Published in final edited form as:

Anal Chem. 2008 November 1; 80(21): 7980–7987. doi:10.1021/ac800860u.

Micropatterned Fluid Lipid Bilayer Arrays Created Using a Continuous Flow Microspotter

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Abstract

We have developed a new method for creating micropatterned lipid bilayer arrays (MLBAs) using a 3D microfluidic system. An array of fluid lipid membranes was patterned onto a glass substrate using a Continuous Flow MicrospotterTM (CFM). Fluorescence microscopy experiments were used to verify the formation of a bilayer structure on the glass substrate. Fluorescence recovery after photobleaching (FRAP) experiments demonstrated the bilayers fluidity was maintained while being individually corralled on the substrate. The reproducibility of bilayer formation within an array was demonstrated by the linear response of membrane fluorescence versus mol % rhodamine functionalized lipids incorporated into the vesicles prior to fusion to the surface. The highly customizable nature of the MLBAs was demonstrated utilizing three different fluorescently labeled lipids to generate a multiple component lipid array. Finally, the cholera toxin B (CTB)/ganglioside GM1, anti-dinitrophenyl (DNP) antibody/DNP and NeutrAvidin/biotin protein-ligand systems were used to model multiple protein-ligand binding on the MLBAs. The multi-component patterned bilayers were functionalized with GM₁, DNP and biotin lipids and binding curves was generated by recording surface fluorescence versus increasing concentration of membrane bound ligands.

Keywords

avidin; biotin; cholera toxin; ganglioside; GM₁; antibody; fluorescence microscopy; lipid bilayers; microarrays; microfluidics; protein binding

INTRODUCTION

There is an increasing interest in the development of microarray based assays for high-throughput analysis and detection of biomolecules such as DNA and proteins.¹⁻³ Array-based analyses have proven extremely useful for DNA detection and sequencing.^{1, 2, 4} The general versatility of microarrays for multi-variable, high-throughput analysis has also been applied to solve analytical problems in the emerging field of proteomics.^{1, 2} One area of chemical and biological analysis which could potentially benefit from the use of microarrays is the study of biological membranes. Building an assay platform which is capable of producing an array of model lipid bilayers which maintain many of the characteristics found in cellular membranes such as fluidity and biocompatibility and allows for the investigation of protein-ligand and protein-membrane interactions in a multiplexed fashion is the target of our studies.

Planar supported lipid bilayers (PSLBs) serve as good models of biological membranes, allowing a diverse assortment of biological and chemical interactions to be explored in a

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controlled manner. PSLBs are also an attractive platform for performing biological assays due to their intrinsic resistance to nonspecific biomolecule adsorption and non-fouling nature.^{5, 6} One of the key attributes of lipid membranes which makes them desirable as biocompatible substrates and as models of cellular membranes is the fluid nature of these 2D macromolecular assemblies. However, this property of PSLBs also makes it challenging to create arrays of lipid bilayers of varying composition, as adjacent bilayers can fuse and mix their contents.⁷

Current methods used to create micropatterned lipid bilayer arrays (MLBAs) include micro-contact printing,^{8, 9} deep ultraviolet photolithography (UV),¹⁰ the use of pre-patterned substrates,¹¹ a combination of pre-patterned substrates with a robotic spotter system,¹² and 2D microfluidics.¹³ Many of these approaches are discussed in a recent review by Castellana et al.¹⁴ Microcontact printing was first introduced by Kumar et al. for creating arrays of self-assembled monolayers.¹⁵ A lithographically patterned poly(dimethylsiloxane) (PDMS) stamp is used to transfer the material onto a substrate. Microcontact printing has since become a common method for patterning PSLBs.^{8, 9, 16} Yang et al. demonstrated that a PDMS mold can also be used to displace portions of a continuous PSLB followed by the generation of addressable compartments above each patterned bilayer array.¹⁷ The use of deep-UV radiation is another way to create lipid bilayer arrays by directing light through a photomask onto a continuous lipid bilayer resulting in localized photochemical degradation of the exposed lipid bilayers.^{10, 18, 19} Small unilamellar vesicle (SUV) solutions can also be manually pipetted into corrals created on pre-patterned substrates¹¹ or the same approach can be mechanized, with the aid of robotics, to create spatial addressed bilayers.¹² The polymer lift-off technique patterns a layer of Parylene, a polyxylylene polymer, on a substrate using photolithography. Vesicles are then fused to the polymer-patterned substrate and after the polymer is removed a patterned bilayer is generated.^{20, 21} The air bubble collapse technique patterns lipids by removing a portion of a bilayer with a clean air bubble, creating a monolayer of lipids at the bubble's air water interface. The lipid covered air bubble is then brought into contact with a substrate where the air is removed causing the monolayer to collapse on itself resulting in the formation of a bilayer.²²

Many of the methods mentioned above, such as micro-contact printing and photolithography, employ backfilling with vesicles to create multi-composition arrays, significantly limiting the number of bilayers with distinct compositions. Majd et al. demonstrated a method to create multi-component arrays for micro-contact printing by manual hand pipetting SUV solutions but a drawback to this is the need for large spot sizes (~1 mm). Robotics can generate multi-component membrane arrays with small spot sizes (250 μ m) but this approach has drawbacks as well. Due to the extremely small volume size (nanoliters), this type of deposition process must be performed in a humidity chamber (~98% humidity) to prevent evaporation during SUV delivery.

