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Using micropatterned lipid bilayer arrays to measure the effect of membrane composition on merocyanine 540 binding

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ABSTRACT

The lipophilic dye merocyanine 540 (MC540) was used to model small molecule–membrane interactions using micropatterned lipid bilayer arrays (MLBAs) prepared using a 3D Continuous Flow Microspotter (CFM). Fluorescence microscopy was used to monitor MC540 binding to fifteen different bilayer compositions simultaneously. MC540 fluorescence was two times greater for bilayers composed of liquid-crystalline (l.c.) phase lipids (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC),1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)) compared to bilayers in the gel phase (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)). The effect cholesterol (CHO) had on MC540 binding to the membrane was found to be dependent on the lipid component; cholesterol decreased MC540 binding in DMPC, DPPC and DSPC bilayers while having little to no effect on the remaining l.c. phase lipids. MC540 fluorescence was also lowered when 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (DOPS) was incorporated into DOPC bilayers. The increase in the surface charge density appears to decrease the occurrence of highly fluorescent monomers and increase the formation of weakly fluorescent dimers via electrostatic repulsion. This paper demonstrates that MLBAs are a useful tool for preparing high density reproducible bilayer arrays to study small molecule–membrane interactions in a high-throughput manner.

1. Introduction

One major function of biomembranes is to provide a selectively permeable barrier for the binding of small solute molecules, such as ions, nonelectrolytes, and pharmaceutical drugs [1]. These types of molecules can adsorb to the surface or partition into the membrane and diffuse across the bilayer to the other side [1]. Understanding these membrane interactions is of interest to many areas of research, in particular, drug discovery. For example, the way in which drugs permeate the cell membrane is important to the pharmacological activity of a drug and its effectiveness in reaching its target site [1–3]. However, cell membranes are complex structures with a variety of phospholipids, sterols and proteins, making them difficult surfaces to work with when investigating the mechanism of small molecule–membrane interactions [1,4]. There has been an extensive amount of work to develop model membranes that possess characteristics of biological structures without the complexity of cellular membranes. Planar supported lipid bilayers (PSLBs) are one of the most extensively used models of cell membranes because they mimic many biological properties such as 2D fluidity and resistance to nonspecific biomolecule adsorption, while allowing control over the membrane composition [5–8]. Placing PSLBs in a microarray format is extremely desirable because a high-throughput platform for the investigation of specific biological and chemical interactions can be realized; with micropatterned lipid bilayer arrays (MLBAs) being particularly useful in the study of small molecule–membrane interaction. The ability to create lipid bilayers containing an assortment of lipid compositions can also accelerate the study of specific membrane components on small molecule association. The aim of the study presented here is to demonstrate the use of MLBAs for the high-throughput investigation of various membrane constituents on small molecule association using the well-studied membrane-associating molecule merocyanine 540 (MC540) [9–15].

A convenient method for preparing lipid bilayer arrays has previously been demonstrated using a 3D continuous flow microspotter (CFM) system [16]. The CFM consists of a poly(dimethylsiloxane) (PDMS) stamp with 96 inlet and outlet wells connected by a series of microchannels embedded within the polymer. The PDMS

Abbreviations: MC540, merocyanine 540; MLBAs, micropatterned lipid bilayer arrays; CFM, continuous flow microspotter; l.c., liquid-crystalline; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt); CHO, cholesterol; PSLBs, planar supported lipid bilayers; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SUV, small unilamellar vesicle; PDMS, poly(dimethylsiloxane); PC, phosphatidylcholine; PS, phosphatidylserine, phase transition temperature

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print-head connects the pairs of inlet and outlet channels when it is placed in contact with a substrate. Each inlet well is individually addressable allowing for the production of a 2D multi-component bilayer array. We previously showed that an array of fluid lipid membranes generated with the CFM could successfully be used as a platform for multiple protein–ligand binding experiments [16]. Polymerized, ligand functionalized lipid bilayer arrays were also generated with the CFM proving a more robust stable bilayer platform for biological assays, as was demonstrated by the application of these novel materials for the simultaneous detection of multiple protein–ligand interactions [17].

