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Patterning Adjacent Supported Lipid Bilayers of Desired Composition To Investigate Receptor−Ligand Binding under Shear Flow

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To achieve efficient targeting, carriers containing either drugs or imaging agents must have surface properties that promote binding to targets yet at the same time block rapid immune system clearance. Here we describe a versatile technique that allows simultaneous comparison of the effects of carrier surface composition on binding properties under *identical flow conditions*. Parallel lanes of supported lipid bilayers that mimic the surface of liposomal delivery vehicles are formed using the vesicle fusion method in microfluidic channels created via standard soft lithography techniques. Vesicle stock solutions are premixed and injected into lanes formed by a poly(dimethylsiloxane) (PDMS) stamp reversibly sealed to a glass slide to create *adjacent* lanes of *distinct* composition. After removing the stamp, an adsorbed layer of bovine serum albumin (BSA) is used to prevent bilayer spreading before assembling the patterned substrate into a flow chamber for binding studies. Advantages of this method include easy and rapid preparation of bilayers with desired compositions from an unlimited number of lipid types, choice of feature size, time-stable features, and low nonspecific binding. Feature sizes on the order of tens of microns allow multiple compositions to be analyzed in one field of view, thereby reducing the number of experiments, ensuring identical flow conditions, and enabling simultaneous incorporation of controls. We show that the presence of a long poly(ethylene glycol) (PEG) tether (MW 2000) between the lipid and ligand results in higher detachment resistances as compared to a short six-carbon spacer.

Introduction

A major challenge in the field of targeted drug delivery is the design of carriers that avoid the immune system and yet retain binding specificity in vivo. One of the most common strategies for preventing rapid immune clearance is to decorate the surface of carriers with poly(ethylene glycol) (PEG). Although PEG significantly increases circulation times presumably through its steric properties,[1,2] these same properties can present a substantial barrier for efficient binding of the ligand to its target.[3] To remain bound, ligand−receptor mediated attachments must also overcome shear flow in the vasculature which decreases contact times and applies forces on bonds that form. It is therefore critical to design carriers with properties that balance specific interactions for efficient targeting with nonspecific interactions to avoid rapid clearance.

Surface properties that will likely affect binding specificity include spacer length, relative molecular weights of liganded and unliganded PEG, and ligand kinetics. While the ultimate test of carriers is their ability to function in vivo, it simply is not practical to perform combinatorial screens of carrier surface properties in vivo. Moreover, the complexity of in vivo systems makes it difficult to identify the biophysical properties that are most important for improving targeting. Supported lipid bilayers serve as models of the surface of liposomal drug carriers and can be patterned into microarray platforms.[4−9] For effective in vitro screening of carrier surface properties, a method for generating patterned lipid bilayers should have the following characteristics: (1) tunable feature size, (2) time stable features, (3) minimal nonspecific binding, and (4) adjacent features with adjustable composition. While there are many methods for patterning bilayers that satisfy some of these requirements, to date, there is no single method that encompasses *all of these features*.

Although no single existing technique can be directly applied specifically for screening drug carrier properties, numerous innovative methods for forming patterned lipid bilayers have been reported. We therefore highlight key features of these techniques that are most relevant for our studies. The majority of methods for patterning supported lipid bilayers use vesicle fusion combined with soft lithographic techniques (e.g., microcontact printing or microfluidics) because molds can be reused hundreds of times and feature sizes can be easily controlled. However, supported lipid bilayers can spread and fuse with adjacent bilayers, thereby destroying the pattern.[10,11]

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Furthermore, the unpatterned regions must be treated to block nonspecific binding. Barriers between bilayers formed by incubating substrates in solutions of proteins such as bovine serum albumin[7] (BSA) are attractive because they not only prevent bilayer spreading but also reduce nonspecific binding.

Another critical feature necessary to determine optimal liposome formulation for binding under flow conditions is the ability to specify amounts of unconjugated, PEGylated, and liganded lipids in adjacent bilayers. Although microfluidic-based techniques[6] and application of electric fields[6,12] have been used to generate gradients in composition, methods that offer choice of composition require either multiple alignment steps[5,13] or careful positioning.[14] The concept of premixing different vesicles is extremely useful as it enables creation of bilayers with tunable amounts of desired lipid types[8] from a limited number of stock solutions. To develop a platform to screen carrier surface properties, we chose to use microfluidic patterning of premixed vesicles to generate compositionally distinct, adjacent bilayers that are closely spaced and separated by BSA barriers.

While micropatterned arrays have been used extensively under static conditions, to our knowledge, multiplexed screening under tunable physiologically relevant flow conditions has not yet been investigated. Here we describe a unique technique that integrates patterned supported bilayers with a parallel plate flow chamber to enable high-throughput screening of specific binding under shear flow. Our technique allows binding interactions to be observed between receptor-coated beads and ligand-containing supported bilayers mimicking the surface of liposomes. While this arrangement is the opposite of the actual case in vivo, reversing the mobile and stationary phases should only alter the frame of reference and is expected to have little or no effect as reported by other flow studies.[15−17] We employ fluorescence recovery after photobleaching (FRAP) to verify that we obtain fluid bilayers and binding studies with fluorescently labeled molecules to confirm that premixing indeed creates bilayers with different compositions. We find that a longer spacer between the lipid and the ligand promotes higher detachment resistance of beads under shear flow.

