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A miniaturized monolayer trough with variable surface area in the square-millimeter range

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Abstract

A new simple concept for a miniaturized monolayer trough is described. The overall monolayer area in the expanded state is approximately 150 mm^2 and can be reduced by a factor of 2. The surface area is a function of the shape of the meniscus formed by the subphase and is controlled by the amount of water in the monolayer trough. The controlled compression of monolayers to a desired area per molecule with simultaneous observation of the lateral distribution of fluorescently labeled molecules is shown. A biological reaction between a specific antibody and lipid anchored peptide demonstrates the feasibility of monolayer experiments, which require only very small quantities of substance (in the pmol range). This trough might also be a valuable tool for the 2D crystallization of proteins at lipid layers via specific binding sites such as metal chelators. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The air/water interface offers unique possibilities for the 2D organization of mono- and multilayers of molecules and particles. The classical tool for the manipulation of such layers is the Langmuir film balance [1,2]. It allows variation of the molecular density of the layers at the air/water interface using a movable barrier (or barriers) and the simultaneous measurement of the surface tension, or equivalently, the lateral surface pressure of the film spread on the surface [3]. This technique has experienced a renaissance in recent years due to a vastly increased interest in 2D systems in a number of scientific disciplines [4–7]: ordering and phase behavior in 2D systems of organic monolayers of lipids, detergents and peptides; 2D crystallization of proteins; biological receptor ligand interaction; fabrication of highly organized functional monolayers containing chromophores, receptors, liquid crystal compounds and nanoparticles for the development of novel technical devices. Simultaneously with the growth of interest in novel 2D systems, many new analytical techniques such as fluorescence [8–10] and Brewster microscopies [11,12], FTIR spectroscopy [13], optical second harmonic generation [14], neutron [15] and X-ray scattering techniques [16] have been developed and

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combined with the Langmuir technique, making this an even more powerful approach.

In general, the number of molecules needed for the preparation of films on a Langmuir trough is relatively small. However, very often, when tiny quantities of peptides or proteins are investigated, very small surface areas or, for adsorption experiments from the subphase to the air/water interface, small trough volumes are required. We present, in this paper, a new concept for a trough with a variable and controllable surface area of some hundreds of square millimeters (see Fig. 1) and a volume of approximately 0.5 ml.

On a small round Teflon trough with a diameter of some millimeters, the water surface is highly curved due to its surface tension. The curvature and the surface area are functions of the amount of water in the trough. The water volume can be easily varied through a syringe which is connected to the subphase. With such a system, a variation of the surface area can be achieved through a change in the shape of the meniscus. A similar principle was recently used in studies of a self-assembled monolayer (SAM) on a mercury drop [17].

Here, we present measurements on such a trough in combination with fluorescence microscopy. First, the area ratio of the condensed phase of a lipid monolayer was measured as a function of the subphase volume on the small trough and as a function of the overall area on a normally sized Langmuir film balance. This allowed a relationship to be established between the subphase volume and the surface area of the small trough. The results are compared with simple geometrical calculations. In a second experiment, the specific binding of a monoclonal antibody —injected into the subphase—to an antigenic lipopeptide, which is incorporated to a floating lipid monolayer, is demonstrated. The peptide consists of part of the sequence of a capsid protein of the Picornavirus causing foot-and-mouth disease in cattle. This peptide segment represents a major antigenic site of the virus [18,19]. Such lipopeptides exhibit excellent carrier and adjuvant properties for antibody production and have also been used in the development of vaccines.

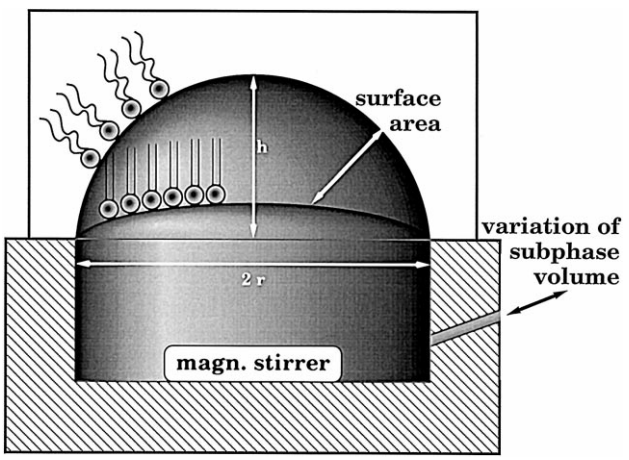

Fig. 1. Schematic view of the miniaturized monolayer trough. The area of the water surface is a function of the volume of the aqueous subphase. This allows compression of a lipid monolayer from the fluid into the condensed phase. A small lid on the trough prevents evaporation of the subphase.

2. Materials and methods

2.1. Substances

The lipid 1,2-dipalmitoylphosphatidylcholine (DPPC) was from Fluka (Buchs, Switzerland), and used without further purification (purity $>99\%$, as tested by thin-layer chromatography) as a stock solution of 2 mg ml^{-1} in chloroform/methanol (9/1). To this solution, 2 mol% of fluorescently labeled 1,2-dimyristoyl-*sn-glycero*-3-phosphoethanolamine (NBD-PE, Molecular Probes, OR, USA) was added. The water used in all experiments was purified in an ion-exchanger purification train (Milli-Q system, Nanopore, Volketswil, Switzerland) and had a resistivity higher than 18 MΩ cm. The buffer used was 5 mM phosphate, pH 7.4. The organic solvents and the salts used for buffer preparation were purchased from Fluka (Buchs, Switzerland), and were of the best quality available.

The NBD-labeled (4-chloro-7-nitrobenz-2-oxa-1,3-diazole) lipopeptide Pam_3Cys-Ser-Ser-(VP1(135–154)) (NBD-LP) was synthesized as described elsewhere [20,21]. The compound was stored as a dry powder at -50°C and dissolved prior to use to give stock solutions of 1 mg ml^{-1} in methanol. The solution was stored at 4°C. The monoclonal antibodies against the VP1(135–154) were produced by Dr. J. Bouvier, Ciba Geigy, St. Aubin, Switzer-

land. The exact NBD-LP and antibody concentrations were determined by their absorbances at 480 nm and 280 nm using molar extinction coefficients of 20.000 and 227.000 M^{-1} cm^{-1}, respectively.

The antibodies were fluorescently labeled with Rhodamine-X-isothiocyanate (Molecular Probes) according to standard procedures [22] to a final ratio of about two Rhodamine-X molecules per antibody, as determined by absorption spectroscopy using the ratio of the intrinsic protein absorption at 280 nm vs. the Rhodamine absorption at 570 nm.

2.2. Monolayer experiments

The monolayer experiments were performed on the miniaturized trough, which is subject of this paper. For the calibration experiments a conventional computer-controlled Langmuir film balance ($A_{max} = 143$ cm^2) was used, which was equipped with a Wilhelmy system for the measurement of the surface pressure (Riegler and Kirstein, Mainz, Germany). Both troughs were made of Teflon. For the fluorescence microscopy experiments, both film balances could be mounted on the object table of a Zeiss Axiotron epifluorescence microscope (Zeiss, Germany). Two different filter sets allowed the separate excitation and observation of the NBD-labeled LP in the monolayer and the Rhodamine-X-labeled antibody. In order to prevent evaporation of the subphase of the small trough it was covered with a small lid. The NBD-LP/DPPC monolayers were spread on the water surface with a Hamilton microsyringe from chloroform/methanol (9:1) solutions. For the antibody binding experiments, a known amount of the antibody stock solution was injected into the buffered subphase and stirred with a small magnetic bar placed on the trough bottom.

3. Results and discussion

A schematic representation of the film trough is depicted in Fig. 1. It is made of Teflon. The inner diameter ($2r$) of the circular trough is 10 mm. The surface area is determined by the amount of subphase in the trough and can be controlled via a syringe connected to the subphase. The maximum area of the water surface is obtained when the meniscus takes the shape of a hemisphere, which corresponds to a value of $2\pi r^2 = 157$ mm^2. The minimal area corresponds to an almost flat surface, which is given by $\pi r^2 = 78.5$ mm^2. Therefore, the compression ratio is 2. Taking some typical areas per molecule for lipids (50 $Å^2$) and for proteins (1000 $Å^2$), the numbers of molecules necessary to cover the water surface are approximately 1.6×10^{14} (or 260 pmol) and 8×10^{12} (or 13 pmol), respectively. The volume of subphase needed is on the order of 0.5 ml.

Neither the direct determination of the area per molecule nor the measurement of the surface pressure is possible on this trough: the paper of the Wilhelmy system is drawn to the Teflon edge due to electrostatic charging. To demonstrate the working principle of this trough we used fluorescence microscopy for the determination of the area ratio of the condensed phase of a monolayer of DPPC. On our normally sized Langmuir trough the area ratio of the condensed phase was measured as a function of the surface area. This allowed the determination of the surface area as a function of the water volume in the miniaturized trough.

Fig. 2 shows the isotherm of DPPC measured on the normally sized Langmuir trough. The kink in the curve indicates the onset of the first order phase transition from the fluid to the condensed phase. It is followed by an extended flat region attributed to the coexistence of the two phases. Using fluorescence

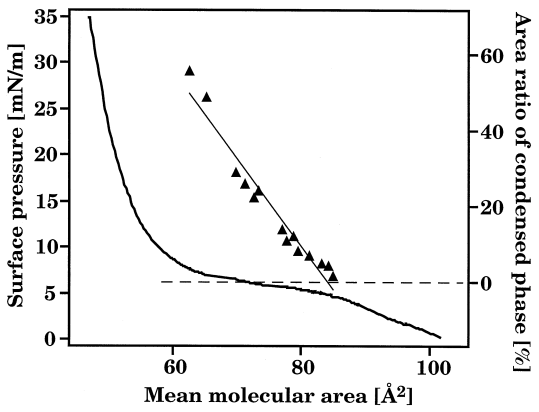

Fig. 2. The surface pressure vs. molecular area isotherm of a DPPC/2 mol% NBD-PE monolayer and the corresponding area ratio of the condensed phase (triangles) as determined by fluorescence microscopy. The straight line is a linear fit. This isotherm was recorded on a normally sized Langmuir trough.

microscopy it is possible to visualize both phases on the water surface: a fluorescently labeled lipid which is added in small amounts has a much higher solubility in the fluid phase than in the condensed phase [8]. Therefore, the condensed domains appear dark in the bright surrounding fluid phase and can be easily identified. As shown in Fig. 2, the area ratio of the condensed phase is a linear function of the area per molecule in the coexistence region. [2] This result is used for the calibration of the surface area on our small film trough: the area ratio of the condensed phase is measured on the miniaturized trough as a function of the subphase volume and this is translated into a surface area volume relation. The result is shown in Fig. 3 (squares). The line in Fig. 3 represents the result of a calculation in which the area–volume relation is derived from a section of a sphere as drawn in the insert. The limiting values of the volume of the meniscus in the calculation are 0 and 250 μl, which correspond to a flat round disc and to a hemisphere. The experimental curve was obtained by transforming the area ratios of the condensed phase measured on the small trough, according to the linear relation from Fig. 2 into a surface area and plotting these values as a function of the volume of the subphase. The only free parameter in this relation determines the absolute surface area for a single measured value (volume = 200 μl and surface area = 133 mm^2). Consequently, the slope of the experimental curve is independent of any fitting procedure.

This demonstrates that the experimental area change vs. volume agrees well with our simple geometrical approximation.

In order to show the kind of applications this trough was designed for, we present a binding experiment using an antibody against a peptide sequence which is immobilized in a lipid monolayer at the air/water interface. The quantity of antibodies which must be injected into the subphase to reach a desired concentration can remain small due to the small volume of the trough. The details of this receptor–ligand system have been described in a recent publication and are summarized here [23]: the NBD-labeled

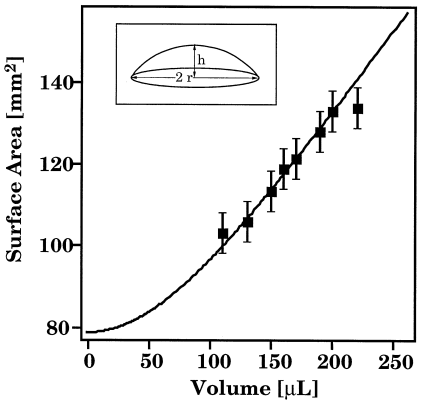

Fig. 3. Surface area vs. water volume of a spherical section (full line). The spherical section (see insert) was used in our model calculation to represent the amount of water above the edges of the monolayer trough. The squares show the area derived from the experimentally measured area ratios of the condensed phase of a DPPC monolayer vs. the water volume (see text for details).

peptide is chemically attached to the lipopeptide Pam_3Cys-Ser-Ser, a chemical analogue of the lipid anchor of membrane-bound lipoprotein in *Escherichia coli* [20,21]. The peptide consists of the amino acids 135–154 of the sequence of the capsid protein VP1 of the Picornavirus. The amphiphilic nature of this lipopeptide allows its vectorial assembly in monomolecular lipid layers at the air/water interface. The monoclonal antibody against the peptide sequence is labeled with Rhodamine-X and has been shown to have a binding constant of 3×10^7 M^{-1}.

A mixed lipid monolayer containing DPPC and 2% NBD-labeled lipopeptide was prepared at the air/water interface of the miniaturized trough. The amount of the lipid mixture spread onto the water surface resulted in a surface density corresponding to the fluid phase of the film (as was verified using our normally sized Langmuir trough). No domains could be observed at this stage. By removing defined amounts of water the monolayer was slowly compressed to a pressure above the phase transition and the formation of solid domains could be observed by fluorescence microscopy. Like normal fluorescent lipid probes, the lipopeptide is only soluble in the fluid phase of the DPPC monolayer and therefore the domains are almost free of labeled lipopeptides and appear dark (see Fig. 4A). After the monolayer had

[2] Here we do not use the degree of crystallization which results from the area ratio when normalized with the areas per molecule in the condensed phase.

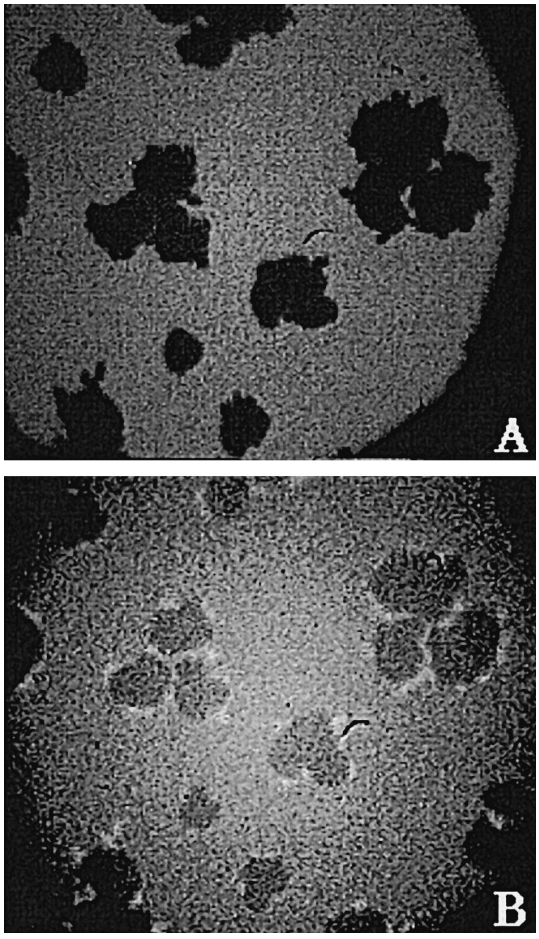

Fig. 4. Fluorescence micrographs of a DPPC monolayer containing 2 mol% NBD-LP at the water surface after incubation with Rhodamine-X-labeled antibodies for 1 h. The image (A) was taken with the NBD filter set and the image (B) was taken with the Rhodamine filter. The images correspond to an area of $150 \times 120\ \mu m^2$.

reached a stable state, the specific antibody against the peptide was injected into the subphase to a concentration of 5×10^{-8} M, which corresponds to a total of 20 pmol antibody. After 1 h, the fluorescence micrographs in Fig. 4A and B were recorded. In (A), a filter set for excitation and emission corresponding to the spectral properties of the NBD was employed; in (B) the filter set for Rhodamine-X was used. It can be seen that the structural features in both images are identical. The specificity of the antibody binding is reflected in the fact that the fluorescence from the Rhodamine-X-labeled antibodies can be observed only in the fluid phase where the lipopeptide is localized. The amount of antibody bound is estimated to be maximally 2 pmol. Particularly bright features of the Rhodamine fluorescence are seen at the edges of the domains. Probably the boundaries can induce some kind of aggregation of antibodies.

We have presented a new simple concept for a miniaturized monolayer trough. The overall monolayer area in the expanded state is approximately 150 mm^2 and can be reduced by a factor of 2. The surface area is a function of the shape of the meniscus formed by the subphase and is controlled by the amount of water or buffer in the monolayer trough. The relationship between water content and surface area can be modeled by simply representing the meniscus by a spherical section with variable height. The experiments performed demonstrate that this trough allows the controlled compression of monolayers to a desired surface area per molecule with simultaneous observation of the lateral distribution of fluorescently labeled molecules. In this way a biological reaction between a specific antibody and lipid anchored peptide was monitored. This shows the potential of this approach for the investigation of surface reactions, in particular when the amount of substance available is very small (in the pmol range). This trough might also be a valuable tool for the 2D crystallization of proteins at lipid layers via specific binding sites such as metal chelators [24]. Multiarrays of such tiny troughs are easily fabricated and can serve for screening for optimal crystallization conditions at the air/water interface.

Acknowledgements

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