In this study we have expanded upon the use of the MLBAs to investigate small molecule–membrane interactions. As mentioned previously, cellular membranes are complex, containing well over 100 unique lipid compositions with different headgroup and acyl group portions whose properties such as charge and lipid packing will impact small molecule penetration into the membrane [1,3,18]. Glycerophospholipids represent the majority of membrane lipids with the zwiterrionic phosphatidylcholine (PC) headgroup being the most common. Cell membranes also contain a significant amount of the anionic phosphatidylserine (PS) headgroup with PS representing ~10% of lipids in human erythrocyte membranes [19].The acyl chains differ in length and degree of saturation which has an impact on membrane segregation, fluidity and the phase transition temperature (T_m). The T_m is the temperature at which the lipids change from the gel phase characterized by dense, tightly packed chains to the liquid-disordered phase which the lipids are randomly and loosely packed [20]. Generally, saturated lipids tend to exist in the gel phase at room temperature with longer chains having a higher T_m as a result of increased van der Waals interactions. Unsaturated lipids exist in the liquid-disordered or liquid-crystalline (l.c.) phase as a consequence of the *cis* double bond introducing kinks in the chain which disorders the packing. Cholesterol is another major component in biological membranes constituting ~30% of the membrane lipids in animal cell plasma membranes and is known to influence the physical properties of cellular membranes [1,21].For example, cholesterol has a condensing effect on membrane lipids decreasing the surface area per lipid molecule [21]. Cholesterol also influences the miscibility of lipid components and modulates the T_m [21,22].

In the work presented here, a MLBA was used for the high-throughput investigation of the various membrane properties mentioned above on the binding of the fluorescent anionic dye MC540 (Fig. 1). MC540 is used as a model amphipathic low molecular weight molecule, a characteristic of many drug compounds, with its nonpolar parts having an affinity for the hydrophobic portion of the lipid membranes. The fluorescence characteristics of MC540 are sensitive to the polarity of the local environment [10].When MC540 is located in the aqueous environment surrounding the polar headgroup, it is in its weak fluorescent dimer form with an absorbance maximum at 530 nm [13]. When the dye is incorporated into a lipid bilayer, it partitions as a monomer slightly above the glycerol backbone with its butyl groups extending into hydrocarbon chain, resulting in a red shift in the absorbance maxima to 568 nm which is also accompanied by an increase in the fluorescence quantum yield [10,13]. Fluorescence microscopy can be used to obtain information on MC540 binding with respect to membrane properties since the fluorescence intensity is related to the location of the dye within the membrane. MC540 has been used to probe membrane properties such as molecular packing of lipid bilayers, with increased MC540 fluorescence in loosely packed (l.c. phase) lipid bilayers as opposed to closely packed (gel phase) membranes [9–12]. MC540 has also demonstrated sensitivity to the membrane surface potential, showing a decrease in MC540 monomer binding to negatively charged membranes [10,14,15,23,24]. MC540 is an excellent candidate to validate the efficacy of MLBAs for monitoring small molecule–membrane interactions due to the large volume of data which have been obtained previously on the system which is available in the literature. In the experiments reported here MLBAs composed primarily of lipids with the zwitterionic PC headgroup and different alkyl chains, along with varying ratios of cholesterol and phosphatidylserine (PS) lipids were examined. Fluorescence microscopy was used to measure the effect of lipid composition on MC540 binding. Lipids with saturated, unsaturated or mixed chains were used to investigate the effects of gel and l.c. phases of the lipid–small molecule interactions using the following glycerophospholipid (chain length:number of double bonds); myristoyl (14:0), palmitoyl (16:0), stearoyl (18:0), oleoyl (18:1) and mixed stearoyl-oleoyl (18:0–18:1) and palmitoyl-oleoyl (16:0–18:1). The lipids mentioned above were also prepared in binary mixtures with cholesterol to study its effect on small-molecule (dye) association. Finally, the PC membranes were doped with varying concentrations of PS to study the effect of charged membranes on MC540 binding.

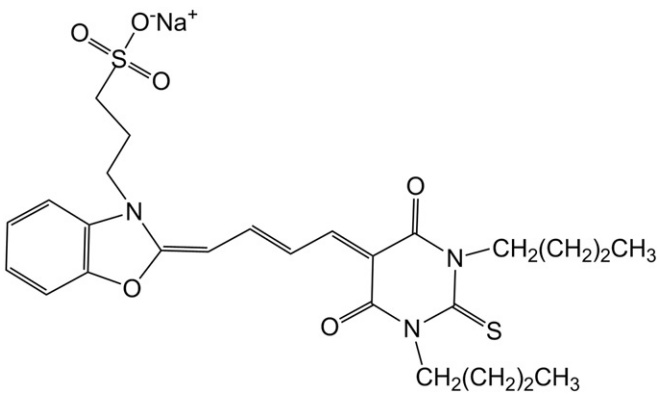

Fig. 1. Structure of merocyanine 540 (MC540).

