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Development of optical immunosensors for detection of proteins in serum

Dimitris Kyprianou, Iva Chianella, Antonio Guerreiro*, Elena V. Piletska, Sergey A. Piletsky

Cranfield Health, Cranfield University, Vincent Building, College Road, Cranfield, Bedfordshire MK43 0AL, UK

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ABSTRACT

The detection of proteins in biological samples such as blood, serum or plasma by biosensors is very challenging due to the complex nature of the matrix, which contains a high level of many interfering compounds. Here we show the application of a novel polymeric immobilisation matrix that helps in the detection of specific protein analytes in biological samples by surface plasmon resonance (SPR) immunosensors. This polymer matrix contains thioacetal functional groups included in the network, and these groups do not require any further activation in order to react with proteins, making it attractive for sensor fabrication. The protein prostate specific antigen (PSA) was selected as a model target analyte. A sandwich format with two primary antibodies recognising different parts (epitopes) of the analyte was used for the detection of PSA in serum. The efficiency of the reduction of non-specific binding achieved with novel polymer was compared with those of other techniques such as coating of sensor surface with polyethylene glycol (PEG), use of charged hydrophilic aspartic acid and surfactants such as Tween20. The detection limit of the polymer based immunosensor was 0.1 ng ml^{-1} for free form PSA (f-PSA) in buffer and 5 ng ml^{-1} in 20% serum. This is an improvement compared with similar devices reported on literature, indicating the potential of the immunosensor developed here for the analysis of real samples.

1. Introduction

The development of analytical methods for quantification of targets analytes in biological samples such as blood, plasma and serum is very challenging. The presence of high amount of interfering compounds responsible for non-specific binding (NSB) makes the detection and quantification of the analytes of interest difficult, imprecise and prone to false positive and negative signals. The optimisation of the procedure for deposition of a biological receptor on sensor surface is a key step for the development of successful biosensors [1]. In biosensors, proteins are either physically adsorbed onto the sensor surface [2] or covalently attached via amino or thiol groups [3]. Common immobilisation methods include direct covalent attachment of receptors/ligands onto gold surfaces or the use of an intermediate matrix, such as polymers or self-assembled monolayers, to which the biomolecules are subsequently attached. Chemical and biological matrices are usually utilised to increase the surface area of sensors and to assist in (ordered) immobilisation of the receptor/ligand. Covalent attachment is applied mainly because it provides a strong and stable binding of the ligand/receptor to the sensor surface and enables regeneration of the sensor surface. Covalent immobilisation includes amino coupling [4], aldehyde coupling [5] and thiol coupling methods [6]. The covalent attachment can occur on gold surfaces modified with polymers such as carboxydextran matrix [7], thioacetal matrix [8] or self-assembled monolayers [9]. Although covalent attachment of biomolecules is usually preferred to simple adsorption, particular care should be taken to protect the immobilised receptors/ligands from denaturing during immobilisation [10]. To achieve low level of non-specific interactions the immobilisation protocol relies on inclusion of polar molecules such as polysaccharides or polyethylene glycol (PEG) derivatives into the immobilisation matrix [11]. This is because proteins are amphiphilic molecules which can interact with surfaces via a variety of intermolecular forces (van der Waals, electrostatic and Lewis acid–base) and also via entropically driven effects such as hydrophobic interactions and conformational changes. The end-result of these effects is an apparent irreversibility of the adsorption process [3], leading to increased background signal in the sensor. The inclusion of the above mentioned polar (neutral) molecules minimises protein interaction with surfaces, minimising adsorption.

In the current work, a three dimensional polymer (3-D polymer), developed previously in our group [8], was applied as a matrix in SPR immunosensors for the detection of PSA in serum. This soluble polymer possesses groups for immobilisation on gold surfaces (disulphide), and groups reactive towards primary amines (thioacetals). The immobilisation of biomolecules (antibodies) on the polymer proceeds via reaction between primary amines and the thioacetal functionality [6,12], the product of the

* Corresponding author. Tel.: +44 1234 758326; fax: +44 1234 758380.
E-mail address: a.guerreiro@cranfield.ac.uk (A. Guerreiro).

