A Novel Hydrogel Matrix on Gold Surfaces in Surface Plasmon Resonance Sensors for Fast and Efficient Covalent Immobilization of Ligands

Stefan Löfås* and Bo Johnsson

Pharmacia Biosensor AB, S-751 82 Uppsala, Sweden

Gold surfaces intended for surface plasmon resonance sensors have been derivatized with a flexible matrix composed of carboxymethylated dextran to provide fast and efficient coupling of proteins and other ligands at low concentrations using the principles of ionic pre-concentration; these hydrophilic and protein compatible hydrogel surfaces allow increased immobilization capacity as compared to monolayer based coatings, for applications in biospecific interaction analysis.

In the increasingly expanding area of biosensor technology, an optical technique based on surface plasmon resonance (SPR) has been found to be potentially suitable for immunodiagnostics and other biomolecule characterizations.[1,2]† By immobilizing one component of a biospecific pair (*e.g.* antibody–antigen), the technique can be used for detection and measurement of the concentration of the counterpart, or for characterization of the kinetics and affinity of binding, by monitoring the change in optical properties during binding and dissociation. This account presents hydrogel modification of gold surfaces and immobilization techniques to give activated surfaces suitable for a wide range of analytical applications.

In earlier papers describing the potential of SPR in this field, the antibody or antigen has simply been adsorbed to the metal surface prior to measurement.[1] Although this approach clearly demonstrates the potential of this technique, there are certain limitations associated with it. Firstly, direct adsorption excludes immobilization of proteins which can undergo denaturation in contact with the metal surface or which do not give well defined and stable adsorption layers. Also, ligands of the hapten type are difficult to bind directly to the sensor surface. Secondly, since the SPR technique is based on changes in dielectric constant in the proximity of the metal–solution interface, the unspecific adsorption of different solutes to the surface must be minimized. With physically adsorbed ligands, there is a risk of undesired interaction with the surface, or exchange processes occurring between solutes and the ligands.[3] In many instances, the ideal situation is a stable covalent binding of the ligand to a surface with the possibility of repeated analyses and maximum exposure of the ligand to the solution containing the biospecific partner.

In this communication we describe the modification of gold surfaces with a novel type of hydrogel matrix, which overcomes the problems mentioned above and which provides surfaces suitable for fast and simple *in situ* covalent binding of proteins and other ligands in a reproducible way. These functionalized surfaces can be used in a wide range of applications, especially in the area of biospecific interaction analysis.

The matrix is constructed from a composite of a metal-protection layer and a covalently bound flexible carboxymethyl-modified dextran hydrogel. On the basis of the concept of self-assembled monolayers created by adsorption of long-chain 1,ω-hydroxyalkyl thiols on gold surfaces,[4] a hydrophilic surface can be formed. This layer serves partly as a barrier to prevent proteins and other ligands from coming into

† In this technique, light is totally reflected at a glass–metal film (preferably gold or silver) interface and the reflectance is monitored as a function of angle. At a certain angle, a minimum in the intensity of the reflected light is observed. This indicates the excitation of surface plasmons at the metal–solution interface. The position of the response is sensitive to changes in the dielectric constant and/or thickness of the layer in the vicinity of the metal surface. The resonance is expressed in an arbitrary scale of Resonance Units (RU). A response is defined as RU difference compared with a baseline, normally fixed at the start of an experimental cycle.

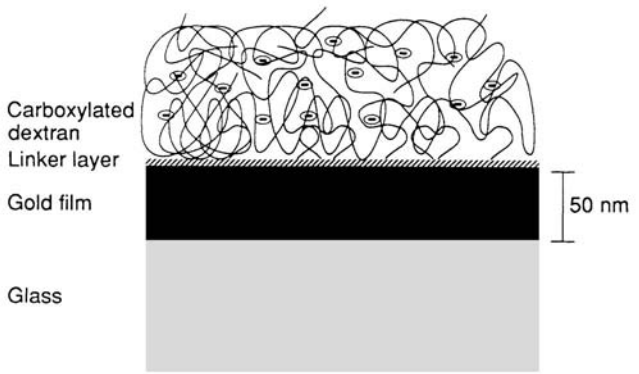

Fig. 1 Schematic illustration of the gold surface modified with the carboxymethyldextran matrix.

