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Comparative study of thiolated Protein G scaffolds and signal antibody conjugates in the development of electrochemical immunosensors

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Abstract

To achieve a high efficiency of analyte capture by a capture antibody attached to an electrochemical immunosensor, we have immobilised an analyte-specific antibody on a self-assembled layer of recombinant Protein G that was thiolated with succinimidyl-6-[3′-(2-pyridyldithio)-propionamido] hexanoate (LC-SPDP). Then two techniques were employed for conjugating a second antigen-specific antibody to alkaline phosphatase (mAb2-AP) using either LC-SPDP or the biotin–streptavidin interaction as the mode of cross-linking the antibody and enzyme. After characterising the two mAb2-AP preparations (mAb2-(LC-SPDP)-AP and mAb2-(Biotin-SA)-AP), they were each used as the signal antibody for immunosensors formatted for two-site immunoassays where the capture antibody was attached to a Protein G-(LC-SPDP) scaffold on gold electrodes. The antibodies and assays were specific for the clinically important hormone, human chorionic gonadotrophin (hCG). Protein G-(LC-SPDP) provided a stable scaffold, while mAb2-(LC-SPDP)-AP and mAb2-(Biotin-SA)-AP performed well as the signal antibodies. Immunosensors with mAb2-(Biotin-SA)-AP were characterised by a limit of detection of 216 IU L^{-1} for hCG and a linear response up to approximately 2000 IU L^{-1}. Conversely, immunosensors with mAb2-(LC-SPDP)-AP exhibited a limit of detection of 240 IU L^{-1} and a linear response up to 4000 IU L^{-1}.

Keywords: Electrochemical immunosensors; Protein G; Self-assembled protein layer; Antibody orientation; Antibody conjugation

1. Introduction

Two important considerations in designing an electrochemical immunosensor are the mode of immobilising the capture antibody on the sensor surface and the efficient generation of the electrochemical signal. In determining a mode of immobilisation of the capture antibody on the sensor surface, it is necessary to consider the orientation of the immobilised antibody with respect to its antigen binding sites. Recently, there have been several reports addressing this aspect in constructing electrochemical immunosensors on carbon electrodes (Diaz-Gonzalez et al., 2005; Micheli et al., 2005). For example, Micheli et al. reported that pre-coating the surface of a screen-printed electrode with anti-IgG antibody subsequently aided in immobilising an anti-aflatoxin M1 capture antibody with its antigen binding sites oriented optimally for binding the analyte. Nonetheless, the majority of methods employed for immobilisation of the capture antibody in electrochemical immunosensors offer limited or no control over the orientation of antibodies with respect to their antigen binding sites (Dong et al., 2006; Wang et al., 2006; He et al., 2007; Ordonez and Fabregas, 2007). Therefore, without incorporating a large excess of antibody, this uncontrolled orientation will result in a compromised dynamic range and sensitivity.

Recently, the direct formation of self-assembled layers of bacterial antibody-binding proteins (Protein A and Protein G) on gold electrodes has been demonstrated (Lee et al., 2003; Oh et al., 2004; Bae et al., 2005). Such proteins will only bind antibodies through their non-antigenic (Fc) regions, leaving the antigen binding sites of the immobilised antibody available to bind to their target antigen. Specifically, Protein G is a cell surface protein of group C and G Streptococci with three Fc binding domains located near its carboxy-terminal and exhibits specificity for subclasses of antibodies from many species (Bjoerck

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and Kronvall, 1984). The amine groups from a series of lysine residues within Protein G can be converted to thiol groups by reacting with 2-iminothiolane (Oh et al., 2004). This results in the protein having high affinity for gold, thus facilitating the direct formation of a Protein G layer on a gold surface.

Our laboratory has recently investigated the properties of a layer of Protein G-(2-iminothiolane) directly formed on a gold electrode and compared it with a layer of Protein G that is covalently bound to a functionalised alkanethiol self-assembled monolayer (SAM) (Fowler et al., 2007). We found that the Protein G-(2-iminothiolane) layer provided faster immobilisation of the capture antibody that had an improved orientation for binding analytes. Thus, a thiolated Protein G layer directly formed on the surface shows promise in providing a more suitable scaffold for an electrochemical immunosensor. We expect the characteristics of this Protein G layer to be further improved by minimising steric interactions near the electrode surface, allowing for more efficient interaction with the capture antibody. This may be achieved using a long heterobifunctional spacer, such as succinimidyl-6-[3′-(2-pyridyldithio)-propionamido] hexanoate (LC-SPDP), in place of 2-iminothiolane, resulting in the layer being positioned a greater distance away from the electrode surface.

