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Dinitrophenyl ligand substrates and their application to immunosensors

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Abstract

We present an approach for the development of highly specific and sensitive antibody based biosensors by chemically tailoring the sensor surface with materials that control specific and nonspecific binding of biologically relevant molecules. As a model system we employed surface immobilized 2,4-dinitrophenyl (DNP)-ligands that bind specifically to anti-DNP antibodies. Self-assembling characteristics and minimization of the nonspecific interactions were used in the ligand design. The redox activity of the DNP-head group was used to calculate the surface density (coverage) of these assemblies using cyclic voltammetry. Quartz crystal microbalance (QCM) and impedance analysis were used to assess the ligand–antibody interaction and estimate the quantity of antibodies bound to the surface. The ligand surface density and the QCM data were useful in determining the sensitivity of our model system. A simple two-step kinetic model was shown to fit the experimental data.
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1. Introduction

In the field of biotechnology there has been considerable interest in using self-assembled monolayers (SAMs) as biosensing platforms. The order and dense nature of the monolayer (surface density 10^{14}–10^{13} molecules cm^{-2}), the ease of generating an environment for immobilization of biological material, and the ease of forming assemblies with metals, especially gold, and providing the possibility of performing surface based electrochemical studies, are some of SAMs' very attractive features. Numerous interfacial biosensor systems are based on specific recognition. Some of the best known examples include biotin-streptavidin (Haussling et al., 1991; Spinke et al., 1993) or avidin recognition, ligand or carbohydrate–protein recognition (Mrksich et al., 1995; Lahiri et al., 1999; Svedhem et al., 2002), nitrolotriacetic acid–histidine-tagged protein (Sigal et al., 1996) recognition and antibody–antigen interactions in immunosensors (Taira et al., 1993).

Immunosensors in which the antibody or the antigen (or ligand) becomes immobilized onto the transducer surface can be detected using optical, electrochemical and acoustic signal transduction mechanisms among others. Immunoassays for these sensors were initially investigated using radioactive labeling (Garvey et al., 1977), but have now advanced detection methods such as enzyme-linked immunosorbant assays (Engrall, 1980), fluorescence-based (Soin and Lovgreen, 1988), electrochemical (Aizawa, 1987; Doyle et al., 1987; Wright et al., 1987), mass loading in quartz crystal microbalance (QCM) (Janshoff et al., 2000) and surface-plasmon resonance (SPR) (Stelzle et al., 1993) measurements.

Numerous groups have studied the binding characteristics of antibodies against a variety of ligands. The 2,4-dinitrophenyl (DNP) group is often used as a model ligand due to its known immunogenic behavior, easy accessibility and availability of antibodies against it. Dialkyl disulfides functionalized with DNP were used in one of the first surface based studies. These ligand bound surfaces were used in an impedimetric immunosensor, where anti-DNP antibodies were detected at the 10–10^3 ng cm^{-3} level (Taira et al., 1993). Willner and co-workers have also published a series of reports on anti-DNP binding to short chain-length alkanethiols of DNP-lysine and dinitrospiropyrans on gold surfaces. They used various electrochemical detection methods as well as QCM to characterize the binding behavior (Blonder et

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al., 1996, 1997a,b, 1998; Cohen et al., 1996; Patolsky et al., 1998).

The QCM has been explored as an alternative technique to optical based methods after the development of resonators capable of operating in fluids (Janshoff et al., 2000; Kanazawa and Gordon, 1985) However, since the physics and general behavior of biofilms in liquids is complex, there is some skepticism in the use of piezoelectric mass sensing devices as biosensing tools. This complexity can make it difficult to obtain an explicit relationship between the adsorbed mass and the frequency change. When an analyte in solution binds a ligand or protein immobilized on the transducer surface, the QCM generates a response that recognizes this binding event. QCM response is a change in the resonant frequency of the crystal that is subsequently used to estimate the mass change using the Sauerbrey equation (Sauerbrey, 1959)

$$\Delta m = -C_f \Delta F \quad (1)$$

where Δm is the change in mass (ng cm^{-2}), ΔF the change in frequency and C_f, 17.7 ng Hz^{-1} cm^{-2}, is the proportionality constant for a 5 MHz crystal. However, the Sauerbrey relationship assumes that the attached mass (film) is thin, rigid and strongly coupled to the crystal. Therefore, this equation is not valid if the deposited mass is viscoelastic. This limits the use of the QCM for precise mass detection of biological materials in a liquid phase, and there is evidence for overestimation of the deposited mass (Janshoff et al., 2000; Muramatsu et al., 1987).