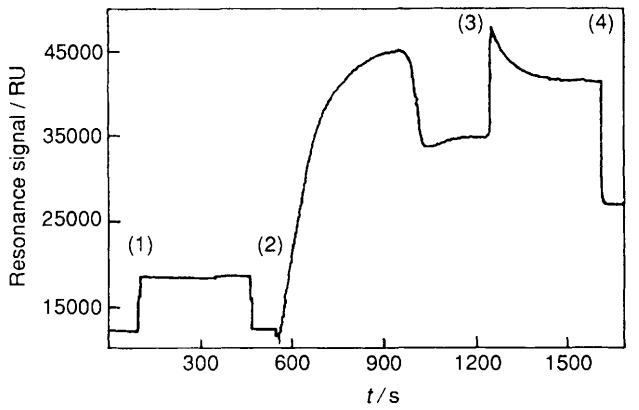

Fig. 2 The change in resonance signal over time during (1) activation of the matrix with EDC/NHS, (2) immobilization of a monoclonal antibody against human transferrin (50 mm^3 pulse, 60 $\mu g\ cm^{-3}$), (3) deactivation with ethanolamine and (4) end of deactivation pulse. The continuous flow buffer was 10×10^{-3} mol dm^{-3} HEPES with 0.15 mol dm^{-3} sodium chloride and 0.05% Tween 20, pH 7.4. The observed resonance signal shifts at (1) and (3) are mainly due to differences in refractive indices between the samples and the continuous flow buffer.

contact with the metal, and partly as a functionalized structure for further modification of the surface. As an example, we have derivatized the first monolayer by activation of the hydroxy groups with epichlorohydrin under basic conditions in order to form epoxides, and then covalently bound a dextran polymer to these. Using this approach a wide variety of flexible matrices of hydrogel-type can be constructed for different purposes.

Typically, a glass substrate coated with a thin gold film was treated with a 5×10^{-3} mol dm^{-3} solution of 16-mercapto-hexadecan-1-ol in ethanol–water (80:20), according to the principles of Whitesides *et al.*[5]‡ The resulting hydrophilic surface (contact angle with water *ca.* 0°) was then reacted with a 0.6 mol dm^{-3} solution of epichlorohydrin in a 1:1 mixture of 0.4 mol dm^{-3} sodium hydroxide and diglyme for 4 h at 25 °C. After thoroughly washing with water, ethanol and water again, the surface was treated with a basic dextran solution [3.0 g dextran T500 (Pharmacia AB) in 10 cm^3 of 0.1 mol dm^{-3} sodium hydroxide] for 20 h at 25 °C. Further functionalization to a carboxymethyl-modified matrix was done by reaction with

‡ Ellipsometric data were in full agreement with the formation of a monolayer of the hydroxyalkylthiol.

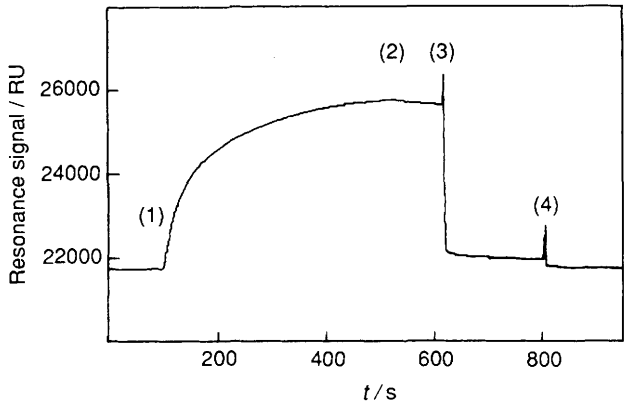

Fig. 3 The change in resonance signal over time during exposure of a surface with immobilized anti-transferrin to (1) a solution of transferrin (100 $\mu g\ cm^{-3}$), (2) end of transferrin pulse, (3) 0.1 mol dm^{-3} glycine (pH 2.5) and (4) end of glycine pulse. The transferrin sample is in the same buffer solution as the continuous flow buffer, see text in Fig. 2.