Another important consideration in designing an electrochemical immunosensor is the mode of generating the electrochemical signal. In the case of electrochemical immunosensors based on a two-site sandwich immunoassay, this is commonly achieved using an enzyme conjugated to a signal antibody, specific for a site different from that of the immobilised capture antibody. Alkaline phosphatase (AP) and horseradish peroxidase are the two most commonly used enzymes for this purpose. In conjugating the enzyme to the antibody, it is necessary to use a cross-linker with an appropriate length to reduce possible steric hindrance that arises from having the two large proteins in relatively close proximity (Bieniarz et al., 1996). An example of a heterobifunctional linker is LC-SPDP, which has a spacer length of 30 Å when the proteins are conjugated (Carlsson et al., 1978). LC-SPDP reacts with primary amines of a protein to form a stable amide bond, and it can link two proteins together by the formation of a disulfide bond. A distinct advantage of such a heterobifunctional reagent is the ease of forming conjugates of different proteins (heteroconjugates), without undesirable cross-reaction products (homoconjugates). Another example of a cross-linking system makes use of the biotin–streptavidin interaction and involves the biotinylation of the antibody on a solid phase using a tetraethylene oxide-functionalised biotin molecule (Strachan et al., 2004). The biotinylated antibody is then mixed with streptavidin-conjugated enzyme, resulting in conjugation with a spacer-arm length of 29 Å. The solid-phase biotinylation format requires less time than that performed in solution, but enables increased efficiency of the biotinylation reaction and the minimisation of dilution, enabling small quantities of antibody to be used.

In this work, we have evaluated the performance of a self-assembled layer of thiolated Protein G as a scaffold for an electrochemical immunosensor, using the longer spacer, LC-SPDP, as an alternative to 2-iminothiolane. Signal antibody–enzyme conjugates employing cross-linkers of LC-SPDP or long-chain biotin–streptavidin were also prepared and the characteristics of each were investigated. Finally, electrochemical immunosensors based on Protein G-(LC-SPDP) and a sandwich immunoassay using signal antibody–enzyme conjugates, employing either of the above-mentioned cross-linkers, were constructed to evaluate the performance of these components. The clinically important hormone, human chorionic gonadotrophin (hCG), was used as a model analyte throughout the development of the immunosensors. hCG is naturally produced by trophoblasts of the placenta during pregnancy, and is also an important marker for trophoblastic tumours (Braunstein et al., 1976; Ozturk et al., 1987).

2. Materials and methods

2.1. Materials and instrumentation

Recombinant Protein G (MW 17,000) was purchased from Amersham Biosciences Pty. Ltd. (Sydney, Australia). Anti-hCG murine monoclonal antibodies (mAb1 and mAb2) were from Bioclone (Sydney, Australia). hCG of 12,500 IU mg^{-1} potency was purchased from Merck (Sydney, Australia). LC-SPDP and an EZ-Link NHS-PEO Solid Phase Biotinylation Kit were purchased from Pierce (Illinois, USA). A Superdex 200 PC 3.2/30 size-exclusion column and a PD-10 column were purchased from GE Healthcare (Uppsala, Sweden). Conjugation grade AP was from MP Biomedicals (Ohio, USA). Streptavidin-alkaline phosphatase ((SA)-AP), a bicinchoninic acid (BCA) QuantiPro Protein Assay Kit, 2-iminothiolane, Chloramine T, dithiothreitol (DTT), bovine serum albumin (BSA), human IgG and 4-nitrophenyl phosphate (4-NPP) were purchased from Sigma–Aldrich (Sydney, Australia). Centrifugal filtration devices with 10,000 MW cut-off were purchased from Millipore (Sydney, Australia). Sodium metabisulfite was from BDH (Victoria, Australia). ^{125}I used for radiolabelling mAb1 and hCG was received as $Na^{125}I$ in NaOH from Australian Nuclear Science and Technology Organisation (Sydney, Australia). Sheep anti-mouse serum used in radioimmunoassays was purchased from Chemicon (Melbourne, Australia). Alumina powder of 0.05-μm diameter was purchased from LECO (Sydney, Australia). The AP substrate, 4-aminophenyl phosphate, was prepared by the reduction of 4-NPP. 4-NPP (250 mg) was dissolved in methanol and Pd/C catalyst was added. The solution was sparged with hydrogen and then sealed. The solution was stirred vigorously for 24 h, while an atmosphere of hydrogen was maintained. The reaction mixture was filtered and reduced to dryness under a stream of nitrogen, yielding a white solid that was characterised by ^{1}H-NMR (data not included).

The following buffers were prepared in Milli-Q water and adjusted to appropriate pH levels with NaOH or HCl: phosphate-buffered saline (PBS), pH 7.6, containing 10 mM phosphate (Na_2HPO_4/KH_2PO_4) and 0.15 M NaCl; PBS-EDTA, pH 7.6, containing 1.0 mM EDTA; BSA-PBS, pH 7.6, containing 0.5% BSA; sodium acetate, pH 4.5, containing 0.10 M sodium acetate and 0.10 M NaCl; glycine, pH 10.4, containing 0.10 M glycine and 0.10 M NaCl.