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reaction being a fluorescent isoindole. The 3-D polymer allows one step immobilisation of biomolecules through their primary amino groups without the need of any activation. In this work widely accepted prostate cancer biomarker prostate specific antigen (PSA) was chosen as a model analyte. High levels of PSA in serum (>4 ng ml^{-1}) can indicate prostate cancer even at early stages of the disease [13,14]. PSA is a 33 kDa single chain glycoprotein, which is found in serum mainly in a complex with α1-antichymotrypsin, α2-macroglobulin, but also in free form [15]. Prostate cancer is one of the main causes of death among male population in several countries. However early detection of prostate cancer, when the carcinoma is still localised in the glands, is vital to assure survival of the patients [16]. Several immunoassays for PSA determination in serum have been developed for diagnosis, monitoring and management of prostate cancer. Currently the detection of PSA at detection limits of <0.1 ng ml^{-1} is performed by Enzyme Linked Immunosorbent Assay (ELISA) or other assays which involved use of antibodies tagged with flurophores or radioactive isotopes [17–22]. Despite high sensitivity and reliability of these techniques, there is a need for the development of new cheaper, faster, and more user friendly methods of detection of cancer biomarkers.

In order to achieve fast and label free detection of PSA in human serum a surface plasmon resonance (SPR) immunosensor was developed. The detection is based on changes of the refractive index at the biointerface on the sensor surface and such changes are directly proportional to the amount of immobilised material [18,23–25]. One of the main disadvantages of SPR immunosensors (as in the case of biosensors in general), is the lack of selectivity and sensitivity when trying to detect an analyte in a real sample such as serum, which contains high levels of many interfering proteins [3]. These disadvantages can be overcome with a sandwich format, where the binding event of the secondary antibody can take place in the absence of complex matrix components responsible for a high background and false positive results. The signal in 'sandwich sensors' can be enhanced by conjugation of antibodies with gold, silver or magnetic nanoparticles [26–28], liposomes [19], or by using an enzyme precipitation strategy [29]. The performance of these types of sensors strongly depends on conjugation procedure [30].

In the present format we are using two primary antibodies which bind to two different epitopes of the same PSA antigen, as described by Jang et al. [31]. The first primary (capture) antibody (C-Ab) is immobilised onto chips modified with 3-D polymer. It binds to PSA present in a real sample (blood or serum). This is followed by elution of matrix components and by specific binding of a second (detection) antibody (D-Ab), which is capable of binding to a different epitope than C-Ab. This detection reaction can be performed with D-Ab dissolved in a buffer solution. The recording of specific sensor signal takes place in buffer, thus minimising problems of nonspecific binding. In addition binding of D-Ab provides an enhancement of the response due to the bigger size of D-Ab (165 kDa) compared with PSA (33 kDa). Several different approaches were tested in our work to assure minimum interference of the blood and serum components. Among these are addition of polymerisable polyethylene glycol (PEG) to the polymer composition, blocking surface with a charged hydrophilic amino acid (aspartic acid) or with amino-PEG and inclusion of a surfactant like P20 (Tween20) into the analytical system. For comparison the same techniques were also applied to commercially available Biacore carboxydextran chips (CM5).

2. Materials and methods

Most compounds were obtained from commercial providers and were of analytical or HPLC grade. Triethylamine (TEA), bovine serum albumin (lyophilised powder) human serum (from male source) and IgG from bovine serum were purchased from Sigma (UK). Monoclonal mouse anti-PSA capture antibody (anti-PSA Ca-Ab), monoclonal mouse anti-PSA detection antibody (anti-PSA D-Ab) and PSA were purchased from Ab-Serotec (UK). Allyl thiol (AT), N,N-bis(acryloyl)cystamine (BAC), and o-phthaldialdehyde (OPA) were purchased from Fluka (UK). 2-Hydroxyethyl methacrylate (2-HEM), ethylene glycol dimethacrylate (EGDMA), poly(ethylene glycol) acrylate M_n 375, 2-aminoethyl poly(ethylene glycol) 3K, 1,1,1-tris(hydroxymethyl)propan trimethacrylate (TRIM) and 2-benzyl-2(dimethylamino)-4′- morpholinobutyrophenone (BDMB) were purchased from Aldrich (UK). Ethanolamine (ETA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), surfactant P20 (10% v/v), NaOH solution (0.2 M), 10 mM glycine–HCl pH 2.0, SIA Kit Au and CM5 chips were purchased from Biacore (Sweden). Solvents were of analytical or HPLC grade and supplied by Acros Organics (UK). The water was purified by a Milli-Q water system (Millipore, Bedford, MA, USA) and all the reagents used for Biacore experiments were filtered using a 0.22 μm filter from Phenomenex (UK).

