The application of polythiol molecules for protein immobilisation on sensor surfaces

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ARTICLE INFO

ABSTRACT

The immobilisation of bio-receptors on transducer surfaces is a key step in the development of biosensors. The immobilisation needs to be fast, cheap and most importantly should not affect the biorecognition activity of the immobilised receptor. The development of a protocol for biomolecule immobilisation onto a surface plasmon resonance (SPR) sensor surface using inexpensive polythiol compounds is presented here. The method used here is based on the reaction between primary amines and thioacetal groups, formed upon reaction of o-phthaldialdehyde (OPA) and thiol compounds. The self-assembled thiol monolayers were characterised using contact angle and XPS. The possibility to immobilise proteins on monolayers was assessed by employing BSA as a model protein. For the polythiol layers exhibiting the best performance, a general protocol was optimised suitable for the immobilisation of enzymes and antibodies such as anti-prostate specific antigen (anti-PSA) and anti *Salmonella typhimurium*. The kinetic data was obtained for PSA binding to anti-PSA and for *S. typhimurium* cells with a detection limit of 5×10^6 cells mL^{-1} with minimal non-specific binding of other biomolecules. These findings make this technique a very promising alternative for amine coupling compared to peptide bond formation. Additionally, it offers opportunity for immobilising proteins (even those with low isoelectric point) on neutral polythiol layers without any activation step.

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1. Introduction

Immunoassay technology is currently growing rapidly due to market demands for low cost, easy to use and sensitive biosensors (Vikholm, 2005; Sadana, 2006). Surface plasmon resonance (SPR), quartz crystal microbalance (QCM), cantilever and electrochemical detectors are the most widespread platforms used with immunosensors. The main advantages of these when compared with immunoassays as ELISA, are the label free detection and the opportunity for measuring biochemical interactions in real time. This way kinetic and affinity constants can easily be obtained (Haga et al., 2008; Katsamba et al., 2006; Regnault et al., 1998).

The ligands (biomolecules) are usually attached on sensor surfaces by physical adsorption (Predki, 2004), covalent attachment (Kusnezow and Hoheisel, 2003; O'Shannessy et al., 1992) or ligand capture, which mainly refers to the strong interaction between biotilynated ligands and immobilised steptavidin or avidin (Craft et al., 1998; Panayotou et al., 1998). Covalent attachment is

used because it provides a strong and stable binding of the ligand/receptor to the sensor surface. This allows easy regeneration of sensors using conditions which can remove the analyte from the surface, but not the attached ligand itself. Covalent immobilisation includes amino coupling (Lofas et al., 1995; Piletska et al., 2001), aldehyde coupling (Abraham et al., 1995) and thiol coupling methods (Johnson et al., 1995). The covalent attachment can also occur on gold surfaces modified with polymers such as carboxydextran matrix (Lofas et al., 1995) and thioacetal matrix (Kyprianou et al., 2009) or self-assembled monolayers (Nuzzo and Allara, 1983). The selection of the immobilisation procedure is a critical point for the development of a successful sensor. This is because the immobilisation may cause denaturation of ligand/receptor or alter the structure of binding sites (Butler, 2000) with consequent loss of bioreactivity. The direct attachment of the receptor on the sensor surface is however unadvisable since it can cause irreversible denaturation of the bound proteins (Su et al., 1998). The application of SAMs or polymers has advantages and disadvantages and selection of one over the other depends on the application. For example, flat surfaces with self-assembled monolayers (SAMs) are beneficial compared to polymeric layers (carboxydextran) both when the analytes of interest are large molecules such as cells and viruses

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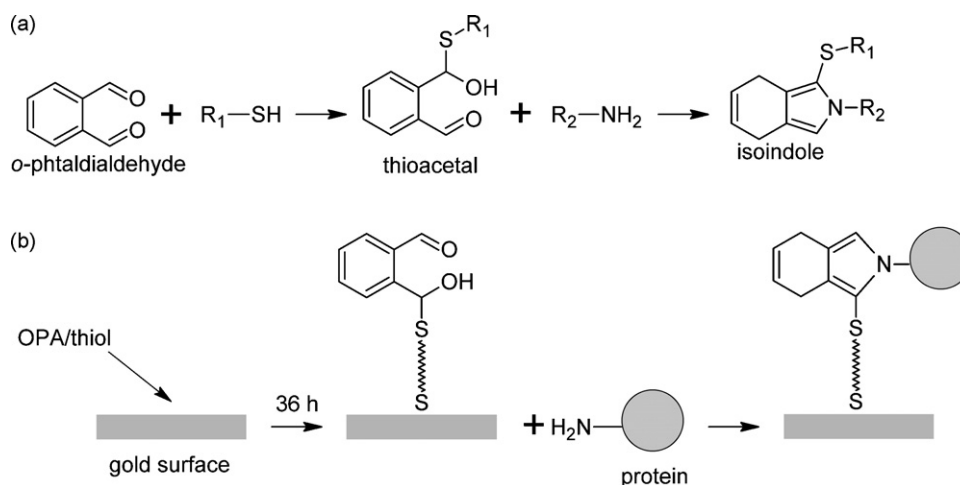*E-mail address:* a.guerreiro@cranfield.ac.uk (A.R. Guerreiro).

Fig. 1. (a) The reaction between o-phthaldialdehyde (OPA) and thiol with formation of hemithioacetal. Further reaction with primary amines will result in the formation of the fluorescent isoindole complex between the hemithioacetal and primary amine. R_1–SH represents a thiol molecule; R_2–NH$_2$ a primary amine. (b) Schematic representation of the process used for the immobilisation of proteins using the reaction described in (a).

and for kinetic parameters determination, when a low amount of non-specific binding is fundamental and low level of immobilised ligand is recommended (Biacore Sensor Surface Handbook). Gels matrices also complicate measurements due to diffusion restriction for large molecules and cells. The achievement of low or negligible nonspecific binding to sensor surface is another significant factor contributing to the success of sensor applications. Nonspecific binding contribution during measurement leads to positive standard errors in analyte determination and causes errors in calculation of kinetic constants, especially for complex sample matrices like serum (Kusnezow and Hoheisel, 2003). Reduction of nonspecific binding can be achieved by creating more hydrophilic sensor surfaces or by including compounds such as polysaccharides/polyethylene glycol derivatives in the immobilisation steps (Masson et al., 2005). Another way for reducing nonspecific binding is the addition of surfactants like P20 to analyte solutions (BIAapplications Handbook, 1994).

Here we report the development of SAMs on which the ligand immobilisation is based on the reaction between primary amines, thiol and o-phthaldialdehyde (OPA), see Fig. 1 (Simons and Johnson, 1978). This reaction takes place without any pre-activation of the surface making it suitable for sensor/array fabrication. In this study several thiols molecules were tested using both a spectrofluorophotometer (recording fluorescence upon isoindole formation at the end of the reaction) and surface plasmon resonance (SPR) with bovine serum albumin (BSA) as model protein. Among the thiols tested, the ones demonstrating the most promising results were applied for kinetic studies and analyte detection. The selected molecule which contains 4 thiol groups (pentaerythritol tetrakis (3-mercaptopropionate)) showed satisfactory protein binding and was unaffected during surface regeneration. In addition the SAMs obtained with this molecule showed stability and negligible nonspecific binding when tested by SPR. As a final study the results were compared to those obtained using corresponding commercially available sensors (Biacore chip, C1). Our novel monolayer proved to possess equal and in some cases improved features compared to the commercially available chips.

2. Materials and methods

2.1. Reagents

All compounds were obtained from commercial distributors and were of analytical or HPLC grade. Bovine serum albumin (lyophilized powder), IgG from bovine serum (95%), trimethy-

lolpropane tris (2-mercaptoacetate) (TMPTMA), pentaerythritol tetrakis (3-mercaptopropionate) (PETMP), 1,6-hexanedithiol (HDT), 1,9-nonanedithiol (NDT), 2,5-dimercapto-1,3,4-thiadiazole (DMTZ) and the enzymes trypsin (lyophilised powder, from bovine pancreas), carbonic anhydrase (electrophoretically purified, dialised and lyophilised), pepsin (lyophilised powder from porcine gastric mucosa) were purchased from Sigma–Aldrich (UK). o-Phthaldialdehyde (OPA) and DL-dithiothreitol (DTT) were obtained by Fluka (UK). Mouse monoclonal antibody anti-PSA and anti *Salmonella typhimurium* (ST) as well as the native human prostate specific antigen (PSA) were purchased from Abd Serotec (UK). ST cells were kindly provided by Dr. Tothill research group (Cranfield Health, Cranfield University).

Ethanolamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxy-succinimide (NHS), sodium dodecyl sulphate (SDS) solution (0.5%, v/v), P20 (10%, v/v), NaOH solution (0.2 M), 10 mM glycine–HCl, pH 2.5, SIA Kit Au and C1 chips were purchased from Biacore (Sweden). Solvents were supplied by Acros Organics (UK). The water was purified by Milli-Q water system (Millipore, Bedford, MA, USA) and all the reagents used for Biacore experiments were filtered using a 0.22 μm teflon filter from Phenomenex®

2.2. Assessment of thiol reactivity

An initial assessment of the reactivity of different thiols was performed by measuring the fluorescence produced by the isoindole derived from the reaction between the thioacetal (after reaction with OPA) and primary amine groups (Fig. 1). Stock solutions were prepared by mixing thiols with OPA in molar ratio of thiol groups/OPA of 2:1 in DMF/ethanol (1:1) solution. Although all stock solutions contained 2.0 mM of thiol molecules the OPA concentration varied in order to maintain the molar ratio thiol groups/OPA 2:1. The resulting fluorescence was recorded every 15 min, after 1:10 dilution of stock solutions in DMF/ethanol (1:1) and addition of 7.5 μL NH$_4$OH 6 M as a source of primary amino groups. The emission of the solutions was measured between 400 and 460 nm in a 3 cm^3 quartz cuvette using a RF-5301 PC spectrofluorophotometer (Shimadzu, Japan) with 360 nm as excitation wavelength. Maximum fluorescence signal was observed between 430 and 440 nm.

2.3. SPR testing

The performance of different thiols on SPR sensor surfaces was evaluated by using Biacore 3000 (Sweden) and Au-coated chips (SIA