Lipid Bilayers   (VIP)

Hydrogel Stamping of Arrays of Supported Lipid Bilayers with Various Lipid Compositions for the Screening of Drug–Membrane and Protein–Membrane Interactions**

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Herein we describe a rapid, reproducible, and straightforward method to form copies of functional membrane arrays with various lipid compositions and the application of these arrays for the screening of drug–membrane and protein–membrane interactions. We employed topographically patterned agarose gels to stamp spatially addressable arrays of supported bilayers on glass and confirmed the fluidity of these membranes by fluorescence recovery experiments. We took advantage of the storage capability of hydrogels and demonstrated that inking posts on an agarose stamp, with extremely small volumes ($\leq 1\ \mu L$) of a solution that contains liposomes, was sufficient to transfer at least 100 copies of a membrane array without the need for reinking. We used stamped membrane arrays for screening the interactions of a protein (annexin V) and an anti-inflammatory drug (nimesulide) with bilayers of various lipid compositions and discovered that the interaction of the prescription drug nimesulide with membranes depends on the membrane cholesterol content.

Interest in supported bilayers[1–3] includes studies of the dynamic structure of membranes,[4,5] their self-assembly,[5] lipid–protein interactions,[5] ligand–receptor interactions,[5–8] development of membrane-based biosensors,[5,9–15] and drug discovery.[16] Furthermore, many pharmaceuticals are known to interact with biological membranes and, as such, assays for testing drug–membrane interactions are important for a better understanding of drug activity, targeting, and toxicity.[17] To use supported bilayers efficiently for the study of the aforementioned processes, the membranes must be fluid[1,16,18,19] and mechanically stable.[19] Techniques that are currently employed to form arrays of supported membranes exploit a) the deposition of droplets of a liposome solution onto surfaces,[6,18] b) vesicle fusion from a bulk solution onto patterned substrates,[20,21] c) delivery of liposomes by microfluidic channels,[22,23] and d) microcontact printing with poly-(dimethylsiloxane) (PDMS).[24]

An ideal fabrication method for the application of membrane arrays for screening protein–membrane or drug–membrane interactions would consist of the rapid creation of many functional copies of an array of different bilayers with minimal consumption of the amount of lipid. Among the existing methods, microcontact printing allows the creation of many spots of membranes in parallel. To prepare arrays with various compositions, however, posts of the stamp used for microcontact printing must be inked individually. Such an inking procedure can be time consuming and can introduce heterogeneity into the stamped arrays. It would therefore be advantageous if a biocompatible stamp, once inked, could store the inking solution and allow multiple transfers without the need for reinking.

Herein, we demonstrate that stamping with hydrogel stamps allows multiple stamping while using minute amounts of material. We fabricated stamps from agarose gel (4%) with a pore size sufficiently large to allow the diffusion of macromolecules and small liposomes (the pore size of 2% agarose gel is ≈ 470 nm[25]). This capability makes it possible to store inking solution within the stamp while replenishing molecules at the surface and, thus, to perform multiple stamping of biomolecules.[25] Recently, agarose stamps[26,27] were applied to pattern arrays of proteins,[28,29] bacteria,[30] and mammalian cells.[31] Herein we present the first attempt to use hydrogel stamps to create functional arrays of fluid lipid membranes.

To form arrays of lipid bilayers, we inked each post on the agarose stamp with a sub-microliter volume of liposome suspension. After the posts had absorbed the solution, we placed the inked stamp in contact with glass slides for 5–10 s (see Supporting Information for details and stamp dimensions). Immediately after removal of the stamp, we immersed the patterned substrates in either deionized water or PBS buffer solution. We hypothesize that during stamping, supported membranes form by diffusion of small liposomes (diameter 20–80 nm)[32] through the pores of the agarose stamp and the subsequent spread of liposomes onto the regions in contact with the glass. As the agarose stamps used in this method consist mostly ($\geq 96\%$ *w/w*) of water, we suggest that the mechanism of bilayer formation is similar to the mechanism of the established method of vesicle fusion from solution.[16]