2. Experimental section

2.1. Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (DOPS) were obtained from Avanti Polar Lipids. Merocyanine 540 (MC540) was obtained from Fluka. Bovine serum albumin (BSA) and cholesterol (CHO) were purchased from Sigma-Aldrich. All materials were used as received. The water used in these studies was obtained from a Nanopure Infinity Ultrapure water purification system with a minimum resistivity of 18.2 MΩ cm. Quartz microscope slides (Chemglass) were used as the supporting substrate for the MLBAs.

2.2. Small unilamellar vesicle preparation

Lipids were mixed in chloroform and dried under a stream of N_2(g) to remove the chloroform followed by vacuum drying overnight. The dried lipid films were resuspended by vortexing in phosphate-buffered saline (PBS, pH 7.4, 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 1 mM NaN_3) above the T_m of the lipid. The lipid suspensions were then bath sonicated until clarity. The resulting solutions were then cooled and stored at 4 °C. The samples prepared in this manner were used over a course of three days.

2.3. MLBA preparation

The quartz microscope slides used for MLBA preparation were cleaned in piranha solution (70%/30%, 18 M sulfuric acid/30% H_2O_2)

followed by a rinse with copious amounts of Nanopure water. (*Caution! Piranha is highly corrosive and a strong oxidant, reacting violently with organic solvents and materials; handle with extreme care.*) The slides were dried under a stream of N_2(g) and then plasma cleaned (Harrick PDC-32G) with Ar for 3 min. Details of the preparation of MLBAs using the CFM have been discussed elsewhere [16]. Briefly, the clean substrates were placed into contact with the PDMS printhead to form a reversible seal. The various small unilamellar vesicle (SUV) solutions at a 0.5 mg/ml lipid concentration were simultaneously introduced through the microchannels at least 15 °C above the T_m and circulated over the substrate for at least 20 min. The vesicles spontaneously fused to the hydrophilic substrate generating an array of multi-component fluid lipid bilayers. The SUV solutions were removed and the channels were rinsed with nanopure water, making sure the channels never completely empty. The substrate was removed from the PDMS printhead in a reservoir of a 0.2% (w/v) BSA solution in PBS and incubated for 20 min. BSA adsorbs to the hydrophobic PDMS residue from the printhead along with any areas of bare glass, corralling the bilayer spots to prevent lipid spreading [25]. The substrate was then transferred in a water bath to a custom built Teflon flow cell.

2.4. MC540 binding experiments

Patterned membranes were prepared with one of the following primary phospholipids (DMPC, POPC, DPPC, DSPC, SOPC and DOPC) containing 0 or 33 mol% cholesterol. Patterned DOPC membranes doped with varying ratios of DOPS (0–25 mol%) were also prepared on the same substrate. The MLBA was incubated with 18 μM MC540 in PBS for 20 min in the dark. The substrate was then reassembled into a new flow cell in a PBS bath to eliminate background fluorescence from MC540 staining the bottom of the flow cell. The binding of MC540 to the different lipid compositions was measured by fluorescence microscopy. All binding experiments were performed at room temperature (25 °C).

2.5. Fluorescence microscopy

An Olympus BX40 microscope equipped with a Photometrics-CoolSNAP$_{cf}$ (Roper Scientific) color camera was used to image the MLBAs under a 10× objective (NA, 0.30). The bandpass filter set used for MC540 has a center excitation/emission wavelength of 550/615 nm with a passband width of 30/45 nm, respectively. The excitation/emission wavelengths of the MC540 dimer and monomer are 530/570 and 568/590 nm, respectively [9]. Fluorescence images were analyzed and background corrected using the software package Voodoo Incantation 1.2 provided by Photometrics. The brightness and contrast of the fluorescent images were adjusted for presentation purposes. The micropatterned bilayer images were pieced together using Canvas X software. The use of a 10× objective allowed for the more precise assessment of MC540 fluorescence within an individual lipid spot, facilitating a more accurate determination of small-molecule binding along with the possibility of observing any inhomogeneous distribution of MC540 as a result of lipid phase segregation or domain formation in the binary mixtures.