Experimental Section

Materials. Egg phosphatidylcholine (egg PC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(poly(ethylene glycol))-2000], ammonium salt (mPEG2000-DSPE), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(poly(ethylene glycol))2000], ammonium salt (biotin-PEG2000-DSPE), are purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red-DHPE), *N*-((6-(biotinoyl)amino)-hexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (biotin-X-DHPE), and Texas Red-X streptavidin are purchased from Molecular Probes (Eugene, OR). Streptavidin-coated polystyrene beads are purchased from Dynal Biotech (Dynabeads M-280, Lake Success, NY). Bovine serum albumin (BSA), reagent-grade chloroform, and methanol are purchased from Sigma. Phosphate buffered saline (PBS) is purchased from Gibco, poly(dimethylsiloxane) (Sylgard 184) from Dow Corning (Midland, MI), and SU8-50 from MicroChem (Newton, MA).

Vesicle Preparation. Vesicle stock solutions consisting of either pure egg PC or egg PC supplemented with Texas Red-DHPE, mPEG2000-DSPE, biotin-PEG2000-DSPE, or biotin-X-DHPE are formed by the sonication method.[18] Briefly, lipids are combined in 9:1 chloroform to methanol, dried with argon, and placed under vacuum for at least 2 h. Lipids are hydrated with deionized water to a final concentration of 1 mg/mL and placed in a 50 °C oven for 20 min. Solutions are then sonicated for 15 min with a Branson 450 tip sonicator (Branson Ultrasonics Corp., Danbury, CT) at 50% duty cycle and three output control in an ice bath to form vesicles. The compositions of vesicle stock solutions are given in figure captions with all percentages representing mole percent. Abbreviations used in figure captions for vesicle stock solutions are as follows: egg PC = 100% egg PC; TR (x%) = x% Texas Red-DHPE and (100 − x)% egg PC; BXD (x%) = x% biotin-X-DHPE and (100 − x)% egg PC; BPEG (x%) = x% biotin-PEG2000-DSPE and (100 − x)% egg PC; mPEG (x%) = x% mPEG2000-DSPE and (100 − x)% egg PC; and BPEG/TR (x%, y%) = x% biotin-PEG2000-DSPE, y% Texas Red-DHPE, and (100 − x − y)% egg PC. In figure labels, percentages preceding abbreviations indicate the total amount of that component in the premixed solutions. For example, 2.1% BXD means that vesicles were premixed so that the total amount of biotin-X-DHPE present is 2.1%.

Patterned Bilayer Formation. Parallel lanes of supported bilayers are formed using the vesicle fusion method[19] in microfluidic channels. The microfluidic channels are formed using standard soft lithography techniques.[20,21] Briefly, SU8-50 positive photoresist is spin-coated onto silicon wafers to a thickness of roughly 100 μm. The coated wafers are then selectively exposed to UV light using a high-resolution mask transparency and developed. PDMS stamps are formed by curing Sylgard 184 at 70 °C for 5 h on the silicon masters. Cured PDMS stamps are removed from the masters, and inlets/outlets are punched with an 18 gauge blunt needle. Glass slides are plasma-etched (PDC-32G, Harrick Scientific Corp., Ossining, NY) for 2 min on high power under vacuum. PDMS stamps are then firmly pressed down against the glass slides to form a reversible, leak-tight seal. Vesicle solutions are premixed in desired ratios to enable formation of lipid bilayers with any desired composition from a limited number of stock solutions. Specific premixing ratios are given in figure captions. The resulting vesicle solutions are mixed 1:1 with PBS supplemented with 140 mM NaCl and vortexed for 1 min prior to use. It is important to note that the vesicles are first formed in deionized water and that salt is added prior to use. The addition of salt induces osmotic stress, which provides a driving force for the formation of homogeneous supported bilayers.[22] The PDMS inlets are filled with various vesicle−salt solutions using a standard pipet and allowed to incubate for at least 5 min at room temperature. Each channel is flushed with deionized water to remove excess vesicles before removing the stamp either in water or in a bath of phosphate buffered saline (PBS) containing 0.2% (w/v) BSA. All transfers are performed under water to prevent exposure of the lipid bilayers to air.

Fluorescence Microscopy. The patterned bilayers are imaged using an Aviovert 25 microscope (Carl Zeiss, Inc., Thornwood, NY) with a CCD camera (Sanyo, Chatsworth, CA) or a Zeiss Axiovert S100 microscope equipped with a digital camera (Princeton Scientific Instruments, Inc., Monmouth, NJ). Texas Red fluorophores are imaged using a mercury arc lamp with a Texas Red filter set (Chroma Technology Corp., Rockingham, VT). Texas Red-DHPE spiked into vesicle solutions serves to confirm the presence of bilayers and to mark lane locations. To verify the presence of biotinylated lipids, bilayers

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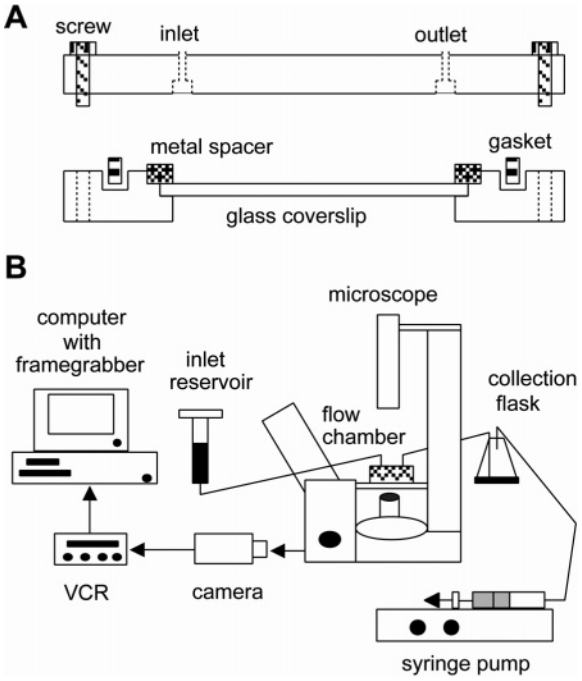

