in the same concentration range of CEA antigen for the RAM-capture assay. Moreover, the non-specific binding of CEA on the control surface was measured as only 3.5 ± 2.7 RU change. Fig. 4B and C represents the sensorgrams of the sandwich assays and RAM-capture assay, respectively. A clear difference was observed between the control and active surfaces through both assay types. All data were control subtracted. However, the recorded RU changes were found to be higher in the sandwich assay (Fig. 4B) when compared to the RAM-capture assay (Fig. 4C) according to the CEA concentration tested. As it is seen in Fig. 5 the obtained correlation coefficient of the sandwich and RAM-capture assays were 1.00 and 0.99, respectively with the 3 ng ml⁻¹ detection limit for both assays.

Though many different immunological methods have been used to detect various types of cancer including radioimmunoassay, enzyme-linked immunosorbent assay, fluorometry, chemiluminescence immunoassay they are time-consuming. On the other hand, biosensor technologies have provided a real-time, label-free assay approach, gain of time and high sensitivity with low detection limits through different type of sensor platforms including optical, piezoelectric and capacitive biosensors. Despite of the common use of these sensors for the detection of various diseases, there have been very few published papers for human CEA protein detection which is a biomarker for common cancer types including lung cancer [17] colon cancer [18] and breast cancer [19]. Diagnosis of these cancers at an early stage is the most crucial point for an effective

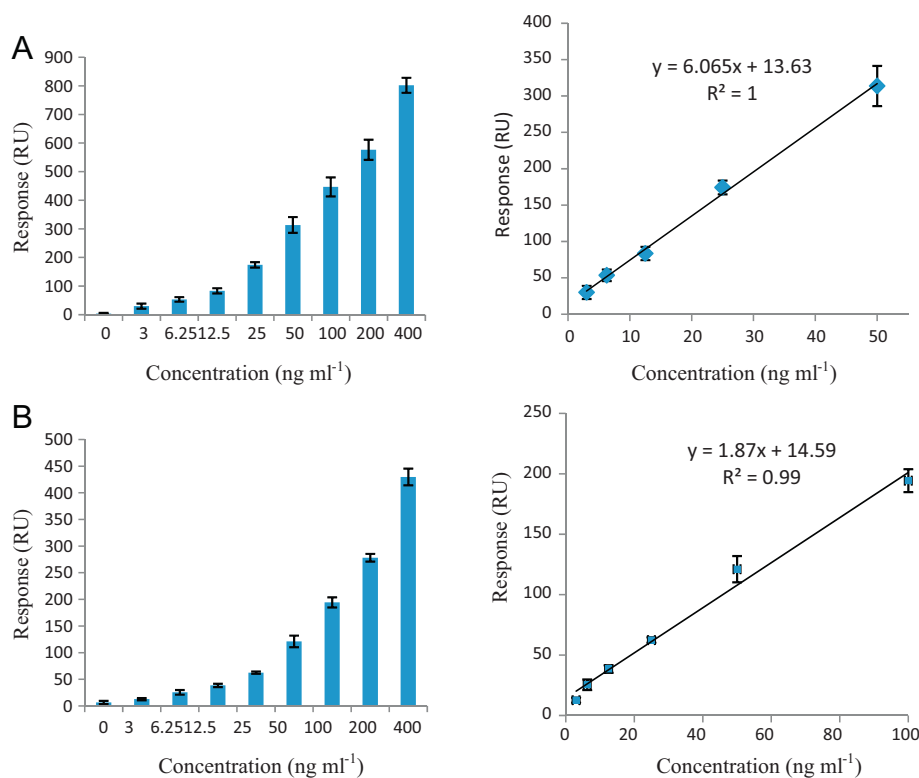


Fig. 5. (A) The overall results of sandwich assay and (B) the overall results of RAM-capture assay (all shown data is control subtracted).

therapy and this aim has been achieved at research level using a strip biosensor, quartz-crystal microbalance (QCM) and surface plasmon resonance (SPR) sensors [20–22].

CEA marker was detected by Zeng et al. through a strip biosensor using gold nanoparticles (Au-NP) and the molecular recognition between specific antigen and antibody [20]. However, the detection limit was at higher level (5 ng ml^{-1}) despite the use of Au-NP and more complex method when compared to our work. In another publication, antibody–antigen interaction was studied to detect human CEA antigen through a quartz crystal microbalance immunosensor based on magnetic composite nanoparticle-functionalized biometric interface. By this approach, the detection of CEA in the concentration range of $2.5\text{--}55 \text{ ng ml}^{-1}$ with 0.5 ng ml^{-1} detection limit was studied and the results were compared with a conventional ELISA method. While ELISA needed a long time with many separate steps and labelling, QCM-based sensor provided approximately same results with reproducible, stable and much faster immunoassay [21] which supports the requirement of developing biosensor technologies for medical diagnostics.

Ladd et al. investigated the direct detection of CEA autoantibodies for clinical serum samples using a SPR biosensor and the results were compared with ELISA that showed the same linear trend. Sandwich assay was performed in this study to enhance the sensor signal in reverse order when compared with our work (CEA antigen was initially immobilized to the surface and polyclonal anti-CEA was then injected to the sensor for measurement) and $\sim 48 \text{ ng cm}^{-2}$ average binding of antibody was observed [22]. An SPR-based sensor was developed by Su and colleagues for the detection of CEA cancer marker with using not only HBS buffer but also 10-fold diluted human serum. A 6.2 ng ml^{-1} and 25 ng ml^{-1} CEA could be detected in buffer and diluted human serum despite of a complex assay method and the use of various mediator proteins for signal amplification [23]. In this study we used a different approach and achieved a detect limit of 3 ng ml^{-1} CEA biomarker in buffer using a simple, reproducible and easily applicable method.

Future work will concentrate on developing the method further for serum analysis.

4. Conclusion

The normal range of CEA in serum for an adult non-smoker is $<3 \text{ ng ml}^{-1}$ and for a smoker $<5.0 \text{ ng ml}^{-1}$. The concentration of CEA more than 3 ng ml^{-1} in non-smoker and 5 ng ml^{-1} in smoker may indicate lung, colon or breast cancer; therefore the detection of this biomarker in the range of 3 ng ml^{-1} or lower level is required for early diagnosis of cancer markers. In this study homogeneous assay approach with two different methods were implemented to detect an important cancer biomarker using SPR sensor. The diagnosis could be measured through real-time, label-free technology. Though the detection of cancer markers in real patient samples is slightly more challenging than in buffer samples, this technology has provided very promising approach. Here, we have achieved a detection limit of 3 ng ml^{-1} CEA concentration with a simple assay design without the use of assay amplifiers such as nanoparticles which we can implement to enhance the sensitivity further. Future research will be carried out on using human serum to obtain an assay in human body fluids for the diagnosis of cancer biomarkers.

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