Dextran modified self-assembled monolayer surfaces for use in biointeraction analysis with surface plasmon resonance

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Abstract: The development of a surface modification method based on self-assembled monolayers on gold surfaces has proved to be a successful basis for the functionalization of sensor surfaces intended for use in biospecific interaction analysis. The dextran coated alkanethiol layer shows high chemical stability towards various conditions employed for different analytical sequences. Optimized synthesis sequences yield highly reproducible sensor surfaces in terms of surface coverage and biomolecule binding capacities. Immobilization chemistries adapted for coupling various biomolecules and other ligands were developed for the dextran coated surface and used in bioanalysis techniques based on surface plasmon resonance. This mass sensitive, real-time analysis technique with label-free detection has developed as a universal tool for analysis of various qualitative and quantitative biospecific interactions, including affinity and kinetic rate constant determinations.

INTRODUCTION

The formation of organized monolayers on surfaces by self-assembly has over the last decades developed as an especially intensive research area. Various aspects of this surface modification procedure, such as choice of surface material, types of assembling molecules, physical organic properties of the formed layers, etc., have long been studied from pure scientific interests (1). The potential of using the self-assembling technique as a construction method for intended interfacial alterations in technological applications has obviously also been a driving force in the interest of this research area (2, 3). We have successfully developed a novel surface modification method based on self-assembly of alkanethiols on gold surfaces. Further chemical derivatization allows the construction of sensor surfaces for use in a newly introduced biosensor based bioanalytical system (4). To the best of our knowledge, this is the first commercial product which includes a chemical modification based on these principles. This article gives an overview of the functionalization strategy of the surfaces in perspective to the requirements put on sensor surfaces for applications in the area of biospecific interaction analysis. The principles of the analytical instrumentation will be described as well as some examples of applications of the analytical technique for studying affinity and kinetics of interactions with biomolecules.

INSTRUMENTATION FOR BIOSPECIFIC INTERACTION ANALYSIS

In the biological sciences the understanding of the relationships between molecular structure and biological function has become a central area of research. Examples of functional parameters that are of interest to study for biomolecules are affinity and kinetics for interactions, but also identity, multisite binding and allosteric effects. A potentially successful way to study these biomolecular recognition events is to make structural variants and systematically investigate how these affect the function. However, these types of structure-function relationships are still very vaguely explored for most biomolecules. This is partly due to the fact that techniques and methods for functional studies not have been developed as far as the techniques for structural studies.
A biosensor based analytical instrumentation, BIAcore™, was recently developed at Pharmacia Biosensor AB and designed for functional characterizations of biomolecular interactions (5). During two years since its commercial introduction, this analytical system has found applications in kinetic analysis of antigen-antibody, receptor-ligand and DNA-protein interactions, as well as in structural analysis of multisite binding and measurement of DNA hybridization (6).

The basic idea of the analytical technique is to immobilize one of the interacting partners on the sensor surface, introduce the other partner under controlled conditions via a flow system and detect the binding in real-time by a surface sensitive detector. In BIAcore, an optical detection unit based on surface plasmon resonance (SPR) is combined with the chemically modified gold surface and a microfluidic cartridge for sample handling. An autosampler and software for system control are also included for increasing the analysis capacity and for result evaluation. From the detector-response data qualitative information as well as quantitative kinetic data can be extracted.

The SPR phenomenon occurs when plane-polarized light is reflected under flat angles from a thin gold film deposited on a glass substrate (7). For a specific angle the photons interact with the free electron cloud in the metal film and causes a drop in the intensity of the reflected light. This angle is highly sensitive to refractive index changes near the metal surface, which in turn is directly proportional to changes in the biomolecule concentration at the surface, Fig. 1 (8). The monitoring of the resonance angle as a function of time is called a sensorgram and is used for direct visual observation or software controlled quantitative evaluation of binding and dissociation events at the sensor surface.

![Fig. 1 Schematic illustration of the SPR detection principle.](image)

Immobilization reagents and sample solutions are delivered via a miniaturized integrated microfluidic system which has been developed for optimizing the sample handling. This flow system holds a flow cell with a volume of 60 nl, integrated loops and valves close to the sensor surface in order to reduce sample dispersion (9).