2.6. UV–Vis absorbance

The results from the MC540 binding experiment were verified by performing a similar binding experiment on SUVs using absorbance spectroscopy. The liposome stock solutions were prepared at a 2.7×10^{-3} M lipid concentration. The MC540 stock solution of 29 μM was prepared in PBS. MC540 was added to the liposome solutions to yield a 400:1 lipid:dye molar ratio with a final MC540 concentration of 5 μM. The mixtures were incubated for at least 20 min while being shielded from light. MC540 association to the different liposomes was analyzed by recording the absorbance spectra using a Lambda 25 UV/Vis Spectrometer (Perkin Elmer Instruments). All measurements were performed at room temperature with the exception of DMPC and DMPC + 33 mol% CHO which were measured at 50 °C. The DMPC/cholesterol mixture is unstable at room temperature resulting in cloudy solutions, altering the absorbance spectrum due to high levels of light scattering. The background samples were obtained by diluting the SUV stock solutions with PBS to yield the appropriate lipid concentration.

2.7. Surface pressure measurements

Surface pressure measurements were performed using the Wilhelmy balance from a KSV Instrument Langmuir–Blodgett trough (Minitrough) and a custom designed glass cell which contained 100 ml of PBS as a subphase. DOPC with varying amounts of DOPS was dissolved in $CHCl_3$ and spread dropwise at the air/water interface until the surface pressure reached a value between 29 and 31 mN/m. Discrete amounts of a 17.9 mM solution of MC540 in water were added while gentle stirring the subphase and allowing the surface pressure to reach equilibrium (approximately 10–20 min) before recording the subsequent change in the surface pressure. The injection volume of the MC540 solution did not exceed 150 μl to ensure the cell volume was not significantly changed.

3. Results and discussion

3.1. MC540 binding to a multi-component MLBA

The CFM has been used here to pattern multi-component lipid arrays to examine MC540 membrane binding. The array was prepared using six primary lipids (DMPC, POPC, SOPC, DOPC, DSPC and DPPC) differing in the acyl chain length as well as the degree of unsaturation. These parameters influence the T_m which determines the phase state of the lipids with DMPC, POPC, SOPC and DOPC existing in the l.c. phase while DSPC and DPPC exist in the gel phase at room temperature (25 °C). Bilayers were prepared from SUV solutions containing these primary lipids with and without cholesterol. The partitioning of MC540 into the twelve unique lipid compositions is represented by the fluorescence image in Fig. 2. The fluorescence intensity from each bilayer spot was obtained by averaging the fluorescence intensity over the entire bilayer square. The measured fluorescence intensities shown in Fig. 2, with their respective errors, represent the average and standard deviation of the pooled normalized fluorescence values from three independent binding experiments where each experiment had two bilayer spots of identical bilayer composition on the same substrate for a total six independent measurements. The fluorescence intensities were normalized to the pure POPC bilayers without any cholesterol present. For the membranes without cholesterol, the fluorescence intensity is approximately two times greater for the more disordered l.c. phase (DMPC, POPC, SOPC and DOPC) lipids than those in the well-ordered gel phase (DSPC and DPPC). The higher fluorescence intensity for the l.c. phase membranes suggests that more dye is penetrating into the hydrophobic core than in gel phase lipid bilayers, in agreement with previous MC540 binding experiments [9–11]. This can be rationalized by the lipid packing in the bilayer relative to its phase. The loosely packed l.c. phase bilayers (60–70 Å^2/molecule) allow MC540 to penetrate deeper into the hydrophobic core than the more tightly packed gel phase bilayers (~50 Å^2/molecule) [1,9,11]. Note that the reported area per molecule values were extrapolated from Langmuir pressure-area isotherms of the appropriate lipid monolayers assuming a surface pressure of 30 mN/m for the MLBAs [26].

When comparing the fluorescence intensities for the bilayers within the l.c. phase, there is a subtle difference of MC540 binding with respect to the different acyl chain compositions. MC540

