SURFACE PLASMON RESONANCE AS A BIOANALYTICAL TOOL

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SUMMARY

It is shown that Surface Plasmon Resonance is capable of detecting submonolayer quantities of proteins; information concerning the affinity of the surface can be obtained using this method.

It is indicated how this method can be used to obtain more detailed information on the conformation of adsorbed macromolecules.

INTRODUCTION

As it is increasingly realized that most biological processes occur at the interface between lipid membrane and environment, the study of surface phenomena is a field of growing importance in biophysics (ref.1). Some examples are protein adsorption, the mechanisms of cell-cell recognition and interaction, and gating of membrane ion channels. Also, from a practical point of view, much effort is put into the construction of so-called 'biosensors' (ref.2), that are intended to determine specifically the concentration of certain biologically important species (H⁺, K⁺, proteins, etc.). One of the conditions for an adequate operation of most biosensors is the preparation of a well-characterized molecule-selective surface.

However, in view of the nature of a relevant bio-surface, often covered with a sub-monolayer of molecules in a water environment, experimental methods to characterize such an interface are scarce. Among the optical methods, Surface Enhanced Raman Spectroscopy has not completely fulfilled the earlier promises; the same holds for IR reflection spectroscopy. Fluorescence spectroscopy has the potential for providing detailed surface information, but needs often elaborate labeling procedures.

In this paper we discuss the relevance of another optical method in characterizing bio-surfaces, Surface Plasmon Resonance (SPR). It will be shown that SPR is capable to detect sub-monolayer coverages of proteins. A possible route will be discussed to extract structural information from SPR experimental data.
fig. 1. The SPR experiment. (a) gives an impression of the field distribution near the silver interface; (b) shows the experimental set-up (cf. text)

SURFACE PLASMON RESONANCE

Here only a very short description will be given; for an extensive treatment we refer to Raether's review (ref.3).

Consider an arrangement as depicted in fig.1a. A prism (refractive index $n_1$) is coated with a thin (~50 nm) silver overlayer ($n_s$, complex) which is in contact with a medium of refractive index $n_2$. P-polarized light with wavelength $\lambda$, falling through the prism, reflects at the silver-prism interface; the intensity of the reflected light is measured. At some angle, $\theta_0$, a sharp minimum is found in the reflectance. At this angle a collective motion of the conduction electrons (a surface plasmon) in the silver is excited (ref.3).

From Maxwell's equations it can be calculated that for this situation a strong evanescent electric field is built up at the interface $n_s/n_2$. This field decays into medium $n_2$ with a characteristic distance in the order of $\lambda/2$ (see fig.1a). If an extra layer is placed between silver and the medium, then it is found that, owing to the strong localization of the field at the interface, the resonance angle is strongly dependent on the thickness ($d_p$) and refractive index ($n_p$) of this layer. We have calculated (ref.4) that a thickness-resolution of ca. 0.05 nm can be obtained for an experimental $\theta_0$ resolution of 0.01 degree.

Thus, if a protein is adsorbed to the surface, this can be considered as a change of the refractive index profile in the immediate vicinity of the surface. The detection limit then only depends on the equilibrium constant $K$ of this adsorption and the molecular mass of the protein under study. For a 150 kD protein, a detection limit of ~ $1/(100K)$ M, corresponding to a surface coverage of the order of 0.01 is found (ref.4).

EXPERIMENTAL

An outline of the experimental arrangement (ref.4) is given in fig.1b. A thin silver layer (thickness ca. 53 nm) is evaporated on a 25x25 mm$^2$ microscope glass slide (Chance); this slide is optically coupled to a prism
with identical refractive index by using a matching oil. A flow-cell is pressed against the glass slide. This assembly is placed on a turntable with angular resolution of 0.01 degree. Light from a 5 mW HeNe-laser (λ=632 nm) passes through a beamsplitter and a half-wave retarder, and is directed onto a prism, where it is reflected at the glass slide/silver interface. The reflected intensity is measured by a large area PIN photodiode. Part of the light that is reflected by the beamsplitter is used to monitor the incident light intensity. A ratio recording of the reflected intensity as well as the control of the turntable are performed by a PC. In view of the small changes of θ₀ (0.01-0.5 degree) it was decided to measure the reflectance change in the region of maximum slope of the reflectance curve. Some experiments were performed using an alternative set-up where θ₀ is measured directly (ref.5).

For a description of the biochemical preparation procedures we refer to our earlier work (ref.4).

RESULTS AND DISCUSSION

Protein adsorption and immune reaction

In fig.2 the reflectance change ΔR as a function of time is shown for the adsorption from solution of a 150 kD antibody. This antibody has the property that it is capable of a highly specific association with one particular type of protein, a so-called antigen. At t=t₁ the antibody solution is added. At t ~ 80 min a plateau is reached. Assuming a layer refractive index nₚ = 1.4 an adsorbed layer thickness of ~ 4 nm can be calculated, consistent with a monolayer surface coverage. However, it should be noted that this number is strongly dependent on the assumed value of nₚ.