**SENSOR SURFACE MODIFICATION**

Surface based bioanalytical techniques have normally used direct binding of the biomolecule to the plane surface. This often diminishes or even abolishes the bioactivity of the bound molecule by uncontrolled orientation of the active parts towards the surface or by denaturation (10). Thus, if passive binding of the biomolecule to a hydrophobic substrate is employed, reorganization of the biomolecule occurs so the hydrophobic parts are oriented towards the surface. Passive binding can also lead to uncontrolled dissociation and exchange during the analysis which can adversely affect the quality of the results. In the development of a sensor surface appropriate for use in biospecific interaction analysis a modification route had to be designed to eliminate the drawbacks of low bioactivity and desorption risks of the surface bound ligand. Thus a reliable immobilization technique for stable and defined binding of various types of ligands combined with high functional activity was a
prerequisite. Furthermore the mass-detecting SPR-technology necessitate a sensor surface with minimized non-specific binding during analysis. A bare gold surface shows high tendencies for adsorption and denaturation of proteins. Another aspect to consider is that the SPR-technique is sensitive to refractive index changes to a distance of approximately 1 μm out into the solution which make it advantageous if the micro-volume near the surface can be fully used during analysis (11).

The surface modification sequence that we have developed to meet these demands is based on the concept of molecular self-assembly of sulphur-containing molecules on gold (or silver) surfaces. This spontaneous monolayer formation is mainly driven by the coordination of thiols or disulphides to gold accompanied with interchain van der Waals interactions. The first example of spontaneous formation of monolayers on gold was shown in 1983 by Nuzzo and Allara who adsorbed dialkyldisulfides on gold (12). We designed 16-mercaptophexadecan-1-ol as appropriate for our purposes which could be synthesized in five steps starting from 15-hydroxypentadecanoic acid (4, 13). The hydroxyl group was intentionally introduced as a reactive handle for further modification as a means for the creation of a neutral hydrophilic interface. A densely packed monolayer is formed within minutes from dilute ethanol solutions of the alkanethiol resulting in an interface which is completely water wetting (14). This layer is then reacted with epichlorohydrin which introduces epoxy groups on the surface as electrophilic coupling sites, Fig. 2. For the applications of biointeraction analysis further modification with dextran was found to be very suitable. Dextran, which is a hydrophilic linear polymer based on 1,6-linked glucose units, was randomly linked to the epoxy groups under basic conditions. Further reaction with bromoacetic acid introduces carboxylic groups on the dextran which can be used as reaction sites for the covalent linkage of the ligand (4).

Using a dextran fraction with a molecular weight of 500,000 resulted in a surface coverage of approximately 2 ng/mm², where each polymer chain is estimated to have one or only a few binding points to the surface. The flexible chains extend approximately 100 nm in water solutions, thus forming a highly hydrophilic surface with little tendency for non-specific adsorption of proteins and other biomolecules. The difference in behaviour on bare gold and the modified surface is exemplified in Fig. 3 for a protein of the immunoglobulin G type, clearly indicating the passivation effect obtained from the surface modification (15).

Fig. 3 Sensorgram of the adsorption of immunoglobulin G (2 mg/ml in 10 mM HEPES + 0.15 M NaCl, pH 7.4) : (a) on a bare gold surface, (b) on a CM-dextran modified surface.
The abilities of self-assembled surface layers to withstand various derivatization and working conditions are of crucial importance for their potential in technological applications. We found that the alkanethiol monolayer is resistant to highly basic conditions such as 1 M sodium hydroxide for 16 hours employed during the dextran coupling without any detectable disintegration of the layer. The integrity of the layer was confirmed by surface analysis methods such as X-ray photoelectron spectroscopy (XPS), attenuated total reflection infrared spectroscopy and cyclic voltammetry. The surface layer is also resistant to acidic conditions and various organic solvents. However, treatment with oxidizing agents such as bromine or periodate solutions tend to disrupt the monolayer. The outlined modification sequence was found to give very reproducible surface coverages, an important aspect to consider for manufacturing purposes. Variations between different surfaces is typically smaller than a 5 % coefficient of variation (CV) as determined with XPS or in BIAcore.

General immobilization sequences have been developed for the coupling of various types of ligands within the analytical system. For bioanalytical applications water based chemistry is employed and starts with the activation of the carboxylic groups using a mixture of N-ethyl-N' -dimethylaminopropyl-carbodiimide and N-hydroxysuccinimide (16). A fraction of the carboxylic groups is converted to active esters whereafter coupling of the ligand is done under controlled conditions. It was found for proteins that electrostatic attraction of the protein molecules to the activated dextran layer enhances the coupling yields considerably. This is done by working under low-ionic strength buffer conditions at a pH value where the protein is positively charged. A much higher local concentration of the protein in the still partly negatively charged dextran layer compared to the bulk concentration is thus obtained and favours the reaction of the nucleophilic groups in the protein over hydrolysis of the esters. The competing hydrolysis reaction of the activated coupling matrix otherwise reduces the efficiency considerably for protein coupling in water solution. Much lower protein concentrations than normally used for surface immobilizations can consequently be employed by working under these conditions. A typical activation and coupling sequence is shown in Fig. 4.

