brief communication

Polymer-supported bilayer on a solid substrate

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In order to be able to apply the broad range of novel highly sensitive experimental techniques developed in recent years for the characterization of ultrathin (organic/polymeric) films also to model membrane studies it is necessary, in many cases, to prepare lipid bilayers on solid supports. The classical technique for this is the Langmuir-Blodgett-Kuhn (LBK) method, in which multilayer assemblies are built up (layer by layer) by dipping and withdrawing a substrate through a densely packed monomolecular layer of the lipids at the water/air interface (1, 2). The resulting bi- or multilayer assemblies are, however, polycrystalline or amorphous in nature and hence do not allow one to mimic those biophysical properties and processes that require a fluid membrane, e.g., the proper function of a membrane-integral protein. In addition, the surface roughness of most materials used as substrates for the LBK-layer preparation prevents the undisturbed (self-) organization of the lipids as a monomolecular layer, which is a necessary prerequisite for the existence of fluid membranes. This roughness with a r.m.s. amplitude of the order of 1–2 nm for most materials used (SiO_2, glass, metals, polymers, etc.) seems to control many structural aspects of LBK-model membranes (3). The only exception to this is mica with its perfectly smooth surface. There, the second lipid layer transferred is already undisturbed in its phase behavior and can, hence, exist in a fluid state (4).

In order to overcome these problems encountered with rough surfaces, we started a program with the aim of forming bilayer membranes bound to, but structurally decoupled from, the solid support by a flexible polymer chain or network. Following up the observation that the adsorption of the positively charged polyethyleneimine to a negatively charged fatty acid monolayer in the Langmuir trough results in the formation of a fluid-expanded state of the monolayer even after transfer to a solid substrate (5, 6), we extended the "spacer concept," developed for polymerizable and prepolymerized membranes (7, 8, 9) to the build-up of polymer-supported monolayers. By synthesizing copolymers with various functionalities one can tailor a macromolecular assembly that serves several purposes at the same time: comonomeric units with reactive anchor groups ensure the covalent attachment of the polymer to a solid substrate; amphiphilic comonomers with long alkyl chains have intrinsic monolayer forming properties or simply can be used as inserting anchor groups if coprepared with low molecular mass amphiphiles; and, finally, comonomers with a polar character allow for the controlled balance between hydrophilic and hydrophobic properties of the polymer.

In an additional paper we reported on the monolayer formation of various copolymers of this family by the self-assembly (SA) process from organic solvents (10). In this paper a methacrylic terpolymer containing a hydrophilic main-chain spacer, hydrophobic lipid-like parts, and a disulfide unit (anchor group) was used to prepare self-assembled monolayers from organic solvents. It will be shown that these functionalized self-assembled monolayers on solid substrates can be used to built well defined single bilayers by fusion with phospholipid vesicles. The copolymer used for these studies is shown in Fig. 1. It is a statistical terpolymer containing a ratio of hydrophilic monomer to hydrophobic monomer to disulfide anchor group of 6 to 3 to 1. The molecular weight as obtained by GPC measurements with tetrahydrofuran as solvent and polymethylmethacrylate as standard was M_w = 27,000 with $M_w:M_n$ = 1.65. This means that, on average, each polymer can be covalently bound to the gold substrate through up to 10 S-Au bonds.

The film and bilayer formation was characterized by surface plasmon spectroscopy (11). A diagram of the experimental set-up is shown in Fig. 2 *a*. A thin Au-layer was evaporated onto the base of a 90° LaSFN9 high index glass prism (n = 1.85 at λ = 633 nm). This Kretschmann coupling scheme (12) allows for the excitation of plasmon surface polaritons (PSP) at the metal-dielectric interface, upon total internal reflection of a laser beam (HeNe, λ = 633 nm, power ~ 5 mW). At the resonance angle θ_0, which is given by the matching condition for energy and momentum between the (evanescent) photons and the PSP mode, the reflected intensity, monitored as a function of a angle of incidence, θ, shows a sharp minimum. The exact angular position of the resonance depends on the (optical) architecture of the interface (13). An example is shown in Fig. 2 *b*. The full circles are the data points for the bare Au substrate in aqueous buffer containing 0.1 M NaCl. From the Fresnel fit to these experimental data (*full curve*) one obtains