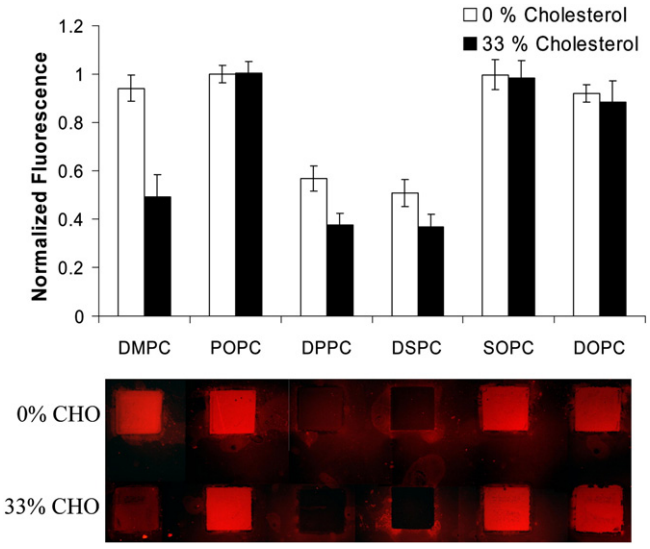

Fig. 2. Fluorescence image of MC540 binding to a MLBA with the membrane compositions stated in the figure. Quantitative fluorescence values were obtained from three separate experiments with each experiment having duplicated bilayer spots of identical membrane composition prepared on the same substrate (not shown). The data presented in the graph were normalized to MC540 fluorescence on a pure POPC bilayer. Each bilayer spot is 400 x 400 μm^2.

fluorescence is ~8% higher in bilayers composed of the mixed chain lipids, POPC (fluorescence intensity = 1.00 ± 0.04) and SOPC (fluorescence intensity = 1.00 ± 0.06), than the bilayers composed of the symmetric acyl chain lipid DOPC (18:1) (fluorescence intensity = 0.92 ± 0.04). A Student's *t*-test was performed on the pooled normalized fluorescence values to determine if the different acyl chains had a significant impact on MC540 binding. There is no statistical difference, at the 95% confidence level, in the fluorescence intensities between the mixed chain lipids POPC (16:0–18:1) and SOPC (18:0–18:1) suggesting MC540 binding is insensitive to the difference in the tail length of the saturated chain for lipids in the same phase state. The fluorescence obtained from the bilayer patches prepared with the short, fully saturated DMPC (14:0) lipids falls within error (fluorescence intensity = 0.94 ± 0.05) of the fluorescence signal obtained from the mixed chain lipid bilayers and DOPC bilayers, at the 95% confidence level. These data along with observed uniform fluorescence within the bilayer spots provide evidence that DMPC exists primarily in the l.c. phase. Between the gel phase lipids, there is a slight but statistically insignificant decrease in MC540 signal in DSPC (18:0) bilayers relative to the bilayers prepared from the shorter chain DPPC (16:0) lipids, at the 95% confidence level. Altogether, these results demonstrate that MC540 binding is sensitive to the phase state of the membrane while not being as sensitive to the changes in chain length and degree of unsaturation of the lipid tails within a certain phase state given the error in our measurements.

The influence of cholesterol on MC540 binding was found to be dependent on the primary lipid composition of the bilayer. Fig. 2 shows a decrease in MC540 fluorescence in all the saturated lipid bilayers when cholesterol is introduced with a 28%, 34% and 50% attenuation of MC540 fluorescence observed upon addition of 33 mol % CHO to DSPC, DPPC and DMPC bilayer, respectively. The decrease in MC540 binding could be explained by the condensing effect cholesterol has on saturated lipids decreasing the area per lipid molecule [21]. The addition of CHO into DMPC membranes yields the largest reduction in MC540 signal because it induces the greatest change in lipid packing in comparison to the gel phase DPPC and DSPC bilayers which already have tightly packed lipids. This physical change in DMPC membranes is apparent in the phase diagram of DMPC + CHO presented by Almeida et al. [27]. When high concentrations of cholesterol (>30%) are introduced into DMPC bilayers above the T_m, there is a large condensing effect on the bilayer, changing the bilayer from the loosely packed l.c. phase to the liquid-order phase exhibiting ordered acyl chains and tighter lipid packing (~50 $Å^2$/molecule) characteristic of the gel phase while still possessing membrane fluidity characteristic of the l.c. phase [28]. In contrast, there was no statistically significant difference in MC540 fluorescence for the remaining l.c. phase + CHO bilayers, at the 95% confidence level. These results were unexpected since a previous study showed a decrease in MC540 fluorescence in SOPC large unilamellar vesicles (LUVs) with increasing cholesterol content [11]. Error bars were not reported in the study by Stillwell et al. [11], but our results do qualitatively track with the previous study. A slight decrease in MC540 fluorescence in the SOPC + CHO bilayer spot was observed, but the error in the measurements precludes any strong statements regarding the differences between these systems from being definitively assigned.