![Resonance Signal (kRU) vs. Time (s)](image)

**Fig. 4** Typical sensorgram of the activation and coupling of proteins under electrostatic attraction conditions: 1) activation by carbodiimide/ hydroxysuccinimide (0.2/0.05 M in water), 2) coupling of immunoglobulin G (50 μg/ml in 10 mM sodium acetate, pH 5.0), 3) deactivation of residual esters with ethanolamine hydrochloride (1M, pH 8.5).

Immobilizations are highly reproducible under the controlled conditions employed in the analytical system. An example is the immobilization of staphylococcal protein A which was done with a CV of 3.4 % over 96 immobilizations, as measured by BIAcore. A range of different water based immobilization chemistries have been developed by extending the initial activation of the carboxylic groups and conversion to e.g. thiols and hydrazides (17). Use of these in-situ immobilization methods

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provides convenient ways for the creation of selected biospecific surfaces for real-time analyses. The stability of the surface coating towards acids, bases and organic solvents also makes it possible to perform repeated measurements on one single sensor surface. Typically, the surface is regenerated by disruption of the binding between the interacting molecules with a pulse of dilute acid and a short reconditioning with neutral buffer solution. It is normally the stability of the immobilized ligand towards regeneration conditions that limits the lifetime of a sensor surface, but 50 to 100 measurements can typically be made on one surface.

APPLICATIONS

A wide range of studies have up to now been performed with this technique, involving protein-protein (18, 19), drug-protein (20), DNA-DNA (21) and receptor-ligand interactions (22). The straightforward qualitative information that can obtained is exemplified in Fig. 5, showing the binding characteristics of three monoclonal antibodies (MAbs) to their antigen, the HIV protein p24 (18). Each MAb was immobilized to the sensor surface and the p24 protein was tested for binding. The overlay plot of the binding curves towards the three MAbs clearly illustrates the different affinities between the three. Steady state binding is obtained for MAb 18 and 28 during the sample pulse, and the higher response level for MAb 18 tells us that this MAb has a higher affinity towards p24 than MAb 28. Another immediate conclusion is that p24 dissociates much faster from MAb 28 than from the other two. These types of qualitative data can often be sufficient for e.g. screening and selection purposes with no need for quantitative determination of the affinity and kinetic constants.

Rate constants can be extracted from the binding curves by applying the proper rate equations for the association and dissociation events respectively. The soluble analyte is constantly renewed by the applied flow over the surface and pseudo-first order conditions can be assumed for most simple binding events. In practice, the interactions are measured for a few different analyte concentrations and the binding rates constants are evaluated from the binding curves (18). Similarly, dissociation rate constants are obtained from analyzing the dissociation event after the sample plug has passed the flow cell. More complex binding events may be analysed using software with non-linear regression analysis methods (23).

A limitation with a mass sensitive technique such as SPR is the detection of the direct binding of low molecular weight (Imw) analytes. A practical lower limit of detection is a mass change of approximately 10 pg/mm² which is hard to reach if the Imw analytes bind to macromolecular

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receptors on the sensor surface. Alternative interaction methods have therefore been developed based on competitive approaches (24). In one format, a derivative of the analyte is conjugated to a large molecule and is allowed to compete with the lmw analyte for the receptor site. The detector response is obtained from the binding of the large conjugate and perturbations are effected by the analyte. The method is exemplified for the binding of theophylline to an immobilized antibody. A conjugate between theophylline (mw. 180) and a protein with mw. 77,000 was mixed with different analogues of theophylline and the response for the binding to the antibody was measured (Fig. 6). A qualitative affinity scale of the theophylline analogues was immediately obtained with aminotheophylline ranking highest, followed by theophylline and last carboxymethyltheophylline. Kinetic data can also be obtained by performing a series of experiments where the concentration of lmw analyte is varied and the response curves are evaluated by non-linear regression methods (24). These examples illustrate the SPR technique as a convenient method for studying interactions involving lmw compounds within e.g. drug candidate characterization. For the future, development within the areas of surface modification, methodology and instrumentation will surely increase the application areas and the quality and types of information obtainable with this novel analytical technique.

Acknowledgements
The development of the SPR technology and surface modifications into the BIAcore system was made possible mainly by the efforts from the research and development people at Pharmacia Biosensor AB which hereby are acknowledged.

References