Figure 1. Flow chamber setup. (A) Side view of partially assembled chamber shown in cross section. Beads introduced into the chamber inlet flow over the lipid pattern on the glass coverslip. (B) Viewing the chamber on an inverted microscope allows bead locations to be recorded onto videotape as shear rates are varied using a syringe pump.

prepared as above are incubated in a PBS solution containing 7 μg/mL Texas Red-streptavidin[23] and 0.2% BSA for at least 1 h at room temperature. Prior to fluorescence measurements, excess solution is removed and bilayers are washed three times with PBS, taking care not to expose the bilayer to air.

FRAP. In the FRAP experiments a small area is photobleached for 1 min using the full spectrum of a mercury arc lamp and a 40× objective. After acquiring an initial image at low magnification (at 10×), the sample is incubated at 80 °C for 20 min and reimaged.

Flow Chamber. A custom-built parallel plate chamber (Figure 1A) is used to monitor interactions between beads and patterned lipid bilayers under laminar, planar Poiseuille flow conditions. Placing the chamber on the stage of an inverted microscope allows observation of interactions that occur between bilayers and streptavidin-coated polystyrene beads of 2.8 μm diameter (Figure 1B). Chamber height is measured optically on the inverted microscope by counting tick marks spanned as the focus knob is moved to focus on the top of the glass slide and the bottom of the chamber top plate. Distance units are obtained by multiplying tick marks by conversion factors obtained from measuring objects of known height. Before each experiment the height of the assembled chamber is measured to determine the volumetric flow rates necessary to achieve desired wall shear rates. The wall shear rate G is calculated from $G = (6Q)/(wh^2)$, where Q is the volumetric flow rate, w is the chamber width of 20 mm, and h is the chamber height. Bead suspensions (43 000/mL in PBS supplemented with 0.2% BSA, pH 4.8) are drawn through the chamber with a syringe pump (Orion Research Inc., Beverly, MA). After allowing the beads to accumulate at a wall shear rate of 5 s^{-1}, the flow is stopped for at least 1 h. After application of a wall shear rate of 1280 s^{-1} the beads remaining bound are imaged. This shear rate is chosen because it is above the highest shear rate normally found in vivo. An indicator of detachment resistance is the percent of beads remaining bound which is calculated as the fraction of beads that were initially present before flow started that remained bound after the highest wall shear rate. All flow experiments are carried out at room temperature on freshly prepared bilayers.

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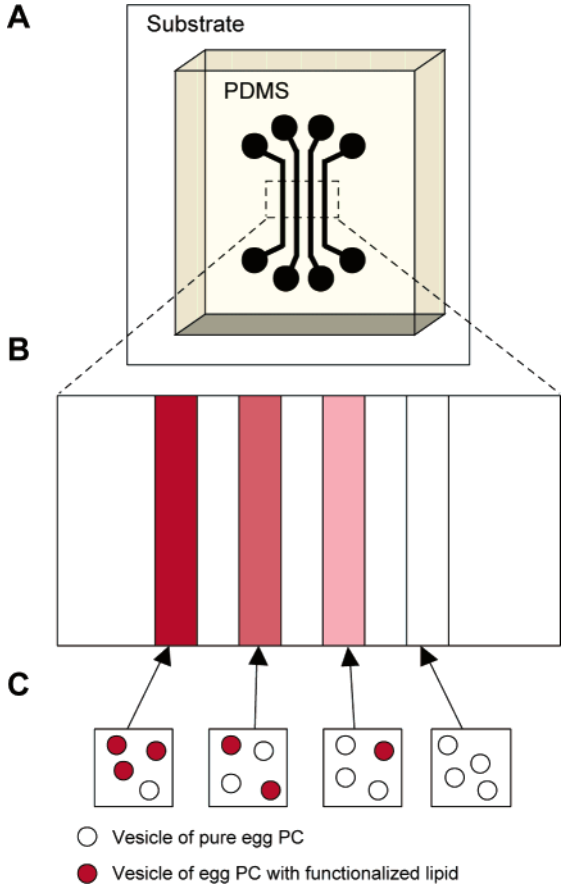

Figure 2. Overview of procedure for creating patterned lipid bilayers of variable composition. (A) A PDMS stamp with individually addressable lanes is pressed against a substrate. Pattern dimensions have been substantially enlarged for illustrative purposes. A typical pattern contains 9−11 lanes 50−200 μm wide with 25−200 μm spacing between lanes. (B) Vesicle−salt solutions of desired compositions are injected into each lane. After incubation, lanes are flushed to remove excess vesicles and the stamp is removed revealing independent supported bilayers of desired composition on the substrate. (C) Bilayers with various concentrations of functionalized lipids, such as biotin-X-DHPE or Texas Red-DHPE, can be achieved by mixing different amounts from stock solutions with vesicles from a stock solution of pure egg PC.

Results and Discussion

Overview of Microfluidic Patterning Technique. The procedure developed for creating patterned lipid bilayers of desired composition is shown schematically (Figure 2). Patterns consisting of long, thin parallel lines are chosen to allow direct comparison of lipid bilayers with different compositions in parallel plate flow chamber experiments. For flow experiments, the patterned bilayer lanes are oriented in the direction of flow. The patterns branch off at the ends to allow enough space for punching inlet and outlet holes (Figure 2A) which prevents mixing between the closely spaced lanes. The versatility of the microfluidic technique allows precise control of the line width and the spacing between lanes to fit multiple lanes into one microscopic field of view (Figure 2B). For flow chamber studies, 50 μm line width and spacing generates four lanes of adequate area to collect sufficient data for statistical comparison.