The decrease in MC540 binding to CHO containing lipid bilayers correlates with cholesterol's ability of increasing the packing density of lipid membranes, most notably in the bilayers prepared with saturated lipids [28–31]. Langmuir film studies have shown that cholesterol at 30 mol% induces a condensing effect on gel phase DPPC monolayers further increasing the packing density of the film [29,30]. It should also be noted that the bilayers presented here were prepared by the vesicle fusion method. Vesicle fused bilayers are known to produce more disordered membranes than ones prepared at the air/water interface on a Langmuir trough [32]. It is likely that cholesterol is increasing the order of the slightly disordered gel phase lipids along with reducing the number of defects present in the bilayer further prohibiting the incorporation of MC540. This is consistent with a study performed by Kim et al. [30], where they characterize Langmuir–Blodgett deposited DPPC monolayers on silanized silicon substrates as a function of CHO content by atomic force microscopy (AFM), ellipsometry and cyclic voltammetry. They found that incorporation of cholesterol (10–30 mol%) into the lipid films yielded more densely packed monolayers with fewer defects than pure DPPC monolayers. Moreover, the hydroxyl group of cholesterol is known to be located at the edge of the polar region of the lipid close to the ester carbonyl group [33]. Thus, cholesterol simply fills up space between the lipids hindering the insertion of the MC540 butyl chains into the tightly packed bilayers. Our results are in agreement with literature reports of tetracaine binding to lipid bilayers [34,35]. Tetracaine is a local anesthetic which locates similarly into the membrane as MC540 [34]. Zhang et al. [34] observed a reduction in drug binding when 28 mol% cholesterol was added to DPPC lipids at 23 °C as a consequence of cholesterol being present at the glycerol backbone where tetracaine would normally locate. It should also be noted that cholesterol can fluidize lipids below their T_m and at high cholesterol concentrations (>20 mol%) [36]. However, it is important to remember that MC540 fluorescence is sensitive to its location in the membrane. Therefore, an increase in fluidity will not directly result in an increase in MC540 binding if cholesterol is preventing MC540 from penetrating deeper into the hydrophobic core of the membrane. Although cholesterol is most likely condensing the fluid phase lipids, there is still ample space between the lipid headgroups at 33 mol% cholesterol for MC540 binding. This is most apparent for DOPC lipids which are condensed only 6 $Å^2$/molecule, from 66 $Å^2$/molecule, upon the addition of 30 mol% cholesterol at a surface pressure of 30 mN/m providing more space than lipids in the gel phase (~50 $Å^2$/molecule) [28].

3.2. Interaction of MC540 with liposomes

Since there is a lack of statistical data in some of the previous studies of MC540 binding to the various lipid systems, we repeated the MC540 binding experiment with liposomes using UV–Vis absorbance as a control in order to validate the use of the MLBA platform. The

absorbance values ofMC540 incorporated into small unilamellar vesicles (SUVs) of the same composition as those examined using the MLBA are shown in Fig. 3. The solution phase vesicle results show similar trends as the MLBA data presented above, namely, an increased monomer concentrations in fluid phase bilayers compared to gel phase lipids. However, the absorbance for the gel phase lipids is only ~30% lower than the l.c. phase lipids as opposed to the ~50% reduction observed from the fluorescence of MC540 within the PSLBs. It should be noted, however, that SUVs have a looser headgroup packing, as a consequence of the small radius of curvature, allowing MC540 to better penetrate the gel phase lipid bilayers in vesicle form than in planar form [9]. For the l.c. phase lipids, there is not an observable difference in MC540 absorbance among the mixed chain POPC and SOPC lipids consistent with MC540 binding measured to the MLBAs. DMPC liposomes have the highest MC540 absorbance out of all the l.c. phase lipids examined, which was not the case for MC540 adsorption to the MLBAs. This is a possible consequence of the elevated temperature used for the DMPC liposomes (50 °C) (see Experimental section 2.6 for more details). DOPC liposomes have the lowest MC540 absorbance out of the liquid phase lipids, agreeing with the MLBA fluorescence data. Similar to MC540 binding to the gel phase MLBAs, the dye penetrates deeper into DPPC liposomes than DSPC liposomes, illustrated by the slightly higher MC540 absorbance for DPPC lipids. With the addition of cholesterol, DMPC liposomes have a significant decrease in MC540 absorbance which is consistent with MC540 binding to the planar bilayers. A considerable decrease in MC540 absorbance was also observed for DPPC and DSPC bilayers when cholesterol is present. Cholesterol in SOPC, POPC and DOPC bilayers did not have a large impact on MC540 absorbance correlating to the MLBA results discussed above. Based on the strong correlation between the absorbance data from the SUVs and the fluorescence data from the MLBA it is argued that a decrease in fluorescence signal is strongly attributed to the decrease occurrence of monomers with absorbance maxima at 568 nm. While there is a shift in the monomer–dimer equilibrium constant towards the increased occurrence of dimers with an increase in lipid packing, the fluorescence signal remains relatively low because of their lower quantum efficiencies [10,13]. Overall, the two separate experiments agree very well with only slight discrepancies which could be a result of SUVs having a high degree of curvature altering small molecule binding [9,13].