bromoacetic acid (1 mol dm^{-3} solution in 2 mol dm^{-3} sodium hydroxide) at 25 °C for 16 h. Analysis by voltammetry[6] showed that the isolating effect of the alkylthiol-layer was fully retained even after the derivatization steps, which indicates the pronounced stability of the monolayer under the highly basic conditions employed during the synthesis. The surface was also characterized by FTIR and XPS, which confirmed the presence of dextran (estimated 1–3 ng mm^{-2}), modified with approximatively one carboxy group per glucose unit.

This negatively charged hydrogel-covered surface (Fig. 1) can be used for covalent coupling of various ligands. Activation–coupling–deactivation protocols have been developed which preferably can be performed automatically in an SPR unit equipped with a liquid handling system with injecting loops, valves and flow channels.[2] An efficient and fast immobilization of proteins is made possible by utilizing electrostatic attraction forces for pre-concentration of a positively charged protein from a solution with low ionic strength to residual negative charges on a matrix, activated with reactive groups. This gives a high local concentration of the protein in the matrix, whereby covalent binding can occur. For example, a defined fraction of the carboxy groups was activated to form reactive *N*-hydroxysuccinimide esters using a solution of 0.2 mol dm^{-3} *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 0.05 mol dm^{-3} *N*-hydroxysuccinimide (NHS) in water. After this activation, a monoclonal antibody against human transferrin (60 $\mu g\ cm^{-3}$) in 10×10^{-3} mol dm^{-3} sodium acetate, pH 5.0, was immobilized *via* reaction of its nucleophilic groups. Excess esters were then deactivated using 1 mol dm^{-3} ethanolamine hydrochloride adjusted to pH 8.5 with sodium hydroxide, which also desalted loosely bound protein. In Fig. 2, this immobilization sequence is followed in real-time by the SPR unit, registering the time-dependent change in optical properties. After the deactivation pulse, a response is observed, which shows that a substantial amount of protein has been covalently bound to the surface. This approach gives efficient immobilization of proteins even at pH 4 if they are below their isoelectric point (pI). Most commonly studied proteins and ligands are there positively charged.[7] Another feature is that small volumes ($\geq$30 μl) of protein at low concentration ($\geq$10 $\mu g\ cm^{-3}$) can be immobilized *in situ*.[8]

Quantisation of the optical response during protein-immobilization using ^{14}C- and ^{35}S-labelled proteins was performed.[2]

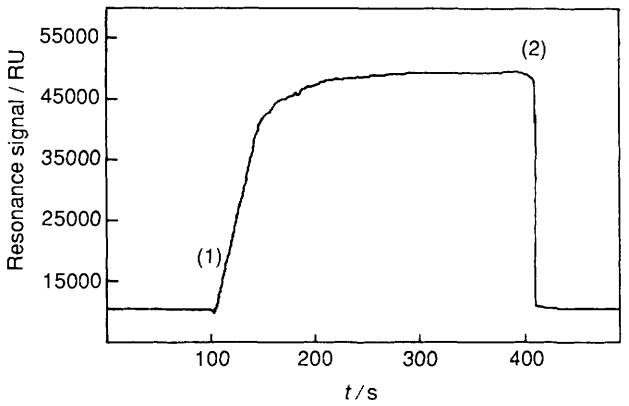

Fig. 4 The change in resonance signal over time during the exposure of the carboxymethylated dextran surface to (1) immunoglobulin G (100 μg cm^{-3}) in 10 × 10^{-3} mol dm^{-3} sodium acetate buffer, pH 5.0. At (2) the continuous flow buffer (10 × 10^{-3} mol dm^{-3} HEPES with 0.15 mol dm^{-3} sodium chloride, pH 7.4) was introduced to the surface.