The type and concentration of functionalized lipids in the bilayers can be easily and rapidly varied by premixing vesicle solutions (Figure 2C). For example, aliquots from a stock solution of pure egg PC vesicles can be combined

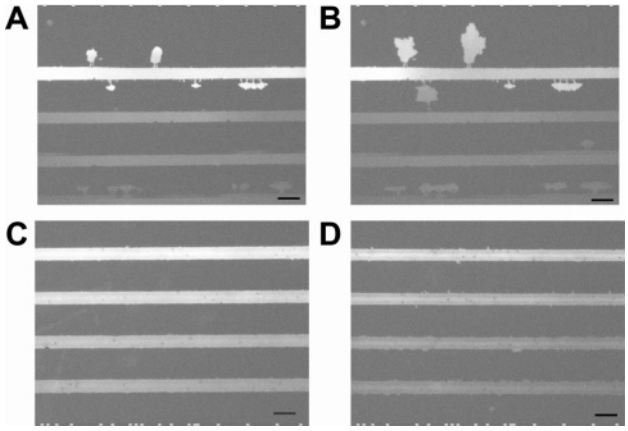

Figure 3. BSA blocks lipid spreading onto glass. Fluorescence images (A) 26 min and (B) 1.75 h after removal of PDMS stamp in water. Fluorescence images (C) 5 min and (D) 48 h after removal of stamp in PBS containing 0.2% BSA. Scale bars are 100 μm.

with aliquots from stock solutions of functionalized lipids such as Texas Red-DHPE or biotin-PEG2000-DSPE in egg PC to create lipid bilayers with different compositions. Premixing is essential as Cremer and Yang[8] observed that the sequential addition of two different vesicle solutions to the same glass area yielded bilayers composed primarily of the first lipid type, whereas premixing two solutions resulted in bilayers composed of both lipid types. Premixing greatly enhances the flexibility of this method by allowing creation of lipid bilayers with desired composition from a limited number of vesicle stock solutions.

BSA Maintains Pattern Shape by Preventing Lipid Spreading onto Glass. When the PDMS stamp is removed from the glass surface in the absence of BSA, we observe lipid spreading that evolves over time to form treelike structures (Figure 3A,B). This is to be expected because the lipid bilayers formed in the large circular areas under the inlets and outlets serve as lipid reservoirs, causing fusion between the closely spaced lanes over time. We find that stamp removal in a solution of PBS containing 0.2% BSA preserves the patterned lanes (Figure 3C,D), indicating that protein adsorption is a much faster process than lipid spreading.[11]

Lipid bilayer spreading with fingerlike projections has also been observed by others[10,11] and is known to produce bilayers with characteristically ragged edges. It has been reported that egg PC spreads to approximately 106% of its original area before spreading ceases due to a balance of intermolecular and surface forces.[11] Hovis and Boxer[5] exploited this so-called "self-limiting lateral expansion" to form lipid patterns which retain their shape by virtue of features spaced far enough apart, e.g., 15 μm wide with 215 μm spacing. However, this spacing would limit the number of lanes which can be observed simultaneously in one field of view.

Prevention of lipid spreading by backfilling with BSA has also been used by Kung et al.,[7] who selectively removed lipid by first blotting with a PDMS stamp and then "caulking" the remaining spaces by adding BSA to the solution several minutes later. Adsorbed proteins such as BSA have been shown to block the lateral diffusion of lipids without disrupting the membrane or reducing lateral mobility as evidenced by fluorescence recovery after photobleaching.[7,11] Additionally, the height of the BSA barriers as measured by atomic force microscopy is ~2−3 nm,[7] which is of the same order as lipid bilayers.[24]

Fluorescence Microscopy Verifies That Premixing of Vesicles Produces Bilayers with Different Compositions. To verify that premixing vesicle solutions could produce bilayers with different compositions, we first tested the incorporation of a common fluorescent lipid probe, Texas Red-DHPE, because its level of incorporation can be determined with fluorescence microscopy. Indeed, we are able to generate patterned bilayers with different levels of Texas Red-DHPE incorporation by premixing various ratios of egg PC vesicles and vesicles spiked with Texas Red-DHPE (Figure 4). Fluorescence microscopy thus verifies that premixing generates bilayers with different compositions of labeled lipids. Such a check is important to confirm that lipids present in vesicles are also found in the resulting supported bilayer. Incorporation may vary for different lipids depending on whether they are in the gel or fluid phase or for lipids with headgroup-attached functional molecules.