The use of 2D microfluidics is a more recent approach for the creation of lipid arrays which involves introducing a solution of vesicles through a single plane of microchannels imbedded within PDMS producing parallel lanes of PSLBs.^{13, 21, 23-28} Microfluidics offers a simple and cheap alternative for creating multi-composition arrays. The use of microfluidics simplifies sample handling and eliminates problems associated with solvent evaporation. The addressable microchannels also allow for the rapid production and interrogation of numerous multi-component bilayers, limited only by the density and number of channels in the microfluidic device.

Microfluidics has proven to be a promising method for producing patterned PSLBs; however, the 2D nature of these devices limits the addressable elements in an array to linear channels on the surface. In this article, we describe the use of a 3D Continuous Flow MicrospotterTM (CFM) system for the preparation of multianalyte PSLB arrays, capable of producing higher

density multi-component arrays compared to traditional 2D microfluidics. The PDMS microspotter consists of a series of inlet and outlet wells connected by pairs of microfluidic channels embedded within the polymer. When the PDMS print-head contacts the substrate, one continuous channel is formed between the inlet and outlet pairs resulting in the continuous flow of solution over the substrate (Figure 1). Each channel is individually addressable, allowing the production of 2D PSLB arrays. The microfluidic system does not require the use of a pre-patterned substrate because PSLBs are effectively corralled into discrete micron-sized domains by the residual PDMS deposited on the silica substrate from the PDMS print-head which prevents the lipids from spreading.²⁹⁻³² Other attractive features include an easy washing process after vesicle fusion and the ability to address each element of the array individually if the print-head is maintained in contact with the substrate, or upon removal of the print-head the entire array can be queried for multiply analytes in a high-throughput fashion.

In this paper, we demonstrate the use of the CFM for creating high density PSLB arrays by vesicle fusion. The PSLB arrays were characterized to ensure bilayer formation and membrane fluidity. The functionality of these arrays was first demonstrated with a multi-component fluorescent array using labeled-lipids incorporated into individual lipid domains. Finally, a rapid multiple protein assays was performed using a multi-component ligand array. The use of the CFM is presented as a straight forward alternative for the production and high-throughput analysis utilizing PSLBs.

EXPERIMENTAL SECTION

Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DOPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DPPE), 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[6-[(2,4-dinitrophenyl)amino]caproyl] (DNP-cap-PE), GM₁ Ganglioside (Brain, Ovine-Ammonium Salt) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(Cap Biotinyl) (Biotin-cap-DOPE) were obtained from Avanti Polar Lipids and used as received. Marina Blue® 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Marina Blue-DHPE) and Oregon Green® 488 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Oregon Green-DHPE) were purchased from Invitrogen. Alexa Fluor 350 labeled NeutrAvidin (~4 mole dye per mole protein), Alexa Fluor 555 labeled cholera toxin subunit B (~5 mole dye per mole protein) and Alexa Fluor 488 labeled anti-dinitrophenyl-KLH rabbit IgG antibodies (~7 mole dye per protein) were also purchased from Invitrogen. The water used in these studies was obtained from a Nanopure™ Infinity Ultrapure water purification system with a minimum resistivity of 18.2 Mohm-cm. Quartz microscope slides (Chemglass) and glass cover slips (VWR International) were also used.

Small Unilamellar Vesicle (SUV) Preparation

The lipids were mixed in chloroform, the bulk chloroform was evaporated under a stream of N₂(g) and placed under a vacuum for at least 2 hours to remove any residual chloroform. The dried lipids were re-suspended, by vortexing, in phosphate buffer saline (PBS, pH 7.4, 140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ and 1 mM NaN₃) to yield a concentration of 0.5 mg/mL. The solution was then bath sonicated for 10 - 30 min to clarify. The solutions were stored at 4°C and used within 3 days.

MLBA Preparation

The quartz microscope slides or glass cover slips used for MLBA preparation were cleaned in 70% -18 M sulfuric acid / 30 % - 30 % H₂O₂ (piranha solution) followed by a rinsing with a copious amount of nanopure water. (Caution! Piranha solution is a strong oxidant and highly corrosive; reacts violently with organic solvents and should be handled with extreme care.) The slides were dried in a 120 °C oven and then plasma cleaned (Harrick PDC-32G) with Ar for 3 minutes.

