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Polyethylene imine-based receptor immobilization for label free bioassays

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ABSTRACT

Polyethylene imine (PEI) based immobilization of antibodies is described and the concept is proved on the label free assay of C-Reactive Protein (CRP). This novel immobilization method is composed of a hyperbranched PEI layer which was deposited at a high pH (9.5) on the sensor surface. The free amino groups of PEI were derivatized with neutravidin by Biotin N-hydroxysuccinimide ester and the biotinylated anti-CRP antibody immobilized on this layer. Direct binding assay of recombinant CRP was successfully performed in the low μg/ml concentrations using a label free optical waveguide biosensor.

1. Introduction

An important aspect of biosensor development is the biofunctionalization protocol [1,2]. Conventional immobilization methods are based on silanization of sensor surfaces with amino (or epoxy, thiol) functional silanes and grafting the receptor molecules (typically antibodies) to the silanized surface by a cross-linker, such as EDC/NHS (or glutaradehyde, BS3, etc.) [3]. Except the well-known dextran layer in SPR applications [4], most detection assays rely on monolayer immobilization methods to graft antibodies to surfaces setting an ultimate limit to the surface coverage of receptor molecules. There is a need for more efficient immobilization methods with potential simplification of this multistep preparative work. It is especially the case when emerging technologies with disposable biochips are considered [5,6]. This contribution focuses on the design of an antibody containing graft architecture (composed of polyethylene imine, PEI) that could be used for biosensor surface preparation. In this work a table top biosensor was used to follow the building up of the molecular layers. Optical waveguide lightmode spectroscopy (OWLS) is a surface sensitive biosensor using evanescent waves. The technique was successfully applied to monitor lipid bilayers, protein adsorption or even living cells [7–9].

Pre-adsorbed polyelectrolyte layers are advantageously used in various applications from biosensors to basic physico-chemical studies of interfaces [10,11]. In such applications the utility of polyethylene imine is mainly to control the surface potential as PEI bears a number of ionizable amino-groups which possesses positive charge with a density depending on the acidity of the environment. Recently [12] used PEI for direct antibody immobilization for the preparation of neuron-adhesive coatings. Our aim was to use the PEI, a cationic polyelectrolyte adsorbed layer as a simple and robust method for surface functionalization with amino groups. The most common and wide-spread surface amination is based on silane reagents for the silica-type surfaces and for the OWLS sensing surface as well [13,14]. Silanization is normally completed by a high temperature curing on the sensing surface, a potential drawback in case of plastic made sensor chips [5,6,15].

The biotin binding proteins (avidin, streptavidin, neutravidin) are from a family of proteins presenting a high affinity and selectivity for biotin. This property permits to bridge a surface coated with avidin and the biotinylated antibody. The system presents a double advantage: an appropriate orientation of antibodies (if biotin is bound to their Fc fragments) and a high affinity of immobilization. Among various technologies being used for linking functional groups to bio-molecules, the avidin/biotin noncovalent linkage has attracted significant focus due to the simplicity and versatility of this system [16,17]. Many proteins can be conveniently biotinylated, as in the case of antibodies (Immunoglobulines; Ig). In this work biotinylated anti-CRP antibody (sheep IgG) was obtained from

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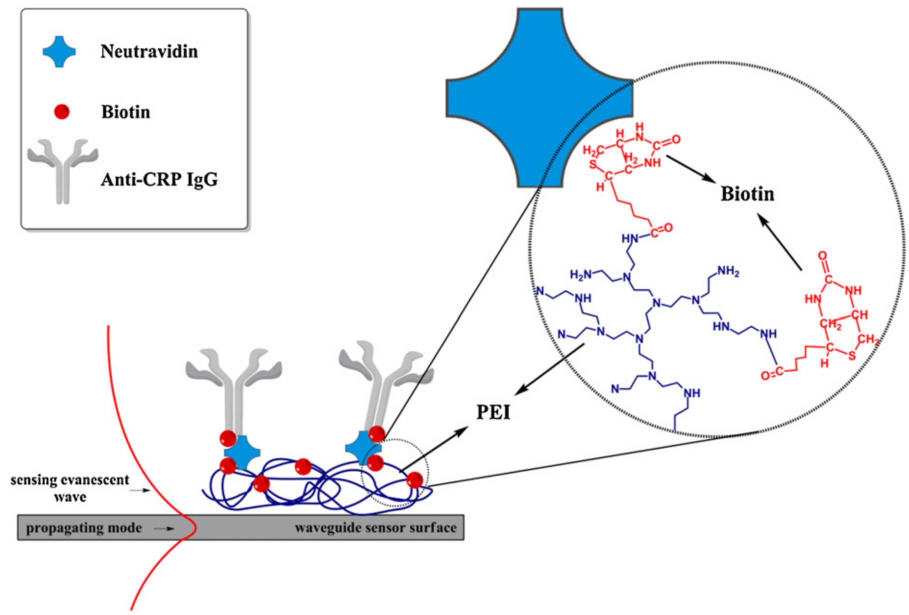