At t~ 90 min the protein solution is replaced by buffer. The reflectance undergoes a stepwise change, presumably because the buffer solution has a refractive index slightly different from that of the protein solution; protein desorption is improbable because of the stepwise character of ΔR.

![graph](https://example.com/graph.png)

**fig.2.** Response of reflectance to addition of antibody at t=t₁; at t=t₂ the antibody-coated surface is exposed to 30 nM antigen.
At \( t = t_2 \) a 30 nM solution of the corresponding antigen (M=40 kD) is added, resulting in a further gradual change of AR until equilibrium is reached.

The selective nature of this reaction is illustrated in fig.3. At \( t = 0 \) the antibody-coated surface is exposed to a 20 nM solution of antigen. After an incubation time of ~50 min and a washing step, the solution is replaced at \( t = t_1 \) by a 300 nM solution of HSA-protein (M=60 kD). A slight decrease of \( \theta_0 \) is the result, probably due to partial desorption of the bound antigen. It is obvious that no HSA-binding takes place. That this is really due to the specificity of the immune reaction is borne out by the finding that upon addition of 60 nM of the antigen at \( t = t_2 \) again a response is found.

These experiments only provide information on possible allowed combinations of \( d \) and \( n \). From these data the adsorbed mass surface density \( \Gamma \) can be calculated using an expression given by de Feijter et al (ref.6):

\[
\Gamma = \frac{d}{d_p} \frac{(n_p - n_2)}{(d_p / dc)}
\]  

where \( d_p / dc \) is the refractive index increment of the protein solution. It is found that \( \Gamma \), calculated according to eq.(1), is constant within 10 \% for the range of allowed combinations of \( d_p \) and \( n_p \).

It is well known that each antibody has two binding sites for antigen binding. Furthermore, it has been shown (ref.7) that a plot of \( \log(G/N-G) \), where \( N \) is the number of binding sites and \( G \) is the average number of bound sites per antibody, versus \( \log(\text{concentration}) \) should result in a straight line with intercept the value of \( \log(K) \) and slope the degree of cooperativity \( A \). Such a plot is shown in fig.4, for \( N=2 \); we find \( K \sim 10^5 \) and \( A=0.8 \). The
found value of A indicates in our case (ref. 7) that the presence of the first bound antigen inhibits occupation of the second binding site. Thus, these numbers give valuable information on the nature of the surface.  

\[
\log \left( \frac{G}{N-G} \right)
\]

\[
\log \text{(conc protein)}
\]

*fig. 4. Plot of \( \log \left( \frac{G}{N-G} \right) \) as a function of \( \log ([\text{antigen}] \) (cf. text)*

and from a practical point of view they provide hints on how to improve the affinity of the surface. As it is beyond the scope of the present paper, we will not discuss this here.

**Characterization of layer structure**

Obviously, SPR experiments would be much more informative if both the refractive index and the thickness of the adsorbed layer could be determined. For proteins these parameters provide information on their conformation in the adsorbed state. In principle, three experimental parameters, viz. \( \theta_0 \), the resonance width, and the value of \( R \) at \( \theta_0 \), can be obtained from one SPR curve. However, it turns out (ref. 8) that in our experimental situation the combination of these parameters does not allow to resolve \( n_p \) and \( d_p \) separately. We are currently investigating alternative procedures by varying the penetration depth \( \lambda_p \), resulting in an adjustable fraction of the optical energy in the adsorbed layer. From Snellius' law it can be deduced that \( \lambda_p \) is given by the following expression:

\[
\lambda_p = \frac{\lambda}{2\pi} \cdot \left[ n_2^2 - n_1^2 \cdot \sin^2 \theta_0 \right]^{-1/2}
\]

From eq. (2) it can be seen that \( \lambda_p \) can be adjusted by any of the following methods:

(a) a change in the refractive index \( n_2 \) of the bulk solution,

(b) a change of the wavelength \( \lambda \),

(c) application of an extra layer with refractive index \( n_a \) between silver and protein, resulting in a different \( \theta_0 \).
Preliminary experimental results show that method (a) indeed results in a separate determination of $n_p$ and $d_p$ for an adsorbed protein layer. However, the optical stability of the silver layer has to be improved before reliable statements can be made on the value of these parameters. Furthermore, it cannot be excluded that in applying method (a) the protein changes its conformation.

CONCLUSIONS

We have shown that SPR is capable of detecting sub-monolayer surface coverages of proteins; information on the affinity of the surface under study can be obtained with this method.

We have indicated how SPR-experiments can be extended to determine the refractive index and thickness of an adsorbed layer. Combined with SPR-microscopy (ref.9), which shows a vertical resolution in the order of 0.1 nm, these experiments can be highly relevant to structural studies of adsorbed proteins.

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