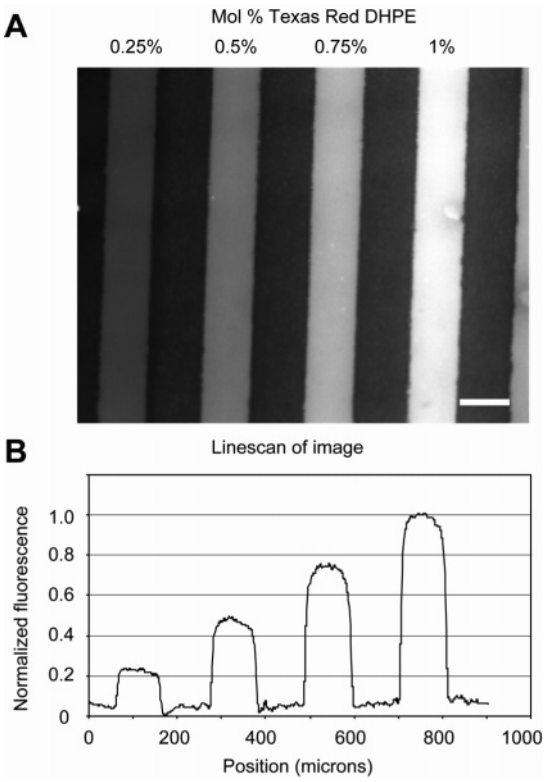

Figure 4. Premixing different vesicle solutions produces bilayers of varied composition. Bilayers were formed from various mixtures of pure egg PC vesicles with vesicles containing 2% Texas Red-DHPE. (A) Fluorescence image is shown of four patterned bilayers where each lane was formed with vesicle solution mixtures containing the indicated overall percentage of Texas Red-DHPE. Premixed vesicle solutions were combined 1:1 (v:v) with 140 mM NaCl in PBS just before injection into lanes. Vesicle stock solutions used are egg PC and TR (2%). Premixing ratios of vesicle stock solutions (v:v) are as follows: 0.25% TR = 1:7 TR:egg PC, 0.5% TR = 1:3 TR:egg PC, 0.75% TR = 3:5 TR:egg PC, 1% TR = 1:1 TR:egg PC. Scale bar is 100 μm. (B) Line scan of image intensity with background subtracted.

Streptavidin Binds Specifically to Biotinylated Lipids Demonstrating Successful Incorporation of Functionalized Lipids into Bilayers via Premixing. Although the incorporation of Texas Red-DHPE into patterned bilayers can be directly probed, ligand−receptor binding studies are necessary to verify incorporation of functionalized lipids. We find that fluorescently labeled streptavidin binds only to lanes containing biotinylated

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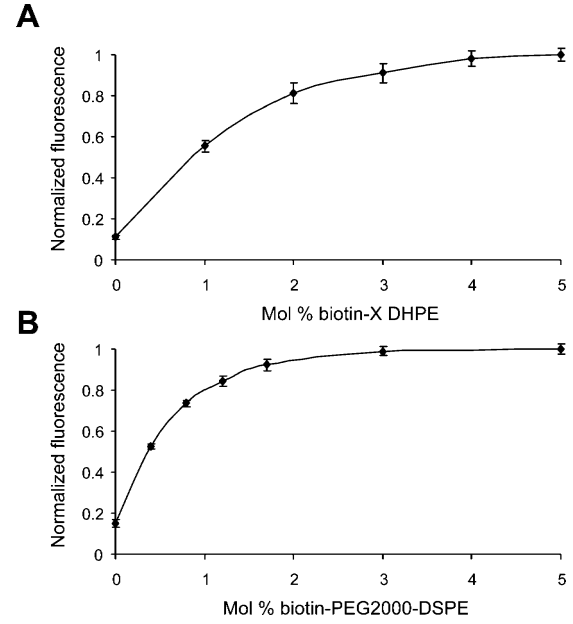

Figure 5. Streptavidin binds specifically to biotinylated lipids. Patterned egg PC bilayers containing (A) biotin-X-DHPE and (B) biotin-PEG2000-DSPE were incubated in PBS containing 0.2% BSA for at least 1 h. The bilayers were then incubated with Texas Red-X streptavidin at room temperature for 1−2 h before washing with PBS to remove unbound streptavidin. Plots depict the normalized fluorescence intensity of the bilayers as a function of the overall amount of biotinylated lipids in the premixed vesicle solutions. Plots A and B are normalized independently to the maximal intensity for that specific set of experiments. Premixed vesicle solutions were combined 1:1 (v:v) with 140 mM NaCl in PBS just before injection into lanes. Vesicle stock solutions are egg PC, BXD (5%), and BPEG (5%). Premixing ratios of vesicle stock solutions were varied to achieve the indicated overall amount of BPEG or BXD, i.e., 4% BXD = 1:4 (v:v) egg PC:BXD and 0.4% BPEG = 23:2 egg PC:BPEG. Each point represents the mean ± standard deviation of duplicate measurements for three separate experiments.

lipids, demonstrating that both biotin-X-DHPE and biotin-PEG2000-DSPE are successfully incorporated into the bilayers (Figure 5). The fluorescence intensity of pure egg PC and glass areas do not change significantly upon incubation with streptavidin, indicating that BSA successfully blocks nonspecific binding. For both spacers we observe higher fluorescence intensity and thus higher levels of streptavidin bound as the concentration of biotinylated lipids in the vesicle solutions increases. However, the saturation occurs at a much lower concentration for biotin-PEG2000-DSPE compared to biotin-X-DHPE: ∼2 and ∼4.5 mol %, respectively. To explain this difference, one can estimate the expected saturation concentration by determining the limiting surface area.

Our streptavidin binding results are in quantitative agreement with predicted surface coverage based on crystallographic data for streptavidin. At least 4.4 mol % of biotin-X-DHPE is needed for a full surface coverage of streptavidin using a projected area of 2900 Å^2 per streptavidin molecule and 65 Å^2 for all lipid headgroups[25] and assuming two biotin molecules are bound to each streptavidin molecule. The concentration of biotin-X-DHPE at which we observe saturation of streptavidin binding (Figure 5) agrees well with this predicted value. Moreover, our results agree with those of Calvert and Leckband,[23] who observed monolayer streptavidin coverage on bilayers with outer layers of 5% biotin-X-DHPE deposited on inner layers of DPPE prepared by the Langmuir−Blodgett technique. The fact that we observe saturation of streptavidin binding at ∼4−5 mol % indicates that it is reasonable to assume full incorporation of the biotin-X-DHPE into lipid bilayers. Because we prepare our vesicles by the sonication technique, we expect the concentration of biotinylated lipids to be the same in both bilayer leaflets.