MLBAs were prepared by vesicle fusion utilizing a Continuous Flow Microspotter™ (CFM). Details of the microspotter construction can be found elsewhere.³³ Briefly, the CFM is capable of producing up to 48 spots, each spot is a $400 \times 400 \mu\text{m}^2$ square with a pitch of 875 μm . However, the pitch may vary slightly due to deformities inherent within the PDMS print-head, variations between print-heads or spreading of the lipid bilayer patches. The PDMS print-head is approximately $5 \times 12 \text{ mm}$. The PDMS microspotting plate was degassed under vacuum before use to help prevent the formation of air bubbles within the microchannels during the experiment. MLBAs were assembled by introducing numerous solutions of DOPC SUVs through the microchannels simultaneously, allowing them to fuse to a silica slide or coverslip with an incubation time of 15 minutes followed by the cycling of vesicle solutions back and forth over the substrate by changing the flow direction. Excess vesicles were rinsed away by flowing nanopure water or PBS buffer through the channels. The rinse cycle was repeated three times by discarding and then refilling the solution in the microchannels for each cycle while ensuring the channels are never completely empty. The CFM print-head was then removed from the substrate in a reservoir of water and then assembled into a custom built Teflon flow cell. Once formed, the MLBA was maintained in an aqueous environment. The MLBAs are stable for at least one day assuming a successful pattern was fabricated resulting in well corralled lipid bilayers.

Verification of Lipid Bilayer Formation

In order to verify a bilayer structure is formed by vesicle fusion using the CFM; fluorescence microscopy was used to quantitatively compare the emission intensity of a symmetric lipid bilayer of DOPC + 0.5 mol % NBD-PC prepared by the Langmuir-Blodgett/Langmuir-Schaefer (LB/LS) method and by vesicle fusion using the CFM. The bilayers were formed on a cleaned quartz slide. For the LB/LS method, the substrate was placed in the water subphase of a KSV Instruments Minitrough, and a 1 mg/mL lipid solution (DOPC + 0.5 mol % NBD-PC) in chloroform was spread onto the air/water interface. The lipid film was transferred onto the substrate by pulling the slide out of the subphase (LB layer). The same glass slide was then horizontally passed through the air/water interface into the same subphase to deposit the LS layer. All depositions were carried out at a surface pressure of 35 mN/m which corresponds roughly to a bilayer surface pressure prepared by vesicle fusion (30-35 mN/m).³⁴ For comparison, an array of DOPC bilayers containing 0.5 mol % NBD-PC was patterned onto a cleaned quartz substrate using the CFM. The fluorescence intensity of the micropatterned lipid bilayer array was measured by averaging two separately prepared arrays, each array consisting of approximately 24 spots. The fluorescence of each spot was obtained by averaging over the entire bilayer area. The fluorescence of the LB/LS bilayer was measured by averaging the fluorescence intensities of two separately prepared LB/LS bilayers. The fluorescence intensities of each bilayer was obtained by averaging the fluorescence intensity over a $400 \times 400 \mu\text{m}^2$ area in the center of the image for approximately 15 different areas on the substrate. The fluorescence measurements were performed on the same day using similar quartz substrates. The samples were all placed in the same flow cell in order to minimize problems associated sample alignment in the microscope, background fluorescence and other sources of errors. All fluorescence images were acquired with the same exposure times. This procedure allowed for the direct comparison of absolute fluorescence intensities between experiments

while minimizing errors. For comparison, the fluorescence intensity of the lipid bilayer prepared by the LB/LS method and the fluorescence of the MLBAs were both normalized to the average fluorescence of the micropatterned lipid bilayer arrays.

Fluorescence Recovery After Photobleaching (FRAP)

A solution of DOPC SUVs doped with 1 mol % Rh-DOPE was introduced through the microchannels of the CFM to create a patterned substrate on a coverslip. After removal from the PDMS print-head the coverslip was placed in a custom built Teflon® flow cell. Using an Olympus 1X71 inverted microscope and a 100x objective (NA 1.4) a 5 µm spot was bleached within a 400 µm bilayer patch using a 488 nm high powered Argon ion laser (50 mW) with an exposure time of between 0.2-0.5 seconds. The fluorescence recovery was monitored by obtaining 3 pre-event and 32 post-event images with a Sony Interline CoolSNAP_{HQ} CCD camera (Roper Scientific) using a Rh filter set and 100W mercury arch lamp as the excitation light source. The fluorescence recovery within the bleached spot was measured over time using ImagePro software to yield the fluorescence intensity profile for each time and the intensity data was fit to the two-dimensional diffusion equation described by Soumpasis³⁵

$$f(t) = e^{-2\tau_D/t} \left[I_0 \left(\frac{2\tau_D}{t} \right) + I_1 \left(\frac{2\tau_D}{t} \right) \right] \quad (1)$$

where I_0 and I_1 are modified Bessel functions, $\tau_D = r^2 / 4D$ is the characteristic diffusion time, and r is the radius at half-height of the bleached area at $t = 0$. FRAP experiments were performed at room temperature in phosphate buffered saline (PBS, pH 7.5), with a phosphate concentration of 0.01M and 0.15 M NaCl. The diffusion coefficient reported represents the average of two different bilayer patches from two different MLBAs with six trials performed within each element.