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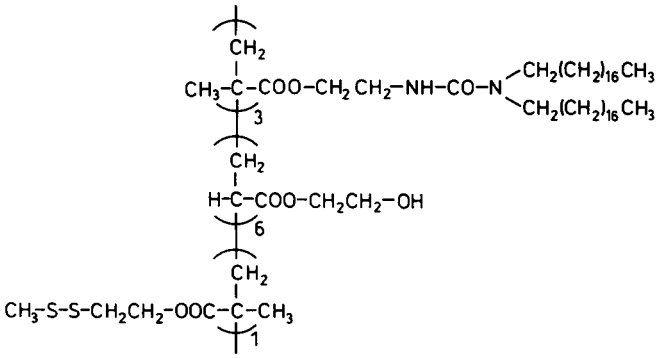


FIGURE 1 Structure formula of the multifunctional, amphiphilic copolymer used in this study.

the dielectric function of the Au layer and its thickness: $\epsilon_{Au} = -12.86 + i\ 1.25$ and $d_0 = 45$ nm. This resonance curve then serves as a reference. Any thin coating, such as a lipid monolayer, typically shifts the resonance to high angles. Fig. 2 *b* shows this for the SA-monolayer of our copolymer. The latter was prepared by immersing the Au substrate in a $CHCl_3$ solution of the polymer for ~20 h. After carefully rinsing and drying the monolayer, the sample was again mounted into the cell and measured in buffer. The open circles are the experimental data. From the Fresnel calculations (*dashed curve*) one obtains the optical thickness of the coating. Assuming an effective index of refraction for the layer of $n = 1.55$, the geometrical thickness is then $d_1 = 1.9$ nm. The error given by uncertainties in the fit procedure is ~10%.

The subsequent vesicle fusion experiment was performed as follows (14, 15): small unilamellar vesicles were prepared by sonication (Branson tip sonicator, 30 W output) of a 20-mg dimyristoyllecithin (DMPC from Fluka) in 2 ml 0.1 M NaCl solution for ~30 min. After dilution to a concentration of 0.2 mg/ml the dispersion was temperature stabilized at $T = 31°C$ and then filled into the sample cell, also held at $T = 31°C$. The fusion process was observed by recording the reflected intensity at a fixed angle of incidence $\theta = 56.00°$ (see *arrow* in Fig. 2 *b*). The time course of the intensity change is given in Fig. 3 *a*. After a small, very fast rise, a slow intensity increase is found which can be well approximated by a single exponential with a time constant of ~1 h. The constant intensity level reached after ~4 h does not change even if one exchanges the vesicle dispersion against pure buffer solution. If the angular reflectivity curve is taken (*full triangles* in Fig. 2 *b*) the Fresnel fit calculation (*dotted curve* in Fig. 2 *b*), with $n = 1.50$ (the known index of refraction for fluid lecithin [16]), yields a thickness increase by the fusion process of $\Delta d = (2.0 \pm 0.3)$ nm which suggests, indeed, the formation of a second monolayer (17) on top of the SA-monolayer. The thickness increase for different experiments was very reproducible provided the vesicles were in a fluid state. Cyclic voltammetry showed that the bilayer formed was able to passivate the gold substrate when used as electrode (Studies in progress).