2.1. Polymer synthesis

The synthesis of the 3-D polymer used for the development of the immunosensor was performed as described previously [8]. Briefly the polymer was synthesised by mixing 2.0 mmol (260 mg) of 2-HEM, 0.3 mmol (60 mg) of EGDMA, 1.5 mmol (507 mg) of TRIM, 1.0 mmol (134 mg) of OPA, 2.0 mmol (150 mg) of AT, 0.1 mmol (26 mg) of BAC, 0.5 mmol (180 mg), BDMB (initiator) and DMF (5 mL) as solvent. A small amount of TEA (40 μl) was added to the monomer mixture, which was thoroughly purged with argon for 5 min. Polymerisation was initiated by placing the mixture under a high intensity Hönle 100 UV lamp (0.157 W cm^{-2}) for 20 min. The synthesised polymer was then precipitated from DMF by adding 20 ml of water and washed several times with methanol. Another 3-D polymer was also synthesised with the same protocol, but with 0.2 mg and 0.5 mg of polymerizable PEG acrylate added to the polymer composition.

2.2. Treatment of gold chips—gold surface modification

Sensor chips, SIA Kit Au (Biacore, Sweden) were used to assess the ability of polymer-coated surfaces to bind proteins. SIA Kit Au chips were cleaned for 3 min using oxygen plasma at 40 W (Emitech, UK). Polymer was self-assembled onto SIA Kit Au by immersing chips in 5 ml acetone/ethanol 50/50 (v/v) containing 10 mg ml^{-1} of polymer for 24 h. The polymer-coated gold chips were rinsed thoroughly with acetone/ethanol, dried with nitrogen and assembled onto the holder.

2.3. SPR experiments

All the SPR experiments were performed using a Biacore 3000 (Sweden) at 25 °C.

2.4. Evaluation of serum adsorption on sensor surfaces modified with antibodies and blocking agents

Biomolecules and blocking agents were immobilised onto 3-D polymer sensor surfaces and the immobilisation was monitored by Biacore 3000 in a continuous flow system. The changes observed in Biacore response in resonance units (RU) are directly proportional to the change of increased surface mass; 1 RU in general is equivalent approximately to 1 pg mm^{-2}. The antibody applied to study the performance of the polymer-modified surface for analyte detection in complex matrixes, like serum, was a

mouse monoclonal anti-PSA antibody (anti-PSA Ab) and the blocking agents applied to reduce the nonspecific adsorption from serum were BSA, ethanolamine (ETA), amino-PEG and aspartic acid. For comparison, the non-specific adsorption of serum on CM5 carboxymethylated dextran chip was also assessed by applying the same biomolecules and blocking agents. For the immobilisation performed on CM5 chips an activation step was carried as suggested by Biacore using EDC/NHS [32]. Activation of CM5 was performed by injecting 30 μl of 0.2 M EDC/0.05 M NHS with a flow rate of 5 μl min^{-1}. Typically protein (antibody and BSA) immobilisation was carried out on polymer-modified sensors by two consecutive injections of 50 μl of 25 μg ml^{-1} of Ab and 100 μg ml^{-1} of BSA in 0.01 M phosphate buffer saline (PBS), pH 7.4, with a flow rate of 10 μl min^{-1}. Immobilisation of Ab and BSA on CM5 chips was performed with the same procedure, but using 0.05 M Na-acetate buffer, pH 5.0, instead of PBS, as suggested by the manufacturer. After protein coupling, the remaining active groups on both surfaces (polymer modified and CM5 chips) were deactivated by injecting 30 μl of 1 M ETA pH 8.5, with flow rate 10 μl min^{-1}. In another set of experiments the sensor surfaces were directly modified with different blocking agents by consecutive injections of 50 μl aliquots in a flow stream with a flow rate of 10 μl min^{-1} until saturation is reached (without the presence of any biomolecules). The degree of adsorption of serum onto all the resulting surfaces was assessed by injecting 50 μl of 10% human serum diluted in PBS pH 7.4 with a flow rate of 10 μl min^{-1}. Furthermore serum adsorption on polymer-modified and CM5 chips was also studied following antibody immobilisation and blocking with ETA by including the surfactant P20 (0.005% v/v) in both serum solution and running buffer.

2.5. PSA detection in PBS with direct and sandwich detection

The immobilisation of anti-PSA capture antibody (C-Ab) on polymer-modified chips was performed by injecting 150 μl of 25 μg ml^{-1} of Ab solution in PBS buffer with pH 7.4 with a flow rate of 10 μl min^{-1}. After Ab coupling the remaining active thioacetal groups were deactivated by injecting 30 μL of 1 ETA at pH 8.5 and several injections (4–6) of 30 μl of BSA (200 μg ml^{-1}) in PBS pH 7.4 with a flow rate of 10 μl min^{-1} until surface saturation. After immobilisation of anti-PSA C-Ab on polymer chips and blocking, detection of PSA was performed in PBS buffer pH 7.4 with 20 μg ml^{-1} BSA and 0.005% (v/v) surfactant P20. This buffer was also used as running buffer for both direct and sandwich detections. For direct detection, PSA was diluted in the running buffer in concentrations ranging from 0.1 to 1000 ng ml^{-1} and injected for 5 min, with a flow rate of 10 μl min^{-1}. For detection of PSA in sandwich format the sensor signal was recorded by injecting 50 μl of 10 μg ml^{-1} anti-PSA D-Ab dissolved in running buffer with a flow rate of 10 μl min^{-1}. A reference channel containing immobilised IgG (from bovine serum) was used for the assessment of binding specificity. Regeneration was carried out by injecting 10 mM NaOH for 30–60 s for both direct and sandwich formats with a flow rate of 30 μl min^{-1}. In this work the limit of detection (LOD) was determined as signal value of PSA response which exceeds three times of standard deviation recorded for the reference channel.

2.6. PSA detection in serum with sandwich format

The running buffer for PSA detection in serum was PBS pH 7.4 containing 0.005% (v/v) P20. For the experiments human serum was diluted five times in running buffer (20% serum). This solution was then used to prepare samples spiked with PSA in concentrations ranging from 0.1 to 500 ng ml^{-1}. Firstly anti-PSA C-Ab were immobilised on polymer-modified chips as described above. Then diluted human serum samples spiked with PSA were injected into the chip with immobilised C-Ab for 5 min with a flow rate of 10 μl min^{-1}. Afterwards the detection of analyte was performed by injecting 50 μl of 10 μg ml^{-1} anti-PSA D-Ab with a flow rate of 10 μl min^{-1}. The signal was corrected by subtracting the values obtained from injections of diluted serum in the reference channel with immobilised C-Ab. In other experiments the specificity of the PSA binding reaction was assessed by injection of PSA in channel with immobilised IgG (non-specific for PSA) which was exposed to the same procedure of blocking and detection as for the working channel.

3. Results and discussion

3.1. Immobilisation of antibodies and blocking agents on sensor surface

The compounds used for blocking in sensor surface from nonspecific adsorption included anti-PSA C-Ab, BSA, aspartic acid, ethanolamine and amino-PEG. The magnitude of sensor response for all modified surfaces is illustrated in Table 1.

Biacore CM5 chips possess a 5 nm thick carboxydextran matrix, which swells to approximately 100 nm in the presence of aqueous solutions. Another important property of CM5 chips is the hydrophilicity and the presence of negative charges, which makes the surface repellent to many proteins and biomolecules. Table 1 shows that higher immobilisations of aspartic acid, amino PEG and ETA were obtained on polymer-modified surfaces than on CM5 chips. For aspartic acid, this is possibly due to electrostatic repulsion. Concerning the PEG and ETA, lower response might be attributed to the swollen matrix and the small size of the ligand, which is immobilised away from the gold surface, leading to reduced effect on the surface plasmons. This is due to the electrostatic repulsion between the negatively charged matrix on CM5 and the negative charged groups of the compounds when immobilisation is performed at pH 4.5–5.5. CM5 chips had higher binding capacity for BSA and anti-PSA C-Ab as compared with 3D polymer because of larger surface area.

To improve the performance on polymer-modified surfaces, polymerisable PEG was included in the polymer composition at 20% and 50% of the total mass of polymerisable material and the study repeated.

3.2. Effect of proteins, blocking agents and PEG polymer on non-specific interactions

The non-specific adsorption on sensor surfaces of complex matrixes such as serum is a limiting factor for direct analyte detection in real samples. Thus, various methods were tested here

Table 1
Biacore responses for polymer-modified and CM5 carboxydextran-coated surfaces to various blocking agents (immobilisation of blocking agent).

	Biacore responses (RU) on polymer-modified surfaces[a]	Biacore responses (RU) on CM5 chips [a]
BSA	1323 ± 166.7	10,126 ± 951.8
Anti-PSA Ca	2563 ± 417.8[b]	13,349 ± 293.7
Aspartic Acid	1260 ± 148.7	0
Amino-PEG	1398 ± 381.7	156 ± 51.3
ETA	774 ± 115.3	147.9 ± 43.8

[a] Standard deviation was calculated from a set of four experiments.
[b] Standard deviation was calculated from a set of 26 experiments.

Table 2
Biacore responses to serum on polymer-modified and CM5 chips with different surface blocking methods.

	Biacore response (RU)			
	Serum (1/10) in PBS on CM5 chips	Serum (1/10) in PBS on polymer-modified chips	Serum (1/10) in PBS with 0.005% P20 on CM5 chips	Serum (1/10) in PBS with 0.005% P20 on polymer-modified chips
ETA	96.4 ± 9.5	2160.4 ± 147.3	33.4 ± 8.2	1106.9 ± 27.8
Amino-PEG	125.9 ± 10.0	1685.5 ± 77.1	26.1 ± 13.6	847.6 ± 87.1
Aspartic acid	Low p*I*	1366.2 ± 102.6	Low p*I*	712.0 ± 62.2
BSA	102.1 ± 11.9	1095.7 ± 73.6	15.7 ± 4.1	431.0 ± 25.7
Anti-PSA Ab	1110.5 ± 61.4	1570.3 ± 113.5	980.7 ± 38.4	376.9 ± 91.4

in order to reduce the nonspecific interactions of serum with the sensor surfaces. In a first instance we have used addition of polymerisable PEG to monomer mixture which should work as a blocking agent to reduce non-specific adsorption of proteins. The reduction of non-specific serum adsorption on PEG-polymer-modified surfaces was ca. 37% when compared with the original polymer composition which was however insufficient for direct measurement of PSA in real samples.

Therefore, several other blocking methods were tested. Polymer-modified and CM5 chips were used for comparison, and the results are summarised in Table 2. It can be seen however that large increase in the level of nonspecific adsorption of serum, both on CM5 chips (980.7 RU) and polymer-modified surfaces (376.9 RU) occurs due to protein–protein interactions between matrix components and immobilised antibodies. This is demonstrated by the increased serum adsorption on CM5 chips following immobilisation of anti-PSA Ca-Ab. The inclusion of P20 both in running and immobilisation buffers reduces significantly the serum adsorption on Ab immobilised on polymer-modified surfaces, whereas it did not significantly improve non-specific adsorption on Ab immobilised on CM5 chips. This suggests hydrophobic interactions, possibly with the Fc region of antibody and serum proteins, which are greatly reduced on the polymer-modified chip by the presence of surfactant. On the bulkier CM5, surface charge is lost after protein immobilisation and subsequent surface blocking with ethanolamine, so this can lead to increased protein interactions, which are not completely suppressed by the surfactant.

These findings lead us to the conclusion that special care should be taken in choosing the material immobilised on a reference surface for a proper assessment of the level of non-specific binding, while measuring real samples. For example if the reference surface is only blocked only with ethanolamine or BSA the level of non-specific serum adsorption would be low, whereas it could be significantly higher if a different antibody is used as control. Also the adsorption of matrix components can differ from antibody to antibody and these can lead to positive or negative error in analyte determination. Thus, the choice of the biomolecules, which are applied for working and reference channel, can have a significant effect on the assessment of nonspecific binding. When detection of analytes is performed in complex matrices like serum, immobilisation of antibody fragments is preferable to whole antibody.

3.3. Detection of PSA in PBS

Detection of PSA in PBS both in direct and sandwich formats was carried out. For the sandwich format Anti-PSA, C-Ab and D-Ab, which bind different epitopes of the PSA, were used as receptor and detection element respectively. The running buffer was PBS containing 10 μg ml^{-1} of BSA. The inclusion of BSA in running buffer decreased dramatically the NSB on the reference channel. Fig. 1 shows the calibration curves obtained in direct and sandwich formats for increasing PSA concentrations prepared in PBS. The figure reports linear correlations between PSA concentration and Biacore responses in the range from 1.0 to 250.0 ng ml^{-1} for both detections systems. The limit of detection, LOD, for PSA in PBS was 0.1 ng ml^{-1} in both detection formats. This LOD is lower than the 10.2 ng ml^{-1} reported in literature by Cao and co-workers [33,36] and still better than the method developed by Besselink and co-workers [34], who achieved LOD of 0.15 ng ml^{-1} of f-PSA in PBS buffer containing 3% BSA by signal amplification with gold nanoparticles. Possible reason for achieving such a low LOD could be due to the application of non-conjugated antibodies. In fact chemical modifications of antibodies can cause complete or partial loss of their bioreactivity due to conformational changes induced by conjugation [30]. Another reason for achieving such a low LOD could also be the use of the 3D polymeric matrix for antibody immobilisation which offers advantages as compared with homogeneous flat surfaces [11]. This can be attributed to an improvement of protein diffusion in