Multiple Protein-Ligand Binding Assay

Patterned DOPC bilayers containing varying concentrations of one of three functionalized lipids (biotin-cap-DOPE, GM₁ and DNP-cap-PE) were prepared. NeutrAvidin labeled with Alexa Fluor 350 was used to monitor avidin/biotin binding. Cholera toxin subunit B (CTB) labeled with Alexa Fluor 555 was used to monitor CTB/GM₁ binding. Anti-dinitrophenyl-KLH (anti-DNP) labeled with Alexa Fluor 488 was used to monitor anti-DNP/DNP binding. Prior to the binding assay, the functionalized MLBA was assembled in a custom built Teflon flow cell and then incubated with a 2 mg/mL bovine serum albumin (BSA) solution to block the hydrophobic PDMS residue from the CFM print-head in order to prevent nonspecific adsorption of protein to the surface surrounding the MLBA. A protein mixture of 500 nM NeutrAvidin, 200 nM CTB and 500 nM anti-DNP all dissolved in PBS was introduced into the Teflon flow cell containing the multi-ligand MLBA and incubated for at least 40 minutes. The cell was then rinsed with PBS and the adsorption of protein to the MLBA elements was measured by fluorescence microscopy.

Fluorescence Microscopy

Fluorescence images were recorded using an Olympus BX40 equipped with a Photometrics CoolSNAP_{cf} (Roper Scientific) color camera. Three filter sets designed to pass each fluorophore's excitation/emission wavelengths of 510/526 nm for Oregon Green/NBD/Alexa Fluor 488, 557/571 nm for Rhodamine/Alexa Fluor 555 and 365/460 nm for Marina Blue/Alexa Fluor 350 were used. Images were taken with a 10x objective (NA, 0.30) which fits one 400 µm spot per field of view and were used to obtain quantitative fluorescence intensity measurements. Typically, images showing more than one bilayer spot were pieced together with images taken under a 4x (NA, 0.10) or 10x objective using Canvas × software. The 10x

objective was used to obtain fluorescence measurements instead of a 4x because of the more uniform light intensity profile located in the center of the image. The use of a 10x objective also allowed for a more precise assessment of lipid and protein coverage within an individual lipid spot, facilitating a more accurate determination of protein binding. In the future a microscope or other high-throughput imaging system, such as that employed by Castellana et al., could be employed to provide increased light gathering capabilities and a larger field of view facilitating the interrogation of multiple bilayer patches simultaneously.³⁶ However, for the sake of characterization of the newly described MLBAs, the imaging method described above was employed. The fluorescence intensity average and standard deviation for each spot were measured using the software package Voodoo Incantation 1.2 provided by Photometrics. All the fluorescence images were background corrected.

RESULTS AND DISCUSSION

Characterization of MLBAs

The MLBAs created using the CFM were characterized to assure both bilayer formation and membrane fluidity. In order to verify that lipid bilayers were indeed created using the CFM, the fluorescence intensity from a symmetric lipid bilayer of DOPC containing 0.5 mol% NBD-PC prepared by the LB/LS method was compared to the fluorescence intensity measured from a MLBA using the CFM prepared by vesicle fusion using the same lipid solution, Figure 2a. The fluorescence intensities in both images were normalized to the average fluorescence of the MLBA. The fluorescence intensities observed from LB/LS bilayer and the MLBA bilayer spots are not significantly different at the 95 % confidence level, strongly supporting the formation of only a single bilayer structure utilizing the CFM deposition methods. The bar graph shown in Figure 2a represents the average fluorescence of two separately prepared LB/LS bilayers and two separately prepared 24 spot MLBAs.

Fluorescence recovery after photobleaching (FRAP) experiments were performed to validate the fluidity of the PSLBs created by the CFM. A lipid array was created using DOPC + 1 mol % Rh-DOPC. A 5 μm diameter spot was bleached into one 400 μm membrane patch. The recovery of the fluorescence intensity was monitored over time (Figure 2b). A diffusion coefficient of $1.4 \pm 0.3 \times 10^{-8} \text{ cm}^2/\text{sec}$ was calculated using a 2D lateral diffusion model (Eq. 1).³⁵ This value represents the average diffusion coefficient measured on two different bilayer patches on two separately prepared MLBAs with six different areas on each bilayer patch being analyzed. The measured diffusion coefficient is in good agreement with previously reported values for the diffusion of similar lipids in PSLBs and are within the same order of magnitude (although several times slower) of solution phase liposomes.^{22, 37-39} These results demonstrate that the intrinsic fluidity of the bilayer is maintained during array formation, with essentially no immobile fraction indicated by nearly full fluorescence recovery ($97 \pm 1\%$).

MLBA Concentration Gradient

The reproducibility of the vesicle fusion method as well as the addressable nature of the CFM method was demonstrated by preparing a concentration gradient of Rh-DPPE lipids in a DOPC MLBA. Lipid bilayer arrays were generated from eight SUV solutions with increasing concentrations of Rh-DPPE ranging from 0.0 to 2.1 mol %. The SUV solutions were introduced through a total of 24 microchannels simultaneously. The array was replicated three times (in three rows) on the same substrate, Figure 3. In addition to the fluorescence image of the MLBA in Figure 3, a graph illustrating the linear trend of fluorescence versus mol % Rh-DPPE is also shown. The data points represent the average fluorescence of three spots prepared with the same SUV solution along with the corresponding standard deviation. Vesicle fusion performed in different microchannels results in approximately the same number of lipids per area as suggested by the reproducibility of fluorescence measured for the various bilayer spots

prepared by the same SUV solutions. The graph also shows that as the mol % of Rh-DPPE doubles, the fluorescence intensity increases approximately two fold. This shows that liposomes created with discrete lipid composition, in this case the addition of Rh-DPPE, are accurately transferred to the substrate with high fidelity and there is no apparent cross-talk between the channels.