We note that streptavidin binding saturates at a much lower concentration for biotin-PEG2000-DSPE than for biotin-X-DHPE. We attribute this difference to the much longer length and flexibility of the PEG tether. In contrast to the rigid spacer of biotin-X-DHPE, the flexible PEG tether allows the biotin−streptavidin complex to explore a larger area which is expected to sterically hinder binding of additional streptavidin to neighboring biotin sites. One can estimate the average projected area of a tethered biotin−streptavidin complex from recent experimental studies and theoretical predictions of end-grafted polymer chains. We assume that free chain ends are located on average at a distance of 0.7 times the Flory radius from the anchoring surface, which is 24.5 Å for PEG2000.[26,27] We then assign an approximate "radius" of streptavidin equal to half the width or 27 Å based on crystallographic data, indicating that the molecule has dimensions of 54 × 58 × 48 Å.[28] Assuming circular geometry and a complex radius equal to the sum of the radii of the PEG tether and streptavidin, or 51.5 Å, the complex will sample an area of approximately 8330 Å^2. On the basis of the area of the biotin-PEG2000-DSPE/streptavidin complex, streptavidin saturation is expected to occur at 0.8 mol % biotin-PEG2000-DSPE, which agrees fairly well with our data. For streptavidin, PEG2000 thus blocks access to binding sites above a grafting density of 0.8 mol %, even though the ligand is attached to the PEG terminus. This is not completely unexpected as it is well-known that PEGylation not only reduces nonspecific protein binding but also reduces specific ligand−receptor binding[3,29,30] even for terminally attached ligands.[31] Similar results were observed by Bendas et al.,[31] who found that increasing the concentration of terminally functionalized PEG2000 lipids incorporated into liposomes above 2.5 mol % did not increase antibody coupling ability.

Osmotic Stress Promotes Vesicle Fusion and the Formation of Lipid Bilayers. We used a combination of fluorescence microscopy and FRAP to verify that we obtain lipid bilayers rather than mixed adsorbed vesicle/bilayer surfaces. Fluorescence microscopy imaging reveals that adding salt to the premixed vesicle solutions promotes fusion with the surface as demonstrated by much higher intensities than in the absence of salt (Figure 6). The osmotic pressure caused by adding salt to vesicles prepared in water is especially important for driving the fusion of PEG-containing vesicles. When pure water instead of salt is added to the premixed vesicle solutions, some fluorescence is observed for Texas Red-DHPE vesicles (Figure 6Ai,ii). Note that the presence of PEG-containing vesicles does not block the adsorption and/or fusion of Texas Red-

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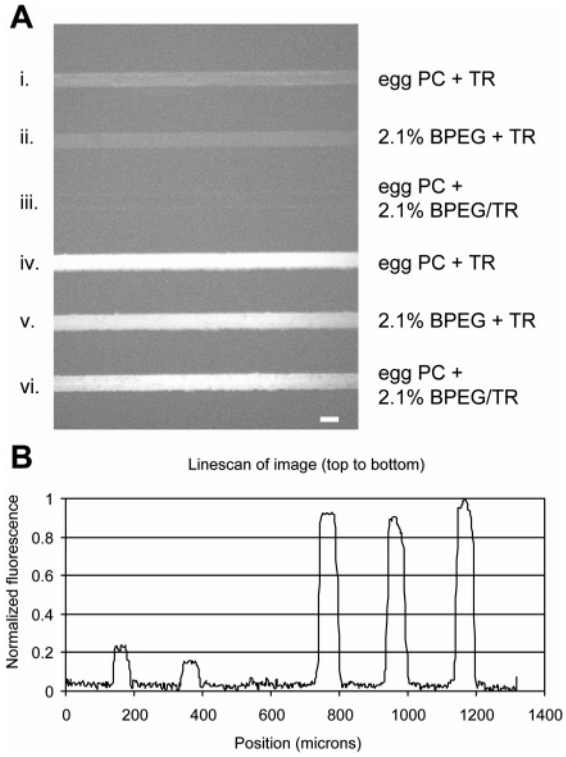

Figure 6. Fluorescence microscopy verifies that osmotic stress promotes vesicle fusion. (A) Fluorescence image of patterned lanes formed in the (i−iii) absence and (iv−vi) presence of salt. Premixed vesicle solutions are combined 1:1 (v:v) with either pure water or 140 mM NaCl in PBS just before injection into lanes. Vesicle stock solutions are egg PC, TR (1%), BPEG (4.5%), and BPEG/TR (4.5%, 1%). Premixing ratios of vesicle stock solutions (v:v) are as follows: egg PC + TR = 1:1 egg PC:TR, BPEG + TR = 1:1 BPEG:TR, egg PC + BPEG/TR = 1:1 BPEG/TR:egg PC. Scale bar is 50 μm. (B) Line scan of the image intensity with background subtracted.

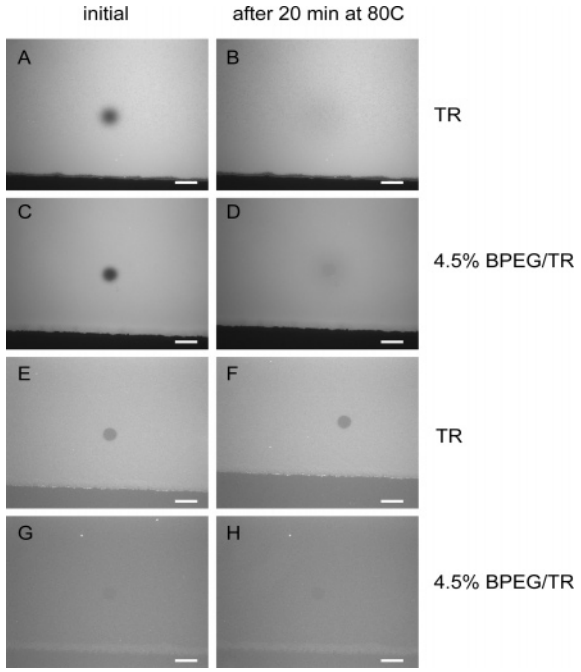

Figure 7. FRAP verifies formation of fluid bilayers.FRAP experiments in the (A−D) presence or (E−H) absence of salt. The column on the left shows images taken just after photobleaching a small circle, while the column on the right shows approximately the same areas after 20 min of incubation at 80 °C. Vesicle solutions were combined 1:1 (v:v) with either (A−D) 140 mM NaCl in PBS or (E−H) pure water just before injection into 1 mm wide lanes. Vesicle stock solutions are egg PC, TR (1%), and BPEG/TR (4.5%, 1%) and are used directly without premixing additional vesicle types. Lane edges are imaged to allow visualization of background intensity. For the images taken in the absence of salt, brightness is scaled to maximize visualization of image features. Scale bars are 100 μm.

DHPE vesicles (Figure 6Aii.) in no salt conditions. In contrast, fluorescence is not observed when using vesicles containing both biotin-PEG2000-DSPE and Texas Red-DHPE, demonstrating that PEG precludes fusion in the absence of salt (Figure 6Aiii.). The fact that fluorescence is not observed above background levels (Figure 6B) for the biotin-PEG2000-DSPE/Texas Red-DHPE lane (Figure 6Aiii) strongly suggests that PEG-containing vesicles are washed away during the rinsing process. Adding salt to the premixed vesicle solutions is thus an important step to obtain lipid bilayers, especially for PEG-containing vesicles. Other studies have also found that osmotic stress provides a driving force for the formation of homogeneous supported bilayers confirmed by both AFM[22] and neutron reflectivity.[32,33]

We performed FRAP experiments to further probe whether we obtain fluid lipid bilayers or mixed surfaces containing bilayers and/or adsorbed vesicles. After photobleaching a small circular area, the samples are incubated above the highest melting temperature of the component lipids and reimaged (Figure 7). As in Figure 6, we observe much lower fluorescence intensities in the absence of salt (Figure 7E−H), especially for PEG-containing vesicles (Figure 7G,H). In the presence of salt we observe almost full recovery for vesicles that contain Texas Red-DHPE (Figure 7B) and both Texas Red-DHPE and biotin-PEG2000-DSPE (Figure 7D). This indicates the presence of fluid lipid bilayers. In contrast, in the absence of salt we see no recovery, signifying a surface containing adsorbed vesicles and/or bilayer patches. Fluorescence microscopy and FRAP experiments thus confirm that osmotic stress is critical for achieving fluid lipid bilayers.

Patterning Enables High-Throughput Screening of Ligand−Receptor Binding under Shear Flow. We tested our technique by observing interactions between streptavidin-coated beads and an array of supported bilayers in a parallel plate flow chamber (Figure 1). Our technique provides direct comparison of binding interactions under identical flow conditions. We observe clear differences between the lanes in terms of the number of beads remaining bound, indicating that the length of the spacer is an important factor that confers detachment resistance (Figure 8). Higher detachment resistance is observed for biotin-PEG2000-DSPE, which contains a much longer spacer between the lipid headgroup and the ligand than biotin-X-DHPE. Low binding to control lanes containing egg PC, 2.1% mPEG2000-DSPE, and glass confirms low nonspecific binding of receptor-coated beads in the absence of ligand.

A longer tether may confer higher detachment resistance for several reasons. The most likely explanation for our result is that more biotin−streptavidin bonds formed in the presence of the PEG tether than with the short spacer of biotin-X-DHPE. Flexible tethers are expected to increase the effective contact area over which biotin and streptavidin are in close enough proximity to bind and thus the number of bonds. In order for beads to remain

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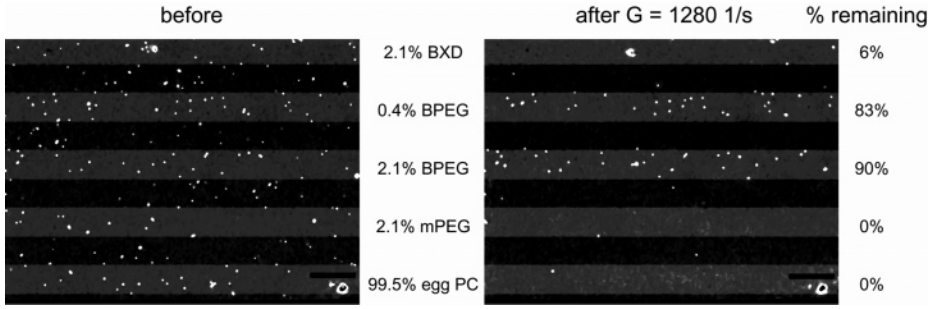