Lowering the temperature to $T < T_m$, i.e., below chain melting temperature of the lipids, resulted in the formation of irregularly thick multi-bilayers. An example of this is shown in Fig. 3 *b*. The first rise of the reflected intensity is an artifact given by the refractive index increase of the cooling buffer solution. Once the transition temperature T_m is reached (see *arrow* in Fig. 3 *b*), a

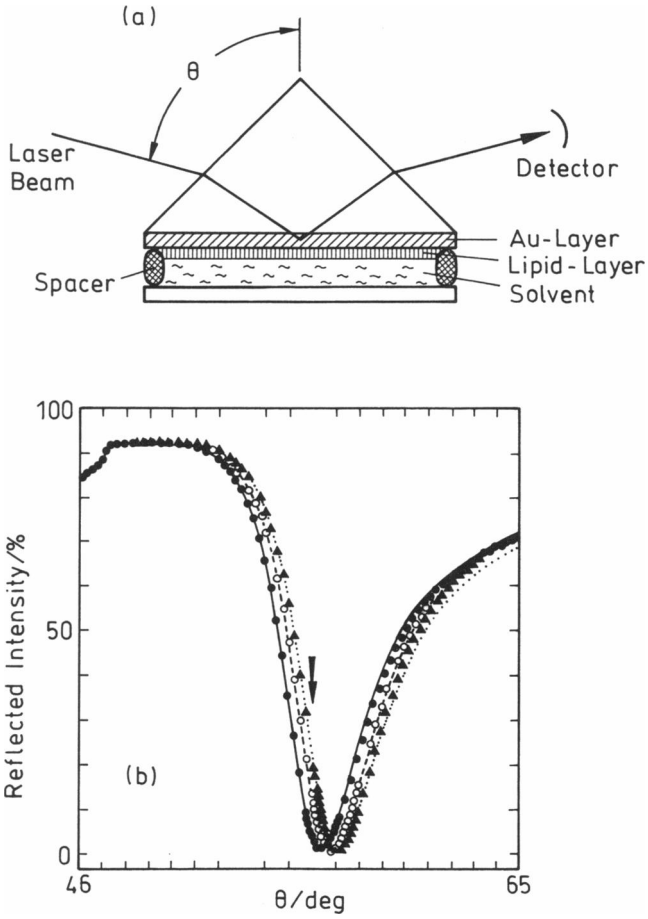
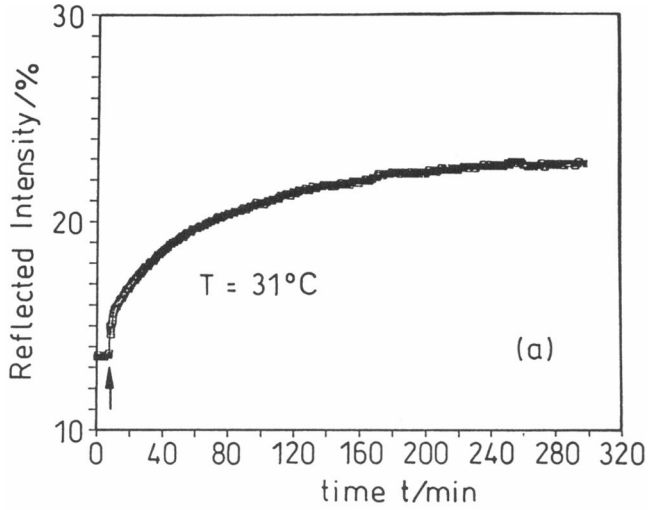


FIGURE 2 (*a*) Schematic of the experimental set-up used for the surface plasmon optical characterization of the mono- and bilayer formation. A laser beam incident at an (external) angle θ is total internally reflected at the Au-layer-coated base of a high index glass prism. The detector monitors the reflected intensity as a function of θ. The polymer-supported monolayer, the bilayer fusion, and the protein binding to a functionalized membrane can be followed with the membrane in contact with the solvent in the cell. (*b*) Reflectivity-versus-θ curves as obtained with the surface plasmon spectrometer for various interfacial architectures. Symbols are the experimental data points, the curves are Fresnel fit calculations: (— ● —) bare Au substrate; (— ○ —), after self-assembly of the copolymeric monolayer; (· · · ▲ · · ·) after vesicle fusion. All experiments were done at $T = 31°C$ in 0.1 M NaCl buffer. The arrow indicates the fixed angle of incidence for the time-dependent measurement of the reflected intensity (cf. Fig. 3 *a*).

strong increase of the intensity indicates the growth of multilayer assemblies onto the initial bilayer. Even after 5 h at room temperature, the thickness still increases. A very similar behavior was reported for the fusion of vesicles onto a SA-monolayer of a lipid analogue with a relatively short spacer in the headgroup (18).