Fig. 1. Schematic view of the proposed OWLS biochip surface showing the molecular structure for immobilization of IgG. The method uses biotin–avidin interaction on a hyperbranched polymeric PEI interface layer.

commercial resource. CRP has been acknowledged as a possible predictor of the risk of an imminent stroke. It has been reliably observed that superior concentrations of CRP are related with larger brain infarcts and poor neurologic outcome. Whether a diminution of CRP quantity could be favourable to stroke patients remains to be elucidated [18]. As part of our project, we selected CRP recognition as a convenient way to demonstrate the immobilization chemistry under development. ELISA workflows were used to evaluate antibody-antigen integrity and affinity. There is a need for advanced label-free detection of biomarkers, and for the case of CRP a sensitivity of 0.1 μg/ml was reported after signal enhancement using NHS-dextran surface chemistry in SPR biosensor [19].

The strategy of this interface chemistry was inspired by a previous work [20], where the researchers built up multilayers by repeated deposition of avidin and biotinylated polymers. Such avidin-polymer layers have not been applied in biosensors to the best of our knowledge. Our proposed immobilization layer is composed of a surface adsorbed PEI (crosslinked by glutaraldehyde), functionalized with a biotin-linker, affinity bound neutravidin and finally the biotinylated IgG as shown in Fig. 1. The proposed PEI-biotin-neutravidin layer is a monolayer, however possesses an extended molecular structure originating from the hyperbranched PEI molecular structure at basic pH.

2. Material and methods

Branched PEI with a molecular weight of 750 kDa purchased from Sigma–Aldrich, was used as obtained. Biotin N-hydroxysuccinimide ester (NHS-biotin) was obtained from Sigma–Aldrich and was dissolved in dimethylformamide, stored at −20 °C in aliquots until use. Phosphate Buffered Saline (PBS) tablets have been obtained also from Sigma-Aldrich which yield a 10 mM phosphate buffer containing 27 mM potassium chloride and 137 mM sodium chloride, pH 7.4, at 25 °C. Ultrapure water was used for all the preparations (Direct-Q system). Borate buffer (12.5 mM) was prepared from borax and sodium hydroxide to give pH 9.5 solution.

CRP – Recombinant human C-Reactive Protein (CRP) was obtained from R&D systems. Polyclonal sheep IgGs against human CRP unconjugated and conjugated with biotin were purchased from R&D Systems. All these products were received lyophilized and they were reconstituted according to the manufacturer's instructions. Rabbit anti-sheep secondary antibody conjugated with alkaline phosphatase (AP) were purchased from Abcam. Streptavidin conjugated with AP as well as AttoPhos® AP Fluorescent Substrate were obtained from Promega. Non-treated 96-well plate (black, polystyrene) as well as neutravidin High Binding Capacity Coated Plates (black 96-well) were obtained respectively from Perkin-Elmer and Pierce. HSCD7 buffer (DB, polyvinyl alcohol, 80% hydrolyzed, Mr 9000–10,000 (Aldrich, Milwaukee, WI, USA), MOPS (Sigma), NaCl, MgCl2 (Sigma), $ZnCl_2$ (Aldrich), pH 6.90, BSA 30% solution, manufacturing grade (Serological Proteins, Kankakee, IL, USA)) were freshly prepared.

2.1. CRP antibody-antigen integrity and affinity evaluation by ELISA

2.1.1. Biotinylated anti-CRP antibody coating

ELISA was performed on commercially available neutravidin coated 96-well plates. Plate wells were pre-wet with 50 μl of borate buffer and then 50 μl of biotinylated anti-CRP antibody diluted in HSCD7 buffer were assayed at seven different concentrations (2000, 800, 320, 128, 51.2, 20.5 and 8.2 ng/ml). The unconjugated anti-CRP antibody was also assayed and used as negative control. The coating step was performed for 1 h at 37 °C. After washing steps with borate buffer (3 times with 100 μl using an automatic washer from Bio-Rad), a secondary antibody conjugated with alkaline phosphatase (diluted 1:5000 in HSCD7, 50 μl/well) was then incubated for 1 h at 37 °C. After a last washing step performed as previously described, 50 μl of AttoPhos® AP Fluorescent Substrate (Promega) was added to each well and the plate was immediately read by fluorescence (excitation 444 nm, emission 555 nm) in kinetic mode (each 2 min during 10 min) using a SpectraMax Gemini XS microplate reader (Molecular Device). Blanks (corresponding to 0 ng/ml of coated biotinylated anti-CRP antibody) were also assayed and defined as the noise. Results were calculated as signal to noise ratio.

2.1.2. Human CRP recognition

ELISA was performed on commercially available non-treated 96-well plates. 50 μl of antigen (recombinant human CRP) diluted