Figure 8. Patterning allows direct comparison of binding to various lipid compositions under identical flow conditions. Bright field images of (A) streptavidin-coated beads accumulated at a wall shear rate of 5 s^{-1} and (B) beads that remain bound after a wall shear rate of 1280 s^{-1} is applied. Lipid mixtures contained 0.5% Texas Red-DHPE to mark lane locations, which are colorized with a brighter intensity than the glass between each lane. Percentages indicate the percent of beads initially present that remain bound after a shear rate of 1280 s^{-1}. Data are a representative result of four experiments. Vesicle stock solutions are egg PC, TR (1%), mPEG (4.5%), BPEG (4.5%), and BXD (4.5%). Premixing ratios of vesicle stock solutions (v:v) are as follows: 2.1% BXD = 1:1 BXD:TR, 0.4% BPEG = 1:4:5 BPEG:egg PC:TR, 2.1% BPEG = 1:1 BPEG:TR, 2.1% mPEG = 1:1 mPEG:TR, egg PC = 1:1 egg PC:TR. Scale bars are 50 μm.

bound as the shear rate is increased, the force must be distributed among enough bonds to prevent lipid pullout. A force and torque balance developed by Hammer and Lauffenburger[34] can be simplified[15] due to the small size of the beads to provide an estimate of the total bond force, F_t, necessary for a bead to remain bound (eq 1). As a result, the total bond force varies linearly with shear stress τ ($=\mu G$), inversely with contact area radius R_{CA}, and exhibits a cubic dependence on particle radius R (eq 1).

$$F_t \approx 100\tau \frac{R^3}{R_{CA}} \tag{1}$$

Assuming all bonds are stressed equally provides an estimate of the force per bond F_b where σ_b is the bond density or number of bonds per area (eq 2).

$$F_b = \frac{F_t}{\pi {R_{CA}}^2 \sigma_b} \approx \frac{100\tau}{\pi\sigma_b}\left(\frac{R}{R_{CA}}\right)^3 \tag{2}$$

In reality, the force per bond will depend on the location of the bond in the contact area of radius R_{CA} with more force applied to bonds on the outer upstream edge. However, assuming a constant force per bond provides a reasonable first estimate.

The effect of PEG molecular weight appears in the contact area radius where L is the maximum length of a spacer and δ is the sphere to surface separation distance (eq 3). For a stationary particle, δ can be approximated by the equilibrium thickness of a compressed polymer layer $\langle x \rangle$. For a chain in good solvent conditions, $\langle x \rangle = aN^{0.6}\sqrt{(\pi/2)} \approx 43$ Å for PEG2000 where a is the length of a monomer unit and N is the number of monomer units.

$$R_{CA} = \sqrt{2(RL - R\delta + \delta L) - L^2 - \delta^2} \tag{3}$$

At a wall shear rate of 1280 s^{-1}, the force per bond (eq 2) is approximately 142, 0.6, and 3 pN for 2.1 mol % biotin-X-DHPE, 2.1 mol % biotin-PEG2000-DSPE, and 0.4 mol % biotin-PEG2000-DSPE, respectively. The force per bond for biotin-X-DHPE is thus an order of magnitude higher than the force for lipid pullout[35] (23 pN) while that for both molar concentrations of biotin-PEG2000-DSPE is an order of magnitude lower. Decreased detachment resistance at a wall shear rate of 1280 s^{-1} is not expected for biotin-PEG2000-DSPE until the concentration is approximately 0.05 mol % or lower. Estimates of the force per bond thus support our experimental findings of very low detachment resistance for biotin-X-DHPE and very high detachment resistance for biotin-PEG2000-DSPE.

An advantage of our technique is that the simultaneous probing of multiple bilayer compositions greatly reduces the number of experiments, in this case by a factor of 4. We can easily apply this technique to examine various combinations of functionalized lipids, including both liganded and unliganded lipids with varying PEG molecular weight. By comparing the dynamic and static binding characteristics as a function of bilayer composition, we hope to elucidate the optimal surface properties for targeted liposomes.

Patterning Technique Can Be Applied To Investigate a Wide Variety of Mobile and Stationary Phases. Our technique is quite versatile as it can also be used to screen binding properties between stationary and mobile phases other than lipid bilayers and beads. Stationary phases that could be patterned include thin films of drug carrier polymers, proteins, linkers that couple ligands such as those used in studies of leukocyte adhesion, and even cells. We have used this technique to pattern cells (data not shown), which is attractive because it provides an opportunity to introduce an additional and relevant level of physiological complexity to flow studies. For instance, binding specificity could be directly compared among different cell types in the flow chamber after first patterning them in microfluidic channels. In addition, cells expressing different receptors could be easily attained by injecting different cytokines or other stimulating agents into the lanes just before removal of the stamp for flow experiments. This might prove to be a useful screening tool in such fields as lymphocyte homing, as it has been demonstrated that certain chemokines mediate the tissue-specific homing of particular lymphoid subsets by inducing the high-affinity conformation of integrins necessary for triggering firm adhesion under shear.[36] Mobile phases such as liposomes, drug carrier micro- or nanoparticles, and leukocytes could be combined with any of the stationary phases mentioned above to investigate a wide variety of binding interactions under shear flow conditions.

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Conclusions

We have demonstrated that microfluidic patterning combined with a parallel plate flow chamber enables the direct quantitative comparison of the effects of varying surface properties on binding under identical shear conditions. Premixing enhances the flexibility of this technique by allowing rapid creation of desired lipid compositions from just a few vesicle stock solutions. In principle, one can employ any functionalized lipid that can be incorporated into a vesicle and fused to a surface to form a supported lipid bilayer. Additionally, our technique can be used with any combination of stationary and mobile phases including cells or proteins. By screening for carrier surface properties that maximize specific binding under flow, this technique can be used to identify key biophysical properties that enhance targeted delivery.

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