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Disposable immunosensor using a simple method for oriented antibody immobilization for label-free real-time detection of an oxidative stress biomarker implicated in cancer diseases

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ABSTRACT

This work proposes a novel approach for a suitable orientation of antibodies (Ab) on an immunosensing platform, applied here to the determination of 8-hydroxy-2′-deoxyguanosine (8OHdG), a biomarker of oxidative stress that has been associated to chronic diseases, such as cancer. The anti-8OHdG was bound to an amine modified gold support through its Fc region after activation of its carboxylic functions. Non-oriented approaches of Ab binding to the platform were tested in parallel, in order to show that the presented methodology favored Ab/Ag affinity and immunodetection of the antigen.

The immunosensor design was evaluated by quartz-crystal microbalance with dissipation, atomic force microscopy, electrochemical impedance spectroscopy (EIS) and square-wave voltammetry. EIS was also a suitable technique to follow the analytical behavior of the device against 8OHdG. The affinity binding between 8OHdG and the antibody immobilized in the gold modified platform increased the charge transfer resistance across the electrochemical set-up. The observed behavior was linear from 0.02 to 7.0 ng/mL of 8OHdG concentrations. The interference from glucose, urea and creatinine was found negligible. An attempt of application to synthetic samples was also successfully conducted.

Overall, the presented approach enabled the production of suitably oriented Abs over a gold platform by means of a much simpler process than other oriented-Ab binding approaches described in the literature, as far as we know, and was successful in terms of analytical features and sample application.

1. Introduction

Immunosensors combine an antibody as biological recognition element with a transducer signaling device that follows the binding of the free antigen (Ag) present in the solution under analysis and the antibody (Ab) anchored to a solid support. The transducer converts electrical, optical, mass or heat changes of the solution into a measurable signal (Byrne et al., 2009; D'Orazio, 2003).

A critical step in immunosensor design is the immobilization of the Ab on the solid support (Matharu et al., 2012). An Ab is a large protein composed of hundreds of amino acids, arranged in a tridimensional order that is recognized as a Y shape and where the carboxyl (−COOH) end of the peptide chain is positioned at the lower end of this Y-shape structure. This region is also recognized as Fc. Each Ab binds to a specific part of an Ag (epitope region), through the two upper-end parts of this Y shape (paratope region) that are amine-terminated. Each Ab is able to bind two Ag species because there are two paratope sites in a single structure. This is also feasible when the Ab is attached to solid-supports, provided that the Ab is attached through the Fc region, therefore leaving both paratope sites free for Ag binding.

Many approaches for immobilizing antibodies on a solid support lead to random orientation of these proteins all over the modified surface, creating supports of low density binding sites and of decreased binding affinity (Song et al., 2012). These include physical/electrostatic non-covalent adsorption or covalent coupling via amine moieties, yielding surfaces highly covered by antibodies with inactive orientation due to steric blocking of the paratope sites (Alves et al., 2012). Despite resulting in a significant loss of Ab activity, these methods are still commonly used due to their simplicity of execution.

Alternative strategies leading to suitable Ab orientation have also been presented (Alves et al., 2012; Kausaite-Minkstimiene et al., 2010; Song et al., 2012). The most common approach includes attaching protein A or protein G to the biosensing surface prior to Ab binding. These proteins bind specifically to the Fc region of the antibodies, providing a suitable orientation of the antibodies all over the surface. However, this additional step upon the surface modification decreases the ordered organization upon

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the biosensing surface, simply because the proteins are also randomly attached to the surface. Other approaches employ the reduction of disulfide bonds between the peptide chains present in the same Ab, using the resulting thiol side chains as reactive sites for conjugation to gold or maleimide functionalized surfaces (Brogan et al., 2003; Lofas et al., 1995; Vikholm-Lundin, 2005). This approach leads to many inactive Ab fragments where side-disulfide bonds may be reduced accidently (Alves et al., 2012). The orientation of these fragments upon the surface is also not effective because in practice it becomes randomly bond to a gold surface, yielding many non-specific regions for protein binding. Other approaches include oxidizing carbohydrate chains that are present on the antibodies, forming reactive aldehyde groups that can bind amine functionalized surfaces (Han et al., 2010). This technique provides a high coupling yield, but the denaturing conditions and oxidative chemicals used can result in a significant loss of Ab activity. Overall, methods reporting oriented Ab binding involve complex chemical procedures, requiring many stages of chemical modification that decrease the technical control over the nanostructures and some of these stages may also hinder Ab activity (Alves et al., 2012). This stresses the need for the development of a simple method for site oriented immobilization.

Thus, this work proposes a different and simpler immunosensor design, by activating the carboxylic residues at the Fc site (via carbodiimide reaction) and coupling these directly to an amine functionalized surface. This carboxylic activation process has been employed for decades for protein covalent binding and is expected to be compatible with protein structures, therefore preventing significant loss of activity of Ab. In addition, although it may affect all carboxylic functions in the outer layer of the protein structure, there is a strong probability that most of the antibodies become suitably oriented. Among the 20 amino acids in nature, only aspartic and glutamic acids have carboxylic acid side chains, and these would only be affected if exposed/directed to the outer surface of the 3D structure of the protein.