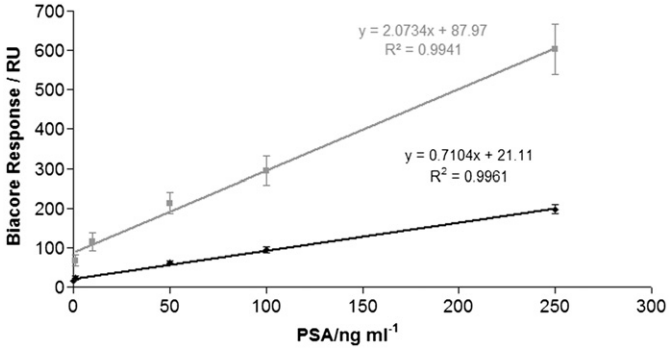

Fig. 1. Calibration curves of PSA detection in PBS in concentration ranging from 1 to 250 ng ml^{-1} obtained by direct (black) and sandwich formats (grey). Responses and standard deviations were calculated from a set of four measurements.

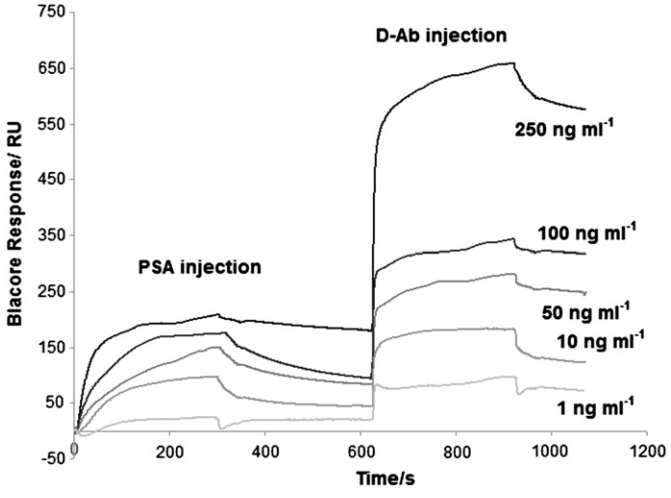

Fig. 2. Detection of PSA by sandwich format in concentrations ranging from 1 to 250 ng ml^{-1}. Firstly the antigen is injected on a 3D polymer surface where anti-PSA (C-Ab) was immobilised. After a reasonable time allowing for antigen dissociation, anti-PSA (D-Ab), which recognises a different epitope of the analyte, is injected and the detection signal is enhanced.

the polymer matrix, together with partial protection of protein structures from unfolding processes. Rigid or solid surfaces often cause irreversible denaturation of the bound proteins [1]. The application of the antibody D-Ab as detection element produces higher nonspecific binding (NSB) on reference surface, but at the same time higher signal on working channel. In this work the values of NSB, assessed on reference channel containing a different type of IgG, were 3.7 RU $\pm$ 3.1 (n=6) and 9.5 RU $\pm$ 4.2 (n=6) for PSA and D-Ab, respectively. One of the advantages of using a sandwich format is the possibility for signal enhancement of the analyte response after the injection of detection antibody. Here the signal obtained with the sandwich format was 2.5–3 times higher as compared with the direct detection which results in higher sensitivity, see Fig. 1. This is because in the present case the detection antibody has a larger molecular weight than that of the analyte. Also, another advantage is the possibility to perform the detection step in the absence of serum proteins.

As depicted in Fig. 1, the direct detection format and sandwich format showed satisfactory linearity for sensor applications, with R^2 values of 0.9961 and 0.9941 respectively. In both cases at concentrations higher than 250 ng ml^{-1} the curve approached saturation and there was significant deviation from linearity (data not shown). The Biacore sensorgrams obtained for PSA detection in concentrations ranging from 1.0 to 250.0 ng ml^{-1} using the sandwich format are reported in Fig. 2. An important step of the sandwich detection format is the choice of the time for the injection of D-Ab. The reason is that early injection of D-Ab during analyte dissociation will lead to errors and high standard deviations.

The regeneration of sensor surfaces after the immunoreaction is of high importance for making immunosensor applications more cost effective. The regeneration step was performed at the end of the sandwich assay by injecting 10 mM NaOH for 30–60 s (see Supplementary information, Fig. S1). The immunoassay showed similar response for three regeneration cycles performed as explained above (Section 3.3).