Multiple Component MLBA

The use of the CFM for the formation of a MLBA, allows for the composition of each bilayer element to be controlled individually, opening up the possibility for multi-analyte assays. A multiple component array was generated using DOPC SUVs containing one of three different fluorescent probes, 1.0 mol% Rh-DPPE, 1.0 mol % Oregon Green-DHPE or 3.0 mol % Marina Blue-DHPE, in order to visually demonstrate the multi-component array capability of the CFM. The three SUV solutions were simultaneously delivered to the substrate surface, producing a multi-color lipid bilayer array with the red, green and blue spots corresponding to Rh-DPPE, Oregon Green-DHPE and Marina Blue-DHPE functionalized bilayers, respectively. A true color fluorescence image of the MLBA is shown in Figure 4. Figure 4 shows 48 high quality bilayers with very few defects indicated by the uniform fluorescence of each spot. The PSLBs in Figure 4 are clearly separated from one another with little spreading, demonstrating the stability of the individual bilayer domains. These experiments demonstrate that MLBAs with spatially addressed compositions can easily be prepared using a CFM.

Multiple Protein-Ligand Binding Assay

PSLBs have long been known to be inherently biocompatible substrates which are ideally suited for performing biological assays.^{40, 41} Bilayer surfaces containing phosphocholine headgroups have been shown to be highly resistant to nonspecific protein adsorption due in part to their charge neutrality and hydration of the headgroups.⁴²⁻⁴⁵ In addition, assays may be preformed utilizing PSLBs by functionalizing the membrane with receptor sites. The avidin/biotin, CTB/GM₁ and anti-DNP antibody/DNP systems were used to demonstrate that these features are maintained with the MLBAs prepared by the CFM in a multiple protein-ligand binding assay. The ligand array was generated by simultaneously introducing DOPC SUVs containing different molar ratios of functionalized lipids through 48 different microchannels, with each ligand type designated to one row (Figure 5). A solution containing CTB (200 nM), NeutrAvidin (500 nM) and anti-DNP (500 nM) was incubated with the surface and fluorescence microscopy used to quantify binding. The high protein concentrations used in this study were employed to ensure surface saturation of NeutrAvidin and CTB^{24, 46} and facilitate rapid equilibrium of the sample. While saturation concentrations of anti-DNP to planar supported lipid bilayers containing DNP-cap ligands has been shown to be in the μ M range, 47 500 nM anti-DNP was sufficient to obtain high fluorescence signal while reducing the cost of performing the assay. Shown in Figure 5 are the representative fluorescence images of a single MLBA imaged with three different filter sets, one for each of the fluorescently labeled proteins, CTB in the red (Figure 5a), anti-DNP in the green (Figure 5b) and NeutrAvidin in the blue channel (Figure 5c). Each row in the image represents a specific ligand, GM₁ for CTB, DNP for anti-DNP and biotin for NeutrAvidin. Each row has two series of increasing concentration of ligand (two adjacent sets) from left to right. Also shown is a control lane containing reference markers used to register the array and pure DOPC bilayers for controls. For each of the proteins a minimal amount of nonspecific adsorption to the solid support is observed. There was some anti-DNP that bound to the PDMS residue in the lower right of Figure 5b possibly due to a leaky spot during MLBA preparation. This does not affect the other bilayer patches and is apparent by the contrast of fluorescence around the patterned spots. The bright spot in Figure 5c is most likely the result of contamination of dust particles which fluoresce upon excitation with ultraviolet light. More importantly, no cross-reactivity or nonspecific protein binding is observed to lipid bilayer elements not containing the protein-

specific ligand. This is apparent by the minimal fluorescence observed for rows containing a ligand other than the one required by the protein-ligand couple of interest.

Also shown in Figure 5 are the binding curves of each protein to increasing concentrations of ligand doped in the DOPC bilayers. The plotted data represent the average of the two reproduced bilayer spots of identical lipid composition whereas the data plotted for the DOPC control represent the average of six spots. The fluorescence of individual bilayer spots were obtained by spatially averaging the fluorescence intensity of the entire bilayer patch. The relative uncertainties of the fluorescence signal within the individual spots were <5 % for the CTB and DNP functionalized bilayers. The standard deviation within each biotin containing lipid patch ranged from 25 to 5 % relative uncertainty from low to high biotin mol % bilayers respectively. The high standard deviation at low concentration of biotin is due to the weaker fluorescence measured with NeutrAvidin versus anti-DNP and CTB. However, the standard deviation within each spot remains relatively constant through the experiment (2.2 ± 0.4 for the unnormalized fluorescence signal), thus explaining the high relative uncertainty at lower signal. The relatively low relative error suggests that the proteins bind uniformly over the bilayers.