In order to provide further evidence that a bilayer is formed if the fusion is initiated at $T > T_m$, we performed a streptavidin binding experiment with a biotinylated lipid layer. This recognition reaction is known to result in a protein monolayer formation with high efficiency only in the gas or liquid analogue phases on the air-water



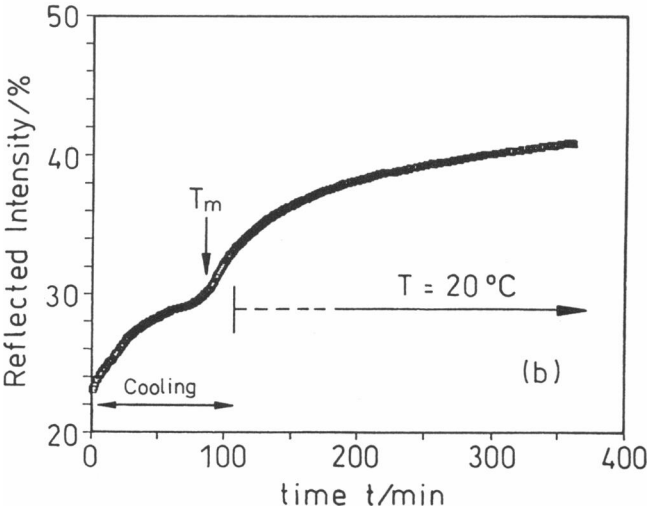


FIGURE 3 (*a*) Reflected intensity versus time after addition of the vesicles dispersion (see *arrow*) at $T = 31°C$. The data are taken at a fixed angle of incidence $\theta = 56.00°$. (*b*) Data taken during the slow cooling and after the sample had reached $T = 20°C$. The first rise of the reflected intensity ($t = 0$–90 min) is due to the refractive index increase of the cooling buffer solution. The rise starting at $T = T_m$ is given by multilayer formation.

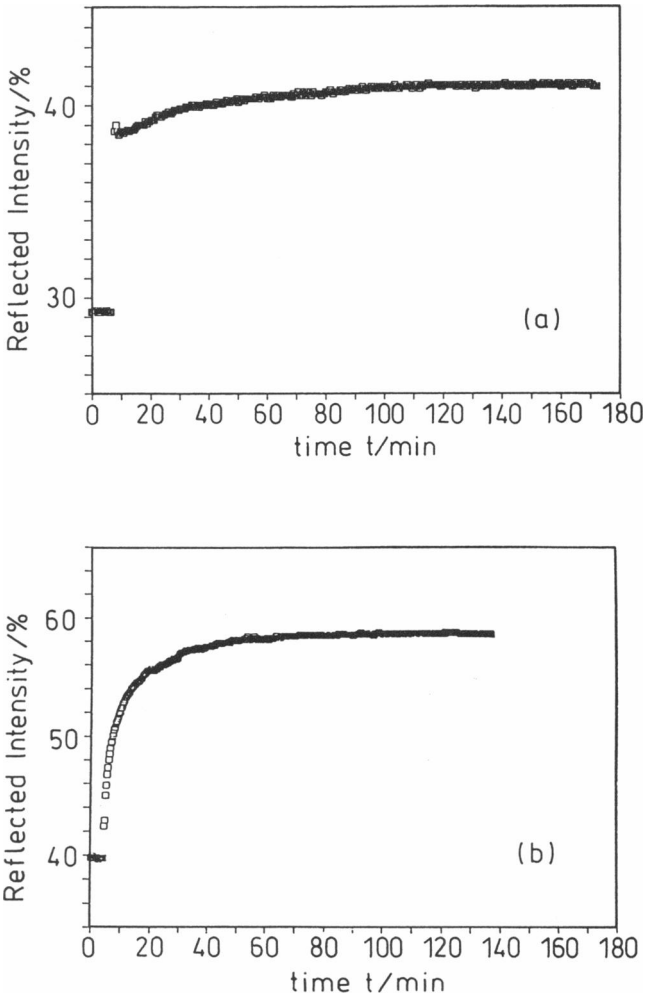


FIGURE 4 (*a*) Reflected intensity versus time after the addition of biotin-functionalized vesicles (see *arrow*). $T = 31°C$, $\theta = 55.70°$. (*b*) Same experiment as (*a*) after the vesicles dispersion had been exchanged against a $5 \cdot 10^{-7}$ M streptavidin containing buffer solution.

interface (19–21). For this experiment we doped the DMPC vesicles with 5 mol% *N*-(biotinoyl) dipalmitoylphosphatidylethanolamine (Molecular Probes) which is negatively charged.

The sonicated vesicles are rather small, resulting in an almost clear dispersion. The fusion, again performed at $T = 31°C$, leads to a very rapid, almost instantaneous bilayer formation (shown in Fig. 4 *a*) with a thickness increase of $\Delta d = (1.6 \pm 0.3)$ nm. This bilayer configuration is very stable for long times. If the vesicle containing solution is exchanged against a $5 \cdot 10^{-7}$ M streptavidin solution (also containing 0.1 M NaCl) a further increase of the reflected intensity shows the formation of the protein monolayer. This is depicted in Fig. 4 *b*. The time course of the first 3 min, can be analyzed to give the diffusion coefficient of the protein $D = 1 \cdot 10^{-7}$ cm^2/s, which is indicative of a diffusion limited adsorption also found for other biotinfunctionalized monolayers (22).

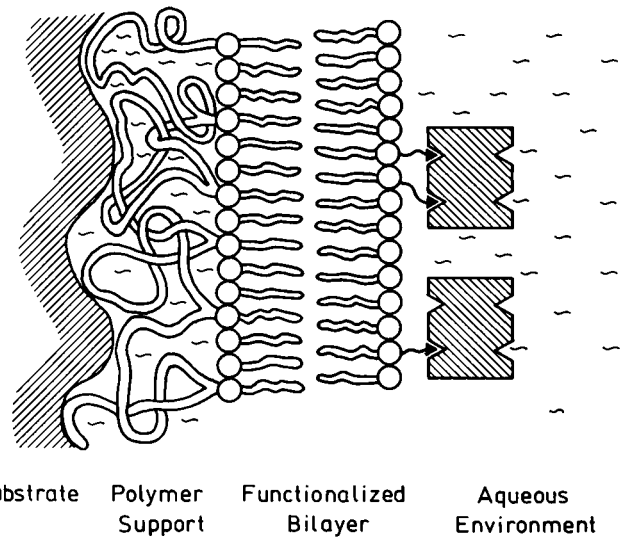


FIGURE 5 Schematic diagram of the polymer supported, functionalized bilayer on a rough solid substrate.

The final thickness increase after washing amounts to $\Delta d = (4.1 \pm 0.3)$ nm (calculated with an effective index of refraction of $n = 1.45$ (23) and indicates a very dense streptavidin monolayer specifically docked to the mobile supported bilayer (see Fig. 5).

In conclusion, we have demonstrated the formation of a polymer-supported monolayer by the self-assembly of a multifunctional disulfide containing amphiphilic copolymer. A fusion process in an aqueous environment, allows the adsorption of a stable lipid monolayer onto the copolymer, provided the lipid is in a fluid state. The stability of this model membrane system is enhanced by the covalent binding of the polymeric support layer to the solid substrate. Its stability and the possibility of varying the composition of the system in a wide range may allow the study of the electrical properties of these stable bilayers in experiments where normally the very fragile black lipid membranes (BLMs) are used. This may open new concepts for sensor applications. In addition, we have shown that such a bilayer can be employed to study specific recognition reactions at functionalized membranes (24).

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