3.4. Detection of PSA in serum

Different strategies were employed to facilitate detection of PSA in serum, including the addition of PEG in polymer synthesis, blocking with amino-PEG and amino acids as well as inclusion of surfactant in running buffer. Unfortunately none of these methods were able to reduce the nonspecific adsorption of serum components on the polymer surface to the level suitable for direct detection and determination of PSA in clinical samples. Thus it was decided to perform PSA detection in serum in a sandwich format. The application of a second primary antibody as a detection element in this case is advantageous, since the 'noise',

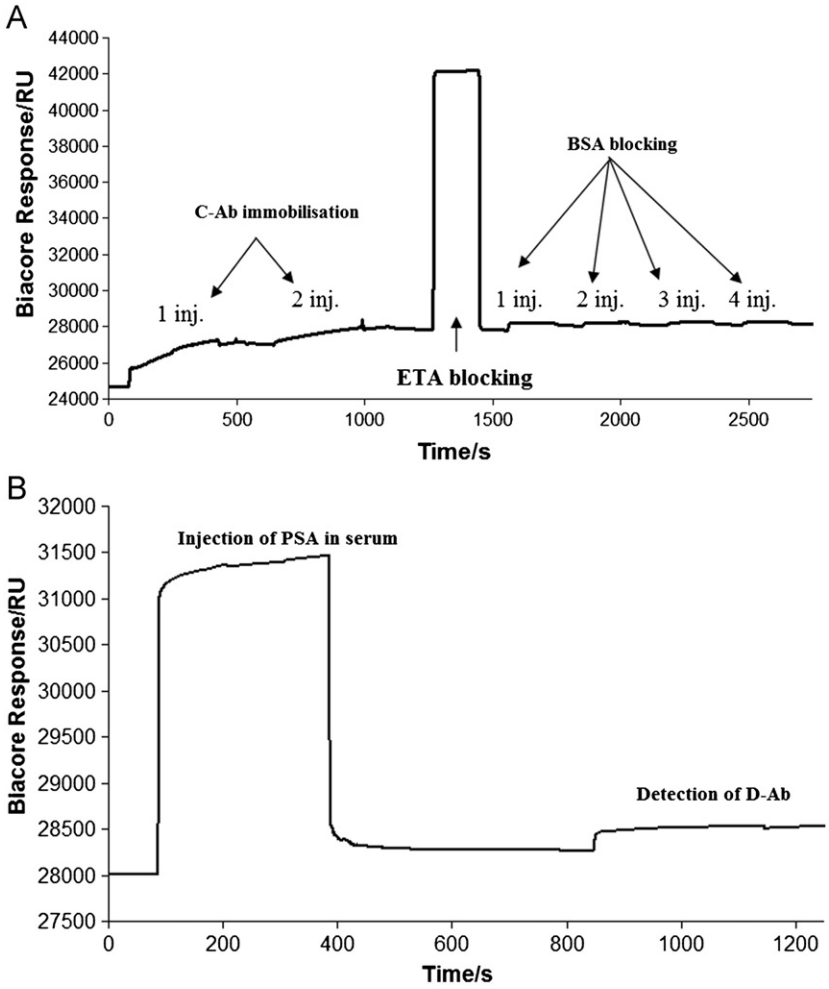

Fig. 3. Biacore sensorgram illustrating detection of 100 ng ml^{-1} of PSA by sandwich format. (A) Surface preparation for PSA detection. The first two injections correspond to the C-Ab immobilisation followed by blocking of unreacted binding sites by one injection of ETA and four injections of BSA for 180 s; (B) PSA detection in serum. Firstly the 20% serum solution spiked with PSA 100 ng ml^{-1} is injected. Afterwards detection is performed by injecting for 5 min 10 μg ml^{-1} of D-Ab prepared in running buffer (PBS with 0.005% P20).

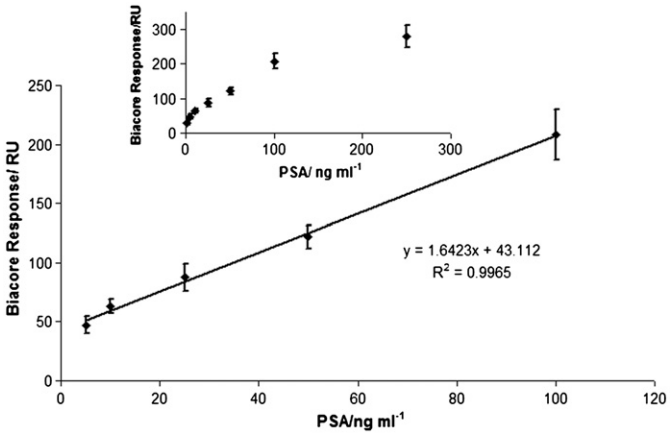

Fig. 4. Calibration curve obtained by plotting Biacore response (RU) after D-Ab injection vs. PSA concentrations added in serum solution. The inset shows the sensors responses to the entire range of PSA concentrations tested.

caused by adsorption of serum components on sensor surface during injection of the analyte, does not influence the detection step. PSA detection was performed in 20% serum (diluted in PBS with 0.005% P20) as described in Section 2. The use of a higher percentage of serum is not recommended for Biacore applications, since it can cause blockage of microfluidic system and further problems. The use of 20% serum for PSA detection is a step closer to real samples analysis, as compared with most applications reported in literature, where PSA is detected in 10% serum [32].