The CTB/GM₁ binding curve presented in Figure 5a shows increasing amounts of CTB binding to DOPC bilayers with increasing mol % of GM₁. The amount of nonspecifically adsorbed CTB to the DOPC controls (0.0 mol % GM₁) was <1 % of the normalized signal. Figure 5b shows that anti-DNP increases in surface coverage up to 4 mol % DNP-cap-PE. The amount of nonspecifically adsorbed antibody to the DOPC control bilayers was ~1 % relative to the fluorescence signal near surface saturation. The surface coverage of NeutrAvidin onto biotin functionalized bilayers increased until saturation at 4 mol %. The fluorescence of the DOPC control spots was at background levels indicating minimal amounts of NeutrAvidin adsorption. There was essentially no cross-reactivity of protein with other ligand functionalized bilayers as shown by the lack of signal when imaged under the remaining two filter sets. The MLBA shows a minimal amount of nonspecific protein adsorption, consistent with the non-fouling nature of the DOPC membranes.

The reproducibility of the multiple protein-ligand assays was tested by performing three independent binding experiments on freshly prepared MLBAs. The data plotted in Figure 6 represent the average of six spots whereas the DOPC controls are averaged from ~18 spots. The three separate experiments were first normalized and then pooled together to determine the reproducibility in the binding trends. These results show good reproducibility of the arrays from day to day demonstrated by the small standard deviations associated with the average fluorescence with the exception of CTB for GM₁ concentrations ≥ 10 mol %. The relatively large scatter could be attributed to the clustering of the GM₁ lipids at high GM₁ concentrations.^{24, 48} The binding curves for CTB, anti-DNP and NeutrAvidin were compared to similar binding curves presented by Cremer and coworkers.^{24, 46, 47} The program g3data (for Linux) was used to extract data from the published graphs in references [24, 46 and 47]. This data was then used to obtain the data points in shown in Figure 6. For the anti-DNP/DNP and CTB/GM₁ data (references 26 and 48), the binding data at the highest protein concentrations (saturation levels), ~28 μ M for anti-DNP and 6 nM for CTB, were used.^{24, 47} For the streptavidin/biotin assay (reference 47), the data from the graph reporting fluorescence data at a saturation concentration (0.01 mg/mL) of streptavidin was used.⁴⁶ All the data presented in Figure 6b and c were normalized to the fluorescence at the highest reported ligand concentration. The data for CTB/GM₁ were normalized to the fluorescence at 5 mol % GM₁ because of the large scatter at 10 mol % GM₁.

The binding curves we have obtained for CTB, anti-DNP and avidin on the MLBAs correlate extremely well with similar binding curves obtained by Cremer and coworkers (see Figure 6)

with the exception of CTB binding to GM₁ at high ligand densities (>5 mol%). This discrepancy is likely due to GM₁ domain formation, as mentioned previously. The similar results obtained for protein binding studies performed using the MLBA and those reported by a different research group using lipid bilayers in a different experimental platform; strongly suggest that the integrity and functionality of the lipid bilayers prepared here are equivalent in nature. In addition, the protein binding experiments reported by Cremer and coworkers were performed within a bilayer coated microfluidic network, while the binding experiments presented in this paper were performed in a flow cell, in which the entire sensing surface could be addressed simultaneously. The important conclusions to be drawn from this comparison are that the lipid patterning process by the CFM has little effect on protein binding to the MLBAs prepared with the CFM and the MLBAs created have the potential to increase the number of assays performed by increasing the usable real estate on the sensor surface.

SUMMARY

We have demonstrated that the CFM provides a powerful way to create fluid and functionalized MLBAs. The fluorescence of lipid membranes prepared by the CFM was nearly identical to the intensity of a LB/LS prepared bilayer, suggesting the formation of lipid bilayers on the substrate surface. The lipid bilayer patches remain nicely corralled on the substrate confined by the PDMS residue of the CFM printhead preventing the spreading of lipids while still retaining their fluid nature, with a diffusion coefficient of $1.4 \pm 0.3 \times 10^{-8} \text{ cm}^2/\text{sec}$ calculated from the FRAP experiments for lipid diffusion in the lipid membranes prepared by the CFM. Vesicle fusion utilizing the CFM has been shown to be a reliable deposition method with for patterning lipid bilayers while maintaining a high level of experimental control and reproducibility. The linear response of bilayer fluorescence vs. mol % Rh-DPPE in DOPC SUVs demonstrates that discrete lipid compositions are successfully transferred from vesicles to the substrate. The standard deviation in the fluorescence between the different bilayer spots prepared from the same SUV solution were small, demonstrating the reproducibility in the packing of the lipids within the $400 \times 400 \text{ }\mu\text{m}^2$ area. Multiple component MLBAs were also prepared utilizing three fluorescently labeled lipid probes for visualization. On average, a 7% relative error in the fluorescence within each of the three sets of functionalized DOPC bilayers, Rh-DPPE, Oregon Green-DHPE and Marina Blue-DHPE was obtained, again illustrating the reproducibility of bilayer formation within an array. A simultaneous multiple protein-ligand binding experiment using, CTB, anti-DNP and avidin, to varying mol % of three functionalized lipids, GM₁, DNP-cap-PE and biotin-cap-DOPE was also obtained. The MLBAs were shown to resist nonspecific protein adsorption and have minimal amounts of cross-reactivity. The high density MLBAs we have demonstrated here have potential applications in many fields such as biosensing, drug discovery, proteomics and clinical diagnostics.

Acknowledgments

The authors thank Wasatch Microfluidics for supplying the CFM and Sriram Natarajan and Adam Miles for their help with modification of the CFM. The authors also thank Prof. Markus Babst and Ms. Elizabeth Ott, Biology Department, University of Utah, for assistance with the FRAP measurements. This work was supported by funds from the NIH (R01-GM068120).

REFERENCES

1. Muller, UR.; Nicolau, DV., editors. Microarray Technology and Its Applications. Springer-Verlag; Berlin: 2005.
2. Falciani, F., editor. Microarray Technology Through Applications. Taylor and Francis Group; New York: 2007.
3. Zhang F, Gates RJ, Smentkowski VS, Natarajan S, Gale BK, Watt RK, Asplund MC, Linford MR. J. Am. Chem. Soc 2007;129:9252–9253. [PubMed: 17622146]

4. Liu, RH.; Lee, AP., editors. Integrated Biochips for DNA Analysis. Landes Bioscience and Springer Science+Business Media; New York: 2007.
5. Brash, JL.; Horbett, TA., editors. Proteins at interfaces: physicochemical and biochemical studies. American Chemical Society; Washington, DC: 1987.
6. Chapman D. Langmuir 1993;9:39–45.
7. Cremer PS, Boxer SG. J. Phys. Chem. B 1999;103:2554–2559.
8. Kung LA, Kam L, Hovis JS, Boxer SG. Langmuir 2000;16:6773–6776.
9. Majd S, Mayer M. Angew. Chem., Int. Ed 2005;44:6697–6700.
10. Yee CK, Amweg ML, Parikh AN. Advanced Materials 2004;16:1184–1189.
11. Cremer PS, Yang T. J. Am. Chem. Soc 1999;121:8130–8131.
12. Yamazaki V, Sirenko O, Schafer Robert J, Nguyen L, Gutschmann T, Brade L, Groves Jay T. BMC Biotechnol 2005;5:18. [PubMed: 15960850]
13. Yang T, Jung S, Mao H, Cremer PS. Anal. Chem 2001;73:165–169. [PubMed: 11199961]
14. Castellana ET, Cremer PS. Surf. Sci. Rep 2006;61:429–444.
15. Kumar A, Whitesides GM. Appl. Phys. Lett 1993;63:2002–2004.
16. Hovis JS, Boxer SG. Langmuir 2001;17:3400–3405.
17. Yang T, Simanek EE, Cremer P. Anal. Chem 2000;72:2587–2589. [PubMed: 10857639]
18. Yu, C.-h.; Parikh, AN.; Groves, JT. Advanced Materials 2005;17:1477–1480.
19. Yee CK, Amweg ML, Parikh AN. J. Am. Chem. Soc 2004;126:13962–13972. [PubMed: 15506757]
20. Orth RN, Kameoka J, Zipfel WR, Ilic B, Webb WW, Clark TG, Craighead HG. Biophys. J 2003;85:3066–3073. [PubMed: 14581207]
21. Moran-Mirabal JM, Edel JB, Meyer GD, Throckmorton D, Singh AK, Craighead HG. Biophys. J 2005;89:296–305. [PubMed: 15833994]
22. Mager MD, Melosh NA. Langmuir 2007;23:9369–9377. [PubMed: 17683151]
23. Burrige KA, Figa MA, Wong JY. Langmuir 2004;20:10252–10259. [PubMed: 15518521]
24. Shi J, Yang T, Kataoka S, Zhang Y, Diaz AJ, Cremer PS. J. Am. Chem. Soc 2007;129:5954–5961. [PubMed: 17429973]
25. Phillips KS, Cheng Q. Anal. Chem 2005;77:327–334. [PubMed: 15623312]
26. Kam L, Boxer SG. Langmuir 2003;19:1624–1631.
27. Kam L, Boxer SG. J. Am. Chem. Soc 2000;122:12901–12902.
28. Perez TD, Nelson WJ, Boxer SG, Kam L. Langmuir 2005;21:11963–11968. [PubMed: 16316139]
29. Glasmaestor K, Gold J, Andersson A-S, Sutherland DS, Kasemo B. Langmuir 2003;19:5475–5483.
30. Groves JT, Ulman N, Boxer SG. Science 1997;275:651–653. [PubMed: 9005848]
31. Ross EE, Joubert JR, Wysocki RJ Jr. Nebesny K, Spratt T, O'Brien DF, Saavedra SS. Biomacromolecules 2006;7:1393–1398. [PubMed: 16677019]
32. Foley Jennifer O, Fu E, Gamble Lara J, Yager P. Langmuir 2008;24:3628–3635. [PubMed: 18303923]
33. Chang-Yen DA, Myszka DG, Gale BK. J. Microelectromechanical Systems 2006;15:1145–1151.
34. Marsh D. Biochim. Biophys. Acta, Rev. Biomembr 1996;1286:183–223.
35. Soumpasis DM. Biophys. J 1983;41:95–97. [PubMed: 6824758]
36. Castellana ET, Cremer PS. Biointerphases 2007;2:57–63. [PubMed: 20408637]
37. Tamm LK, McConnell HM. Biophys. J 1985;47:105–113. [PubMed: 3978184]
38. Przybylo M, Sykora J, Humpolickova J, Benda A, Zan A, Hof M. Langmuir 2006;22:9096–9099. [PubMed: 17042516]
39. Lapinski MM, Castro-Forero A, Greiner AJ, Ofoli RY, Blanchard GJ. Langmuir 2007;23:11677–11683. [PubMed: 17939695]
40. Sackmann E. Science 1996;271:43–48. [PubMed: 8539599]
41. Brian AA, McConnell HM. Proc. of the Natl. Acad. of Sci. U. S. A 1984;81:6159–6163.
42. Murphy EF, Lu JR, Brewer J, Russell J, Penfold J. Langmuir 1999;15:1313–1322.
43. Glasmaestor K, Larsson C, Hook F, Kasemo B. J. Colloid Interface Sci 2002;246:40–47. [PubMed: 16290382]