This concept is here applied to an electrochemical impedance spectroscopy (EIS) transduction, a direct and label-free method for probing protein binding events such as those from Ab–Ag interactions (Guan et al., 2001; Holford et al., 2012; Ronkainen et al., 2010). It offers fast, accurate, sensitive, selective and quantitative responses, coupled with portability, low cost and minimal power requirements. EIS is also independent from sample turbidity or optical pathway, widening the range of samples to which it may be applied.

As target analyte, a biomarker of oxidative stress will be employed. Oxidative stress (OS) is the imbalance between oxidant-producing systems and antioxidant defense mechanisms (such as, for example, superoxide dismutase—SOD, and glutathione), resulting in an excessive production of reactive oxygen species (Mancuso et al., 2012; Ziech et al., 2010). This condition is widely recognized as a central feature of many biological processes and diseases, due to their impact on cell injury and death, being involved in aging, neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and cancer (Cui et al., 2012; Repine et al., 1997; Zwart et al., 1999). Oxidative adducts derived from DNA (Hwang, 2004), proteins, lipids, and sugars, are widely used to measure OS levels in biological samples (Ogino and Wang, 2007). This includes 8-hydroxy-2′-deoxyguanosine (8OHdG) which acts as an indicator of DNA oxidation (Ogino and Wang, 2007; Valavanidis et al., 2009; Zwart et al., 1999). Nowadays, the methods used to quantify this biomarker involve complex, expensive and non-portable techniques (Fan et al., 2012; Li et al., 2011; Wang et al., 2011; Zitka et al., 2011), which impair a more effective utilization of OS biomarkers as a predictive tool for degenerative disease installation. Only an immunosensor is presented very recently in literature for 8OHdG, but the overall procedure is very complex (Darwish et al., 2013).

Thus, this work describes a novel and simple approach for designing an immunosensor presenting an Ab for 8OHdG (anti-8OHdG) suitably oriented over a gold support, here tested for the detection of 8OHdG, a cancer biomarker in urine related to nucleic acid oxidation.

2. Experimental section

2.1. Apparatus

The electrochemical measurements were conducted with a potentiostat/galvanostat from Metrohm Autolab, controlled by NOVA 1.9 software. Atomic force microscopy measurements were made in using tapping mode using a Veeco Metrology Multimode, Nanoscope IVA. Quartz crystal microbalance (QCM) studies were conducted in a QCM E4 from Q-sense, Biolin, with Dissipation monitoring (QCM-D). A DXR™ Raman Microscope from Thermo Scientific™ was used for Raman spectrometry with confocal microscopy.

2.2. Gold supports

Electrochemical studies used Au-screen printed electrodes (Au-SPEs) purchased from DropSens (DS-C223BT). These had working and counter electrodes of gold and reference electrode and electrical contacts made of silver, interfaced in a switch box from DropSens to enable its potentiostat/galvanostat reading. The gold layer of the electrode was washed with alcohol (70%) and de-ionized water before any use. All chemical modifications attempted with these electrodes were made only by applying the reacting solution solely on the working electrode.

AFM studies were conducted in Au-glass slides, where the Au layer was deposited by sputtering to ensure its flatness. This gold layer was cleaned by gentile procedures before use, with Piranha solution.

QCM-D studies were conducted with commercial chips from Q-sense, Biolin. These had 14 mm diameter, 0.3 mm thickness quartz glass with a flat Au-layer on top and were cleaned with de-ionized water before use.

2.3. Reagents and solutions

All chemicals were of analytical grade and water was de-ionized or ultrapure Milli-Q laboratory grade. Potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$) and potassium hexacyanoferrate II ($K_4[Fe(CN)_6]$) trihydrate were obtained from Riedel-deHaen; bovine serum albumin (BSA), creatinine, urea, *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 8-hydroxy-2-deoxyguanosine 98% (8OHdG), and 8-hydroxyguanosine, GTX (Ab-8OHdG) from Sigma; cysteamine and ethylenediamine from Merck; and dextrose anhydrous from Fisher.

Piranha solution was prepared by mixing in a cabinet H_2SO_4 and H_2O_2, in 5:1 ratio, using concentrated H_2SO_4 solution (95–97%, Scharlau) and a 30% H_2O_2 (VWR). The Ab-8OHdG solution was used after a 150 × dilution of the commercial product. A 1 × PBS buffer, pH 7.3 (Amresco, E404, Biotechnology grade) was used throughout this work. Synthetic urine was prepared as indicated in Martinez et al., 2007.

2.4. Antibody immobilization

The reactions used for Ab immobilization are shown in Fig. 1. A solution of cysteamine (50 mM) was placed on clean Au for 1 h. Only the necessary volume to cover the working electrode area