The results demonstrated that protein loading up to 50 ng mm^{-2} can be obtained by electrostatic attraction–concentration to the matrix, following the principles outlined above. For most proteins, this implies a coating of up to several monolayers, which illustrates the high capacity of the matrix. This agrees with a picture of a matrix where the surface bound dextran polymer gives a highly flexible, uncrosslinked, open structure with a thickness of *ca.* 100 nm in buffer solution (depending on pH and ionic strength), where ligands can bind all through the matrix.[2] Although the surface is covered with a small amount of dextran (1–3 ng mm^{-2}), the high water solubility of the polymer gives it this high extension. The SPR effect is, for the conditions applied here, sensitive to changes in the dielectric constant approximately one micrometre from the surface. For certain applications, the high capacity of the matrix can be useful in order to exploit fully this technique.

For practical purposes, the amount of immobilized protein is optimized for the type of analysis to be performed.[2] An example of an interaction analysis is given in Fig. 3, where *ca.* 10 ng mm^{-2} of the transferrin-antibody has been immobilized and a solution of transferrin (100 μg cm^{-3} in 10 × 10^{-3} mol dm^{-3} HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid] with 0.15 mol dm^{-3} sodium chloride, pH 7.4) was injected *via* the liquid handling system of the SPR unit. A response was registrated as the binding of transferrin to the antibody occurred. A pulse of glycine solution, pH 2.5, disrupted the bond and the surface with the antibody was then ready for a new analysis. This demonstrates the advantage of a stable, covalent binding of the ligand to the surface. With proper choice of regeneration conditions, multiple analyses of more than 50 cycles has been performed without the loss of response.

Low molecular weight ligands have been attached to this matrix for use in different types of assays. For a theophylline assay, the surface was modified with the theophylline analogue 8-(3-aminopropyl)theophylline.[9] Thus, by activation with the EDC–NHS-solution (described above) and treatment of the activated matrix with a 1 × 10^{-3} mol dm^{-3} solution of the theophylline derivative in a 0.1 mol dm^{-3} sodium hydrogencarbonate buffer, pH 8.4, for 7 min and deactivation as above, the surface could be used in an inhibition type assay for theophylline.[2] Under these conditions the matrix showed a binding capacity for theophylline antibody of approximately 30 ng mm^{-2}.

The chemical stability of the matrix for acidic and alkaline solutions is obvious from these data and from the reaction conditions during the synthesis. Another feature is the low unspecific binding to the matrix of components in various samples under conditions of high ionic strength, which can be attributed to a combination of the high hydrophilicity of the surface and entropic effects due to the flexible polymer chains.[10] This is exemplified when a protein in a solution with low ionic strength is concentrated to an unactivated carboxymethyl–dextran surface by electrostatic attraction. The protein is subsequently displayed by a buffer with 0.15 mol dm^{-3} sodium chloride (Fig. 4). The return of the baseline to its initial value demonstrates minimal non-specific adsorption to the surface.

In contrast to adsorption to solid substrates, the ligand is more accessible for interaction when it is bound to the flexible polymer chain. This can be of importance for certain applications. However, the flexibility can also cause both chemical crosslinking of the ligand and a biochemical crosslinking when an analyte with more than one interacting site is studied. This has to be taken into account when the interaction analysis is designed.

This communication has presented the modification of gold surfaces with a novel hydrophilic matrix and its potential for biospecific interaction analysis with the SPR technique. In addition, this type of derivatization has potential use in piezoelectric and other sensor techniques, where highly hydrophilic surfaces suitable for covalent immobilization of various ligands are desired.

We are grateful to Jan Bergström and Lennart Söderberg for valuable discussions and suggestions. We also express sincere thanks to the members of the Chemistry Department and others at Pharmacia Biosensor AB for analysis help, technical assistance and other valuable contributions to this work.

Received, 5th July 1990; Com. 0/03031G

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