We examined the structure of the stamped lipid bilayer spots on the glass substrate by atomic force microscopy (AFM) experiments (see Supporting Information). The AFM results revealed a smooth surface of bilayer with scattered defects. The area of the defect sites was $\leq 23\%$ of the total imaged bilayer area. Cross-section analysis of the defects revealed a thickness of 4.3 ± 0.8 nm, which corresponds to the height of a single lipid bilayer.[6]

To investigate the capability of agarose stamps to store small liposomes, we used a stamp that was inked once and stamped 100 times without intermediate reinking. This stamp was inked with a solution of liposomes composed of L-α-

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phosphatidylcholine derived from chicken egg (egg PC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine-rhodamine B sulfonyl) (rh-PE; 1% *w/w*). We found no significant loss in the fluorescence intensity of the spots over 100 stamping events (Figure 1a). Figures 1b and c show
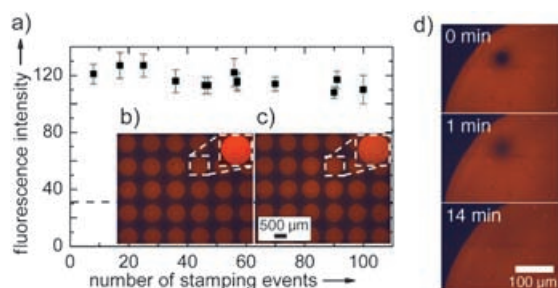

Figure 1. Fluorescence intensity after stamping of 100 arrays of membranes by using a hydrogel stamp (without intermediate inking), and test of bilayer fluidity. The bilayers composed of egg PC and rh-PE (1% *w/w*) were stamped on glass slides. a) Mean fluorescence intensity of supported bilayers as a function of the number of stamping events. The error bars represent the standard deviation of the intensity of all spots in each array. The dashed line represents the mean fluorescence intensity of the background. b) Micrograph of spots of supported bilayers after the 6th and c) 100th stamping event. d) Fluorescence images from a FRAP experiment performed on the array from the last (100th) stamping event after photobleaching for 8 min.

fluorescent micrographs of membrane arrays on glass that were obtained after 6 and 100 stamping events, respectively. To test the fluidity of the stamped bilayers, we performed fluorescence recovery after photobleaching (FRAP) experiments (see Supporting Information for details). Figure 1d shows the recovery of a photobleached spot in a bilayer of the last (100th) array. Fluorescence recovery of the bilayers in this array was similar to that in the 6th array (difference in diffusion constant $\leq 5\%$) which therefore indicates that the quality of the stamped arrays remained constant over ≈ 100 stamping events. The fluorescence intensity in the photobleached spot recovered to $\approx 90\%$ of its original intensity, a value typical for supported membranes of high quality.[6]

We used a stamp with 200-µm-diameter posts to explore the potential of this method for the production of membrane arrays with a high density. Figure 2a shows such a resultant array with a density of 600 membrane spots per cm². If such a stamp were inked by using robotics, it should, in principle, be possible to stamp multiple copies of high-density arrays with various membrane compositions.[29]

To demonstrate the ability of this method to create, in parallel, bilayers with different lipid compositions, we transferred arrays of supported membranes by using liposomes that contained 0–50% negatively charged lipids and 0–50% cholesterol. Remarkably, hydrogel stamping resulted in high-quality membrane arrays on glass substrates even when the bilayers contained 50% negatively charged lipids (Figure 3a). Figure 2b shows an array of bilayers with two different fluorescently labeled lipids. These spots were transferred in parallel from the same stamp.
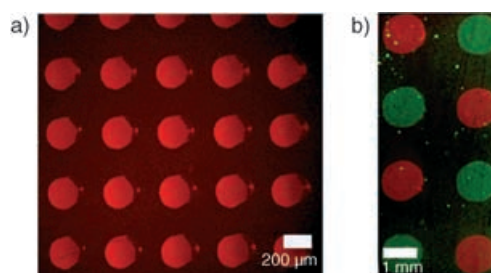

Figure 2. Stamped high-density array of supported bilayers and membrane arrays with various compositions. a) Fluorescent micrograph of a patterned array of bilayers composed of egg PC with rh-PE (1% *w/w*) by using an agarose stamp with a post size of 200 µm. b) Fluorescent micrograph of an array of bilayers that contains egg PC and rh-PE (1% *w/w*; red) and NBD-PE (3% *w/w*; green).
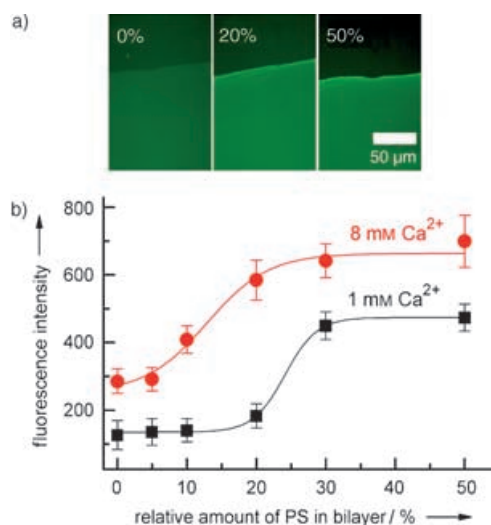

Figure 3. The protein annexin V binds to bilayers with different DOPS content. a) Micrographs of fluorescently labeled annexin V bound to bilayers that contain 0, 20, and 50% (*w/w*) DOPS. b) Increase in fluorescence intensity due to the binding of annexin V to an array of bilayers with a gradient in DOPS. The binding of annexin V is calcium-dependent; these data were obtained in (■) 1 mM and (●) 8 mM Ca²⁺. The error bars represent the standard deviations in fluorescence intensity.

To determine if liposomes with different lipid compositions are transferred differentially from the agarose stamp to the glass slide, we inked the posts of a stamp with mixtures of two different liposome populations (the first population was composed of egg PC (99% *w/w*) and rh-PE (1% *w/w*) and the second was composed of egg PC (50% *w/w*) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS; 50% *w/w*)). We used this stamp to create a membrane array and subsequently studied the compositions of the resultant bilayers (see Supporting Information for details). Epifluorescence microscopy images showed that the fluorescence of the transferred bilayers was proportional to the ratio of the two liposome populations in the inking solution. This result suggests that there is no significant preference in transfer between the neutral and negatively charged liposomes.

To explore the possible applications of membrane arrays with various compositions for the screening of protein–

membrane interactions, we formed arrays from egg PC with a gradient in the negatively charged lipid, DOPS, and measured the binding of annexin V to these bilayers. Annexin V is a calcium-dependent binding protein that interacts with negatively charged lipids.[33] Figure 3a shows micrographs of the binding of fluorescently labeled annexin V to supported bilayers with three different concentrations of DOPS. Figure 3b illustrates that the binding of annexin V to supported membranes increases with an increasing concentration of both DOPS in the bilayer and calcium ions in solution. These results show that stamped arrays of supported membranes can be used effectively to quantify protein–membrane interactions.

Further to the investigation of protein–membrane binding, membrane arrays may be useful for screening drug–membrane interactions. These interactions can depend on the composition of the lipid membrane[17] (e.g. the content of cholesterol) and can induce a change in the fluidity of the bilayer.[34] The therapeutic and toxic effects of many drugs are affected by their interactions with lipid membranes.[17,34] To demonstrate the influence of the lipid composition on drug–membrane interactions, we studied the fluidity changes that are introduced by the non-steroidal anti-inflammatory drug (NSAID), nimesulide, in bilayers with various cholesterol content. NSAIDs, (e.g. aspirin or ibuprofen) are the most-important drugs in the treatment of inflammation, pain, and fever.[34]

