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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