44. Chen S, Zheng J, Li L, Jiang S. *J. Am. Chem. Soc* 2005;127:14473–14478. [PubMed: 16218643]
45. Ross EE, Spratt T, Liu S, Rozanski LJ, O'Brien DF, Saavedra SS. *Langmuir* 2003;19:1766–1774.
46. Mao H, Yang T, Cremer PS. *Anal. Chem* 2002;74:379–385. [PubMed: 11811412]
47. Yang T, Baryshnikova OK, Mao H, Holden MA, Cremer PS. *J. Am. Chem. Soc* 2003;125:4779–4784. [PubMed: 12696896]
48. Vie V, Van Mau N, Lesniewska E, Goudonnet JP, Heitz F, Le Grimellec C. *Langmuir* 1998;14:4574–4583.

Figure 1. Schematic of the CFM apparatus and close-up of the CFM print head in contact with a silica substrate used for bilayer formation. The arrows represent the direction of fluid flow in the sealed channel created by contacting the PDMS print head with the silica substrate.

Figure 2.

Characterization of a bilayer created with the CFM: (a) is a comparison of the normalized fluorescence intensity of CFM patterned DOPC membranes containing 0.5 mol % NBD-PC (1.00 ± 0.05), and a symmetric DOPC + 0.5 mol % NBD-PC LB/LS bilayer (0.98 ± 0.05) with the fluorescence images of both shown in the insert. The scale bar represents 400 μm . (b) is the FRAP recovery curve with corresponding fluorescence images at times $t = 0$ and $t = 15.05$ seconds. The line is the fit to the data using a 2-D diffusion model (Equation 1). The scale bar represents 3 μm .

Figure 3.

Micropatterned concentration gradient of Rh-DPPE doped in DOPC at 0.0, 0.5, 0.8, 1.0, 1.2, 1.4, 1.7 and 2.1 mol %, corresponding to the images starting from left to right, respectively. The concentration gradient was reproduced over three rows (top to bottom). The scale bar to the left of the image represents the raw fluorescence intensity. The graph represents the linear trend of fluorescence versus mol % of Rh-DPPE in the DOPC SUVs. The graph was normalized to the maximum fluorescence value of 800 at 2.1 mol % Rh-DPPE, seen in the fluorescence image. The data points are the average fluorescence intensity with the standard deviation obtained for three spots located in the same column. Scale bar corresponds to 400 μm .

Figure 4.
A 48 element MLBA of DOPC containing either 1.0 mol % Rh-DPPE (red), 1.0 mol % Oregon Green-DHPE (green) or 3.0 mol % Marina Blue-DHPE (blue).

Figure 5.

A multi-ligand MLBA containing: (row 1) GM₁ at 0.5, 1.0, 3.0, 5.0, 10.0 and 13.0 mol % in DOPC, (row 2) DNP-cap-PE at 0.2, 0.3, 0.5, 1.0, 2.1 and 4 mol % in DOPC and (row 3) biotin-cap-DOPE at 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 mol % in DOPC. Each ligand concentration is present in two sequential sets going from the left to right in each row. Row 4 contains six control DOPC spots with no ligands present. There are also four DOPC + 1 mol % Rh-DOPE spots which were used as position finders at the beginning of the experiment (outlined by open white squares). In order to increase the concentration range of biotin relative to the other ligands, two 6.0 mol % biotin-cap-DOPE spots (outlined by green open squares) were also prepared in row 4. The array was imaged under the red (a), green (b) and blue (c) filter sets to obtain the images shown. The representative binding curves to the right of the images represent the average fluorescence intensity of the duplicate bilayers from rows 1, 2, 3, and 4 obtained under the red (open triangles), green (open circles) and blue (open squares) filter sets. The scale bar represents 600 μ m.

Figure 6.

The average binding curves of three independent MLBAs (open shapes) for CTB/GM₁ (a), anti-DNP/DNP (b) and NeutrAvidin/biotin (c). The data obtained from Cremer and coworkers^{24, 46, 47} for similar systems (see text) are overlaid as closed circles.