3.3. MC540 binding to charged membranes

The negatively charged lipid DOPS was incorporated into DOPC membranes to study the effect on MC540 binding. The image in Fig. 4a shows MC540 associating into DOPC bilayers with 0, 5, 15, and 25 mol % DOPS. The quantitative fluorescence of each bilayer patch is shown in the plot in Fig. 4b. The data represent the average of three independent experiments with each MLBA containing two identical bilayer patches prepared from the same SUV stock solution. The graph shows that with increasing negative surface charge density of the membranes there is a correlated decrease in the fluorescence of the anionic dye with a 10%, 24% and 40% reduction in MC540 binding when 5, 15 and 25 mol% DOPS is incorporated into DOPC membranes, respectively. These results support previous studies which found that negatively charged membranes decreases the absorbance and fluorescence of the dye [14,15]. These observations could be explained by the negatively charged membrane repelling MC540 monomers from penetrating deep into the membrane as well as changing the amount of dye binding. However, MC540's fluorescence yield is dependent on its surrounding environment and therefore, the fluorescence intensity is not entirely related to the amount of MC540 binding to the lipid bilayers.

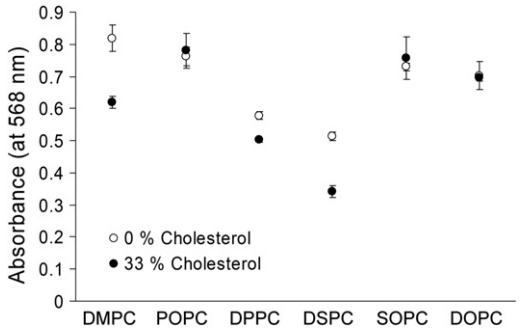

Fig. 3. Absorbance of MC540 monomer bound to different lipid SUVs with and without cholesterol. The data points represent the average values obtained from three liposome solutions prepared from the same SUV stock solution.

Since fluorescence measurements do not quantify the surface concentration of MC540 molecules at the bilayer surface, surface pressure measurements were performed in attempts to correlate the observed changes in MC540 fluorescence to the surface concentration of the dye. Surface pressure measurements are a convenient technique for providing information on molecular adsorption to lipid monolayers [37–39]. The amount of MC540 incorporating into a DOPC monolayer containing varying DOPS concentrations was measured by recording the change in surface pressure with respect to the bulk concentration of MC540 (Fig. 4c). To determine the surface excess concentration (Γ) from the surface pressure data the Gibbs adsorption equation for an electrolyte was used [40,41]:

$$\Gamma_{MC540} = \frac{1}{2RT}\left(\frac{\partial \pi}{\partial \ln C_b}\right) \quad (1)$$

where C_b is the bulk concentration of MC540, Γ_{MC540} is the surface excess of MC540, and π is the surface pressure. By assuming a Langmuir adsorption process it is possible to obtain an integral form of the surface pressure as a function of bulk concentration, described by:

$$\pi = \Gamma_{max} 2RT \ln(1 + K_a C_b) \quad (2)$$

where Γ_{max} is the maximum surface excess concentration of MC540, R is the molar gas constant, T is temperature and K_a is the binding constant. Eq. 2 was used to fit the data in Fig. 4c and to determine the binding affinities of MC540 and Γ_{max}. The K_a for MC540 binding to a DOPC monolayers with 0%, 5%, 15%, and 25% DOPS was determined to be 56 ± 6, 11 ± 2, 10 ± 4 and 6 ± 1 μM^{-1}, respectively. The decrease in K_a with increasing DOPS means a less favorable free-energy of adsorption of MC540 to the membrane as the PS concentration is increased. The surface excess concentration of MC540 (Γ_{MC540}) as a function of the bulk concentration of the dye was determined using the Langmuir isotherm using the values of Γ_{max} and K_a determined from the fits to the data in Fig. 4c using Eq. 2, and are plotted in Fig. 4d. The maximum surface excess concentration was found to increase in the order 34 ± 2, 45 ± 4, 62 ± 1 and 64 ± 4 pmol cm^{-2} with increasing concentration of DOPS from 0 to 25 mol%. An inverse relationship is observed between K_a and Γ_{max}. However, a direct correlation between K_a and Γ_{max} is not necessarily expected because K_a describes the fractional surface coverage with respect to the bulk concentration and therefore does not depend on the absolute concentration (Γ_{max}) in the membrane. The fluorescence intensities of MC540 from the MLBA reported in Fig. 4b were related to the surface excess concentration of dye (at $C_b = 18$ μM) in Fig. 4e. Fig. 4e shows an inverse relationship between the fluorescence signal and the surface concentration of the dye obtained from surface pressure measurements. This could be explained by the binding mechanism of MC540 in response to a change in the electric field applied across a membrane. It was first proposed by Ross et al. [42], who performed MC540 binding experiments on squid axons, that a change in the applied electric field would shift the monomer–dimer equilibrium constant. The authors observed that when squid nerve axons were depolarized there was an increase in absorbance around 570 nm and

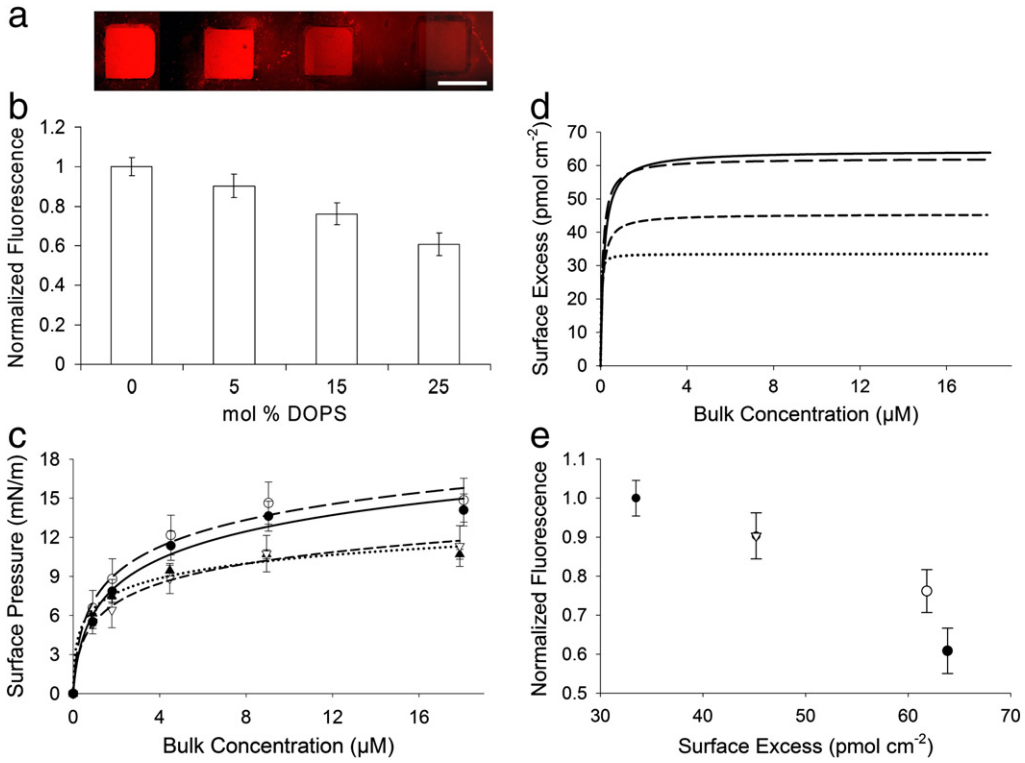

Fig. 4. (a) Fluorescence image of MLBA prepared with DOPC containing 0, 5, 15 and 25 mol% DOPS from left to right. (b) Fluorescence intensity measured for the different bilayer compositions with the fluorescence normalized to the signal obtained from the pure DOPC bilayers. The data represent the average of three independent experiments performed on freshly prepared MLBAs with each MLBA having two reproduced bilayer spots of identical lipid compositions. (c) Change in surface pressure with increasing bulk concentrations of MC540 for increasing DOPS concentration of 0 (▲), 5 (▽), 15 (○) and 25 (●) mol%. The lines represent the fits to the data for 0 (------), 5 (– – – – –), 15 (— —) and 25 (————) mol% DOPS using Eq. 2 with the calculated MC540 surