brief communication

Surface-plasmon microscopic observation of site-selective recognition reactions

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ABSTRACT We demonstrate that surface-plasmon microscopy allows one to monitor the specific binding of streptavidin to biotinylated lipid molecules selectively enriched in one of the two coexisting phase domains of a phospholipid monolayer transferred in its phase transition region from the water-air interface to a solid support.

INTRODUCTION

Plasmon surface polaritons (surface plasmons or PSP for short) are surface electromagnetic modes bound to and propagating along the interface between a metal and a dielectric (1, 2). This specific surface "light" recently was introduced as a powerful tool for the optical characterization of surfaces and thin films (3): examples of PSP diffraction (4), interferometry (5), and, in particular, surface plasmon microscopy (SPM) (6), all demonstrated the high sensitivity of these novel optical techniques, which for solid/gas as well as for solid/liquid interfaces can be investigated equally well. However, PSP can be resonantly excited by laser light only by using a plasmon coupler (grating or prism) and only at a well-defined angle of incidence, given by the condition for energy and momentum matching between photons and surface plasmons (2). This resonance behavior, quantitatively described by Fresnel's theory, depends sensitively on the actual optical architecture of the interface, e.g., on the thickness (and the index of refraction) of any thin film coating deposited on top of the metal film that carries the PSP mode (7). In the usual ATR (attenuated total internal reflection) experiment, e.g., in the Kretschmann prism-coupling configuration (8), the angle of incidence is varied until a minimum in the reflected laser light intensity indicates the resonance condition. Any change of the optical thickness of the thin film, e.g., by an adsorption process from the bulk aqueous phase to a lipid monolayer deposited onto the metal by the Langmuir-Blodgett-Kuhn technique (9), can be seen as a shift of this resonance angle. So far, this information could be obtained only averaged over the laser spot. But recently, we could show that the same thickness sensitivity ($\Delta d \sim 1$ Å) can be combined with a high lateral resolution ($\Delta \ell \sim 5$ μm) by taking surface plasmon microscopic pictures as a function of the angle of incidence (10).

The system (strept)avidin-biotin (11) is currently investigated in many laboratories as a model system for a highly specific recognition reaction ($K_m \sim 10^{-15}$ M) which is stable, commercially available, easy to handle, and which can be chemically modified in various ways. It is therefore an ideal test system for many techniques aimed at improving the sensitivity of biosensors: if biotin is coupled, e.g., via a hydrophilic spacer group to the headgroup of a lipid molecule, monolayer techniques can be employed to study the specific binding of (strept)-avidin to such a functionalized well-organized target surface (12). If the (strept)avidin is labeled by a suitable chromophore fluorescence microscopy, in particular, can reveal many details of this recognition reaction. Our model system consists of a mixture of 95 mol % dimyristoylphosphatidylethanolamine (DMPE) and 5% of the biotinylated lipid (Molecular Probes) given in Fig. 1 *a*. If spread and compressed at the (0.5 M NaCl containing) water-air-interface in a home-built Langmuir trough the pressure area (π-A) isotherm indicates a first-order phase transition (Fig. 1 *b*) with a coexistence of fluid matrix and condensed domains (13). If a fluorescent lipid analogue 1 mol % nitro-benzoxadiazol (NBD-DPPE) is added this coexistence can be directly visualized in a fluorescence microscope through the selective enrichment of this label in the fluid phase because it is squeezed out of the growing (dark) crystallites (14, 15). An example is given in Fig. 1 *c*.

The heterogeneous distribution of the biotinylated lipid component in such a monolayer can be, at least qualitatively, demonstrated by the same experimental technique: if (strept)avidin with a fluorescent chromophore attached is injected into the subphase its selective binding to the fluid matrix can be observed. Fig. 1 *d* shows a fluorescence microscopic picture obtained with sulphorhodamine-labeled streptavidin (SR-

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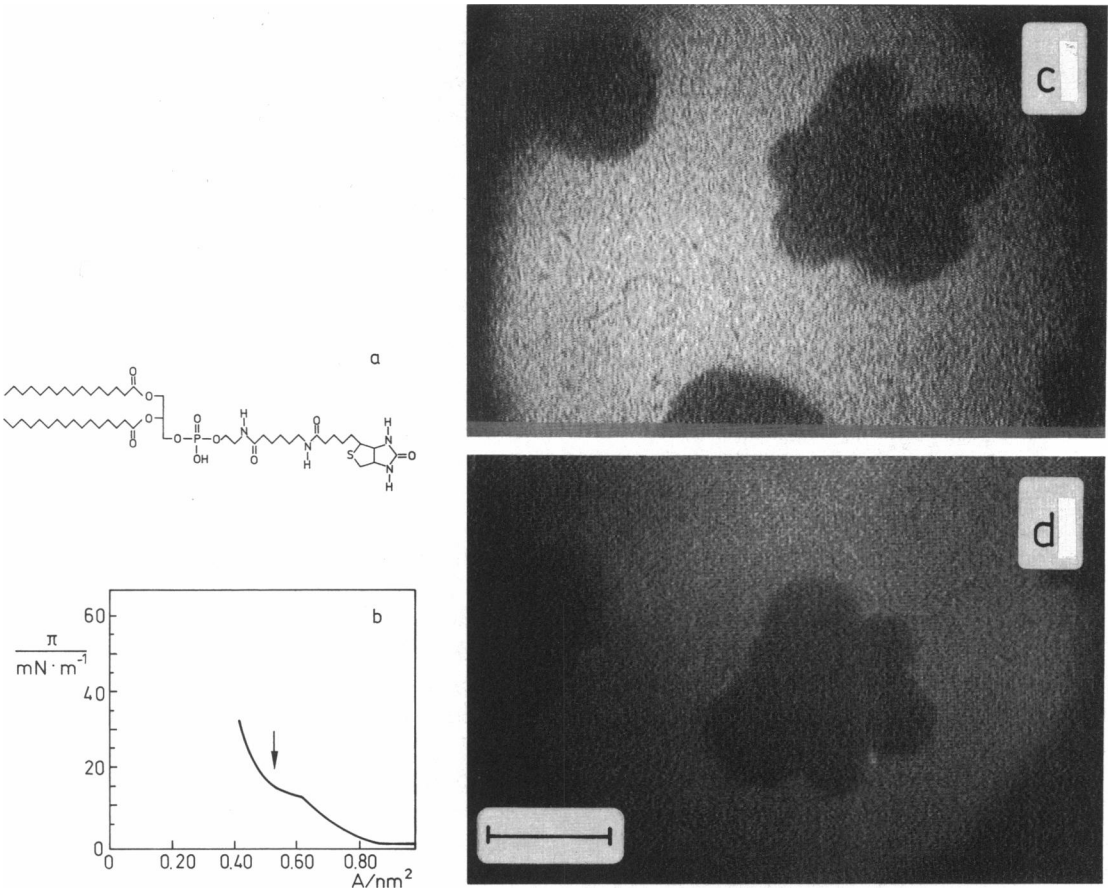

FIGURE 1 (*a*) Structure formula of biotinylated spacer-lipid. (*b*) Pressure area ($\pi - A$) isotherm of DMPE monolayer mixed with 5 mol % of the biotinylated lipid shown in *a*. Subphase: 0.5 M NaCl, T = 20°C. The arrow indicates the pressure of monolayer transfer for surface plasmon optical studies of streptavidin binding. (*c*) Fluorescence micrograph of a DMPE/biotinylated lipid (5 mol %) mixed monolayer doped with 1 mol % NBD-DPPE. $\pi \approx 15$ mN · m^{-1}. The bar corresponds to 50 μm. (*d*) Same monolayer as in *c* but after injection of SR-StA observed in the spectral range of the sulphorhodamine fluorescence emission (which can be clearly discriminated against NBD fluorescence).

StA, kindly provided by Boehringer, Mannheim). The bright areas indicate a high (interfacial) concentration of SR-StA. The lower contrast in this printout compared with the picture obtained with NBD-DPPE indicates a certain solubility of biotinylated lipid also in the ordered DMPE domains, but our setup does not allow for a true quantification, and unspecific binding (see below) would somewhat screen this concentration distribution anyway. The shape of the dark domains clearly indicates that streptavidin binds to its biotin label predominantly present in the fluid matrix. To prove this unambiguously we performed a double-label experiment with NBD-DPPE and SR-StA. The emission characteristics of these two chromophores is sufficiently different so that by the use of suitable filters in the optics of the microscope a clear spectral discrimination of both molecules and hence of their lateral distribution is possible. In our case here, the two pictures obtained were (except for the somewhat different contrast) identical.

Now, for the surface plasmon microscopic observation of this recognition reaction a fresh mixed lipid monolayer in its phase transition region (see arrow in Fig. 1 *b*) but with no label added was transferred to the sufficiently hydrophobic (Cr/Au) metal coated (by evaporation) top of a flow cuvette (made out of LaSF 9) by the horizontal dipping technique (16). Together with the bottom part immersed into the trough subphase before this deposition the cell now contained a closed volume of aqueous medium in contact with the lipid monolayer (see Fig. 2) and could be mounted into the SPM set-up (schematically given in Fig. 2). Fig. 3 shows surface-plasmon microscopic pictures of the lipid monolayer taken at Θ = 58.30 deg (*a*) and Θ = 58.80 deg (*b*). Illumination was done with a HeNe laser beam (2 mW at

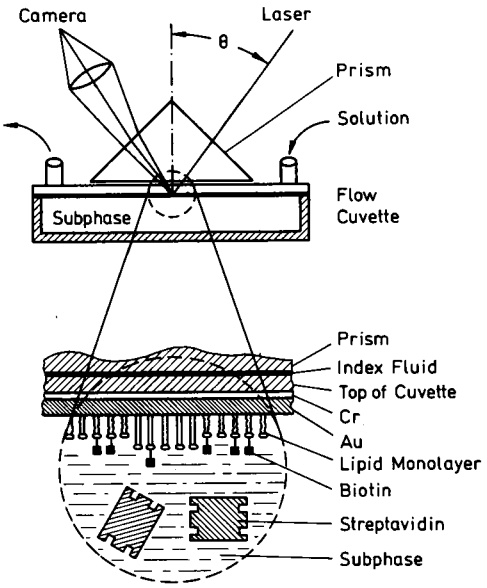

FIGURE 2 Schematic drawing of the experimental setup used for surface-plasmon optical characterization of specific protein binding to a monomolecular lipid layer transferred to the top part of a flow cuvette.

λ = 633 nm). As expected, the crystalline domains are tuned into PSP resonance (appear dark) at higher angles because they are thicker (17). A quantitative evaluation of this thickness difference is given in Fig. 4, *a* and *b*, respectively. Plotted are the averaged reflected intensities from selected matrix or domain areas of $\sim 20 \times 20$ μm^2 as obtained by an image analysis of SPM pictures like those given in Fig. 3, but taken at various angles of incidence, Θ[10]. These curves are equivalent to ATR scans and hence can be compared, in the usual way, with Fresnel calculations. Assuming for both coexisting phases the same index of refraction n_{eff} = 1.50 (19) we obtain from the shift of the two curves (Fig. 4, *a* and *b*, respectively) a thickness difference of $\Delta d_{ab} = 0.6 - 0.7$ nm.

For what follows it is important to note that the two curves have an identical shape, or more generally speaking, that an increasing coating thickness (for small thicknesses) shifts PSP resonances only to higher angles (and does not show any additional broadening due, e.g., to adsorbed aggregates). This is an important feature for the surface plasmon microscopic observation of interfacial adsorption reactions like the binding of streptavidin to a partially biotinylated lipid monolayer like in our case: to follow the kinetics of this adsorption we took SPM pictures every minute after the streptavidin free (sub-)phase in the flow-cuvette had been slowly replaced by a $5 \cdot 10^{-7}$ M unlabeled (!) protein solution which took $\sim$2 min because we wanted to avoid any shear–flow-induced changes at the target monolayer. The angle of observation was Θ = 58.10 deg. The obtained pictures were again image analyzed to derive from individual areas the average intensity values. Given the curves in Fig. 4 we thus obtained the shift of the PSP resonance curve with time, but separately resolved for different areas. In particular we could discriminate between binding to crystalline domains and to fluid/amorphous matrix areas. In principle, we could have analyzed areas as small as 5×5 μm^2 and with a 20 ms time resolution (given by the 50 Hz frame transfer rate of the video system that stores the SPM pictures on magnetic tape [Sony, U-matic]). This shift as a function of time for the first 30 min after the solution exchange was initiated is given in Fig. 5 for the biotin-rich matrix areas (*full circles*). After a time lag of $\sim$2–3 min given by the mixing time the reflected intensity rises because the resonance curve shifts under the influence of the binding. The thickness increase for the crystalline domain areas is considerably less pronounced (not shown) which eventually results in a cross-over of the angular positions of the two corresponding resonance curves. As a result, at $t \sim 25$ min no contrast in the pictures is seen whereas for $t > 25$ min pictures with an inverted contrast are found (see below).

Later on, the thickness increase by adsorption is sufficiently slower so that again SPM pictures taken as a function of the angle of incidence can be analyzed to give the full ATR information. (Such a series of pictures takes $\sim$5 min which would considerably smear out the resonance curves for the faster early stage of the streptavidin binding.) An example taken at t = 5 h is added to Fig. 4 *c* and shows the resonance for the crystalline domains, Fig. 4 *d* for the matrix areas which occurs clearly at higher angles. (The identical shape of the resonance curves compared with those found before the streptavidin binding Fig. 4 *a* and *b* justifies again the fast data sampling technique discussed above!). All thus obtained minimum positions taken every half hour are also plotted in Fig. 5 for both crystalline domains (*open triangles*) and matrix areas (*open circles*). As mentioned already, after half an hour, the formerly thinner matrix areas now are thicker because the streptavidin has bound preferentially to the biotin-rich matrix areas. An example for such an inverted-contrast picture at t = 5 h is given in Fig. 3 *c* taken at Θ = 58.70 deg and in Fig. 3 *d* at Θ = 59.40 deg. Clearly, now the matrix areas are thicker and therefore tune into PSP resonance at a higher angle of incidence. We should stress explicitly that a laser spot averaged normal ATR scan, of course, never could have revealed such details of this site-selective recognition reactions.