The measurement procedure for PSA detection in serum using the sandwich format is depicted in Fig. 3A and B. Ab immobilisation was carried out using two injections in order to saturate sensor surface with antibodies. ETA and BSA were used to block the unreacted binding sites. The injection of PSA and D-Ab is shown in Fig. 3B.

Several PSA concentrations ranging from 0.1 to 500.0 ng ml^{-1} were injected in order to determine the LOD. The Biacore responses obtained after injecting D-Ab vs. PSA concentrations ranging from 1.0 to 250 ng ml^{-1} prepared in 20% serum are shown in Fig. 4. As illustrated in the figure inset, the linear range for PSA determination in serum was between 5.0 and 100 ng ml^{-1}. The slope of the curve was reduced from 2.07, for detection of PSA in PBS (Fig. 1) to 1.64, which indicates a reduction in sensitivity. This result is in agreement with the work of Cao and colleagues, who also observed reduction in sensitivity when the determination of PSA was performed in serum [32]. In the work described here, the lowest concentrations of PSA could not be measured with high confidence due to non-specific adsorption of D-Ab recorded on the control surface, which gave a signal of 12.6 RU $\pm$ 6.1 (for experiments repeated six times). This control (reference) channel was prepared by immobilising C-Ab on polymer-modified chips followed by blocking. The non-specific binding to this channel was assessed by injecting 20% serum with following injection of D-Ab. The LOD of the sensor was 5.0 ng ml^{-1} in serum. The sensitivity achieved in these experiment is however insufficient for practical application since the concentration of PSA level for healthy men varies between 0.1 and 3.0 ng ml^{-1} and the concentration range of clinical interest is 1.0–10.0 ng ml^{-1}.

Nevertheless the results obtained for PSA detection in serum are promising and a step forward on the development of a low cost, simple and fast immunosensor suitable for testing clinical samples. The LOD for PSA detection in serum, which was calculated as 5.0 ng ml^{-1}, can be considered as improvement compared with similar applications reported in literature [32]. The lower detection limit achieved in this work is due to optimised performance of the polymeric matrix used in immobilisation. It can potentially be further improved by using more sophisticated detection tools such as surface plasmon fluorescence spectroscopy (SPFS) [34], combining antibodies with gold colloidal nanoparticles [33], or using enzyme precipitation [35]. With this method the detection of PSA is made with Au nanoparticles conjugated anti-PSA antibody-HRP (Horse radish peroxidase). The amplification of the signal is achieved by precipitation of the labelling enzyme with DAB (3,3′-diaminodbenzidine) H_2O_2 substrate solution [35]. On the other hand, these signal enhancement strategies make the immunosensor more complicated, increasing time of analysis, cost and decreasing the reproducibility, which, again points towards the use of serum without dilution, which can be achieved with a compatible SPR flow-cell.

4. Conclusions.

In this work different strategies were tested for the development of a fast and simple SPR immunosensors for PSA detection in serum by employing a novel polymeric matrix for immobilisation of antibodies combined with use of different blocking agents such as PEG derivatives, ethanoloamine and aspartic acid. A significant reduction of serum adsorption on polymer surfaces was observed using P20. Addition of surfactant, however, did not reduce adsorption of serum on CM5 chips, where the antibody was immobilised. A sandwich immunosensor was developed for PSA detection in serum on polymer-modified surfaces. The detection limit of PSA in PBS was 0.1 ng ml^{-1} and the linear concentration range of the curve was between 1.0 and 250 ng ml^{-1}. Sensor demonstrated detection of PSA in 20% serum solutions with detection limit 5.0 ng ml^{-1} relative to the original serum concentration. The use of novel 3D polymers in immunosensors allowed substantial reduction in detection limit as compared with similar immunosensors reported in literature. The developed immunosensor is therefore a step forward towards a simple test, which could be successfully applied to detect proteins in real samples and in the case of PSA detection for early diagnosis of prostate cancer.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.10.042.

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