Another issue with the QCM arises when large biomolecules (e.g. proteins, cells) are directly immobilized on the sensor substrate. This, results in a random orientation of the molecules on the surface creating many unreacted sites that can result in nonspecific adsorption. Especially in the case of antibodies, the antigen binding domains should preferentially be oriented towards the external medium.

As a prototype of an antibody-based sensor, we have synthesized a DNP-ligand (DNP-PEG4-C11thiol) which specifically binds to anti-DNP antibodies. Using the self-assembling approach, thiols were used as the surface reactive head groups to form a monolayer assembly onto gold substrates. In order to enhance molecular organization and packing, an 11-carbon alkyl chain was added as a hydrocarbon segment (Porter et al., 1987). An oligo(ethyleneglycol) linker was incorporated into the system to introduce an interfacial water layer that could orient the functional group away from surface. The DNP-ligand, served as the functional group and, in addition, the nitro groups on DNP were useful as redox-active sites (Tsutsumi et al., 1995) that could be characterized by cyclic voltammetry (CV). This gave us the opportunity to determine the surface density or coverage of the ligand assembly on the gold. Subsequently, the QCM was used to observe the real-time surface immobilization of anti-DNP antibody mediated via the DNP-groups at the surface. The relatively small volume of the ligand should significantly increase the surface density of ligands compared to an antibody-bound surface and hence, not affect the QCM measurements. QCM and impedance analysis were used to determine the antibody coverage on the sensor. We were also able to describe the kinetics of this binding with a simple two-step model and estimate kinetic and equilibrium constants by fitting the experimental QCM data to this model.

2. Experimental

Synthesis of **DNP-PEG4(IV)** and **PEG3(VII)** and the instruments and methods used for analysis are described in detail in the supporting information (Scheme S1, Fig. S1).

2.1. Monolayer preparation

The SAMs were prepared by immersing gold substrates (QCM disc or electrodes) into a solution of 11-(2-(2-(2-(2-(2,4-dinitrophenoxy)ethoxy)ethoxy)ethoxy)ethoxy)undecane-1-thiol, **DNP-PEG4(IV)** or 11-(2-(2-(2-methoxyethoxy) ethoxy)ethoxy)undecane-1-thiol, **PEG3(VII)** 1 mM in anhydrous CH_2Cl_2 or ethanol for 24 h at room temperature (Fig. S1). Samples were rinsed first with CH_2Cl_2 (or ethanol, respectively), acetone then ethanol, followed by 1:1 ethanol:water and finally water. The modified gold substrates were immediately used for characterization and analysis. Mixed assemblies were formed by either sequential or simultaneous immersion as depicted in Fig. S1. In the case of sequential immersion, a surface modified with **DNP-PEG4(IV)** was immersed in a 1 mM **PEG3(VII)** solution in CH_2Cl_2 for 18 h and rinsed as described before. In the case of simultaneous mixing, the surface was immersed in solutions containing both **DNP-PEG4(IV)** and **PEG3(VII)** in different molar fraction of DNP (χ_{DNP}; 0.9, 0.6, 0.5, 0.1 and 0) with the total concentration of thiols in each solution being 1 mM. These mixed assemblies were used for voltammetric studies (Fig. 1) and immunosensor characterization (Fig. 3).

2.2. Kinetic modeling of QCM data

We modeled the binding of a bivalent antibody to surface associated ligands using a two-step model (Fig. 2). The model and the rate laws used for fitting experimental data are described in detail in the supporting information. We fitted the experimental data for three different antibody concentrations to this model and extracted the rate constants that gave the best fits. Also, based on this model we calculated the affinity constants for the two binding steps and an overall effective affinity constant for the two-step reaction.

2.3. Apparent affinity constant (K) and site density

The change in frequency (ΔF) achieved after reaching a steady state at each antibody concentration in solution can be used to determine an apparent affinity constant of this antibody–ligand system. By assuming that there is a homogeneous surface distribution of ligands and that the binding of antibody occurs with a single apparent affinity, constant K can be estimated by fitting the parameters of a simple Langmuir

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