Lucio et al. reported that nimesulide increases the fluidity of membranes composed of egg PC.[34] In the studies described herein, we incubated stamped membrane arrays of egg PC with various cholesterol content in solutions that contain 0, 50, and 100 µM nimesulide for 2 h, and monitored the changes in fluidity by FRAP experiments.[35], [36] Figure 4 shows the fluorescence recovery of bleached spots in the presence of nimesulide. In agreement with Lucio et al.,[34] we found that nimesulide (50 µM) increased the fluidity in egg PC membranes. We discovered the same effect in bilayers with a moderate cholesterol content ($\leq 20\%$). Surprisingly, however, nimesulide had the opposite effect, namely a decrease in fluidity, on bilayers with a high cholesterol content ($\geq 50\%$). Furthermore, nimesulide decreased the fluidity of all the examined bilayers when it was added at a concentration of 100 µM (Figure 4c). The diffusion coefficients of fluorescently labeled lipids in these bilayers are summarized in Table 1.

Figure 4. Influence of nimesulide on the fluidity of stamped lipid bilayers with various cholesterol content. Recovery curves of supported lipid bilayers of egg PC and rh-PE (1% *w/w*) which contain (■) 0% cholesterol, (●) 20% cholesterol, and (▲) 50% cholesterol in the presence of a) 0, b) 50, and c) 100 µM nimesulide.

In conclusion, we present a strikingly simple and reproducible method to obtain copies of functional membrane arrays from a range of lipids (including up to 50% negatively charged lipids). This method allows the fabrication of at least 100 copies of a bilayer array while using only picomolar amounts of lipids per spot—a characteristic that may be particularly beneficial for stamping precious membrane preparations that can often only be obtained in limited quantities, such as cellular membrane fragments. Binding assays with these arrays elucidated an unknown effect of cholesterol on the interaction of a prescription drug with bilayers, and as such, we expect liposome stamping to be useful for the rapidly growing interest in drug–membrane interactions,[17,34] protein–membrane interactions,[5] and arrays of membrane proteins.[6]

Table 1: Diffusion coefficients, *D* of rh-PE in supported membranes composed of egg PC and rh-PE (1% *w/w*) with various cholesterol content in the presence of nimesulide.

Cholesterol content [%]	*D* [cm²s⁻¹][a]		
	0 µM nimesulide	50 µM nimesulide	100 µM nimesulide
0	2.0×10^{-9}	2.7×10^{-9}	1.4×10^{-9}
20	1.8×10^{-9}	2.4×10^{-9}	1.2×10^{-9}
50	1.5×10^{-9}	0.7×10^{-9}	0.4×10^{-9}

[a] The errors of the diffusion coefficients are $\leq \pm 7\%$ and were obtained through measurement of the differences between diffusion coefficients of fluorescently labeled lipids in bilayers made from the same lipid composition, but stamped on different days.
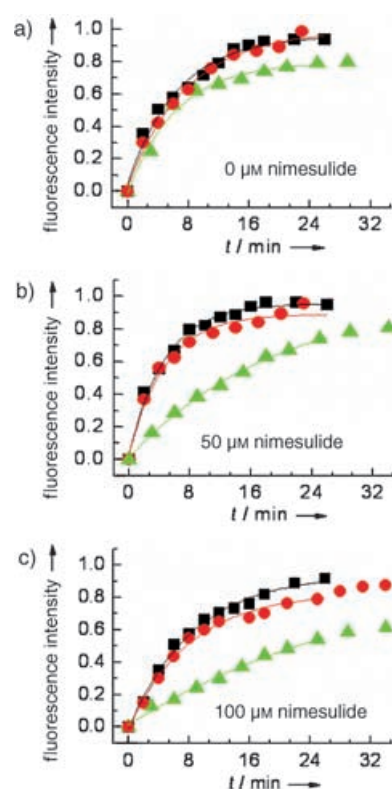
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