The final thickness increase is reached for both areas

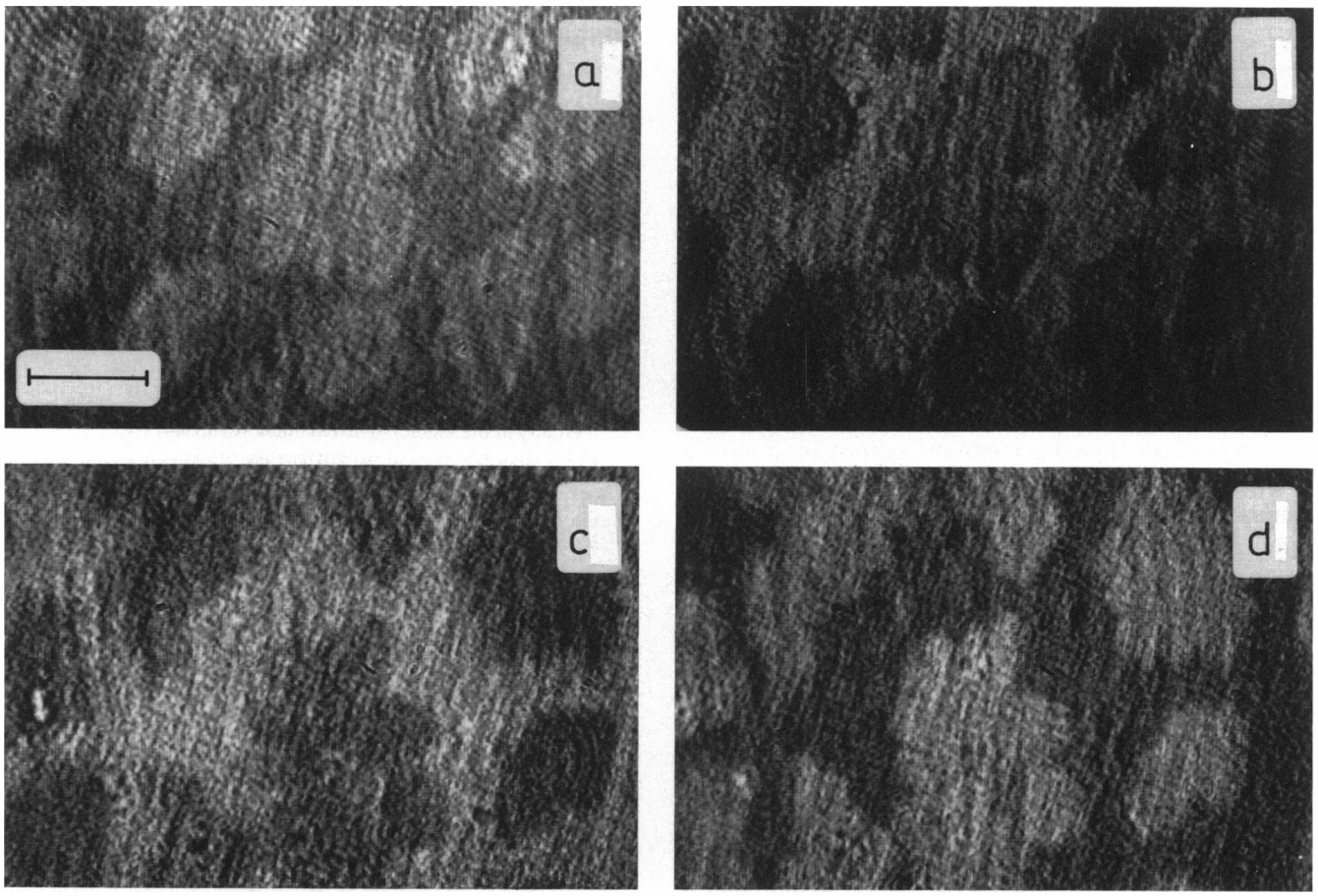

FIGURE 3 Surface-plasmon microscopic pictures obtained from a lipid monolayer transferred in its phase transition region before *a* and *b* and after *c* and *d* binding of streptavidin. *a* was taken at Θ = 58.30 deg, *b* at Θ = 58.80 deg, *c* at Θ = 58.70 deg, *d* at Θ = 59.40 deg. Note the contrast inversion between amorphous matrix and crystalline domains after protein binding! Bar corresponds to 100 μm.

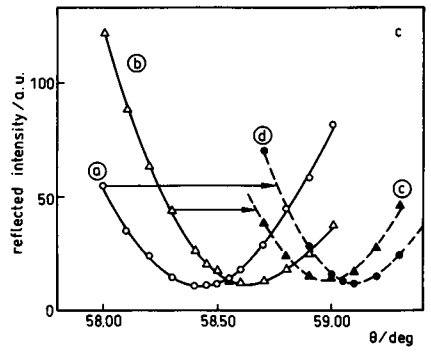

FIGURE 4 ATR-data i.e., reflected intensity-v.-angle of incidence, Θ obtained by a quantitative evaluation of surface plasmon microscopic pictures taken at different angles. Amorphous regions were analyzed simultaneously with the crystalline domains *a* and *b*, respectively, before protein adsorption and after binding of streptavidin injected into the flow cuvette *c* and *d*. Note the larger angular shift of $a \rightarrow d$ (thickness increase of amorphours regions) compared with the crystalline domains $b \rightarrow c$.

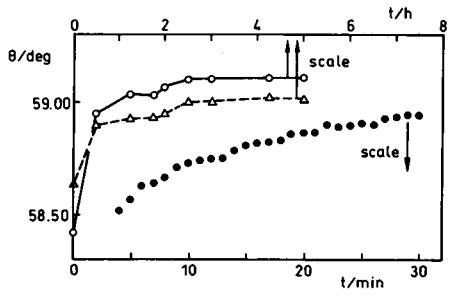

FIGURE 5 Angular shift of ATR resonance curves (indicating the thickness increase) as a function of time after the exchange of the aqueous phase against $5 \cdot 10^{-7}$ M streptavidin solution at $t = 0$. Open circles obtained from amorphous regions, open triangles from crystalline domains. Full circles show the fast binding of streptavidin to the amorphous areas within the first 30 min recorded with a modified technique (see text).

TABLE 1 **Thickness increase Δd of lipid monolayers by specific *a* and unspecific *b* binding of streptavidin**

System	Δd/nm	
	Crystalline domain	Fluid/ amorphous matrix
(*a*) DMPE + 5 mol % biotinylated lipid	1.8	3.4
(*b*) DMPE	0.6–0.8	0.7–0.9

For details see text.

after ~2–3 h and is then stable for several days. Also an exchange of the aqueous medium (against protein-free buffer solution) does not influence the interfacial architecture nor its stability. From Fresnel calculations (assuming for the protein layer an index of refraction of $n = 1.50$) we obtain the thickness increase of each area as it is given in Table 1. The 3.4 nm found for the fluid regions should be compared with the 4.5 nm found by electron microscopy analysis for the densely packed monocrystalline layer of streptavidin (18). In view of this upper limit the coverage of the fluid regions in our experiment is surprisingly high. We should mention that on the lateral resolution scale of the technique which is 5 μm we found no indication for a multilayer formation. The site selectivity of the process given by the preferential incorporation of the biotin label into the fluid phase is seen as a thickness increase about twice as large as that of the crystalline domains. Both values, however, clearly exceed the values obtained for unspecific adsorption in another experiment with identical protein concentration in the aqueous phase but with a monolayer that had no biotin-labels incorporated. This adsorption, in addition, was the same for both fluid and crystalline areas (see Table 1). The biphasic thickness increase seen in Fig. 5 with a time constant of the fast rise of $\tau_1 \approx 20$ min and a slow "relaxation" of $\tau_2 \approx 60$ min is considerably slower than estimated for a diffusion-controlled process: assuming for the unstirred layer a thickness of $\Delta d \sim 100$ μm and a protein-diffusion coefficient of $D \approx 5 \cdot 10^{-7}$ cm^2/s we obtain a diffusion time of $\tau \approx 10^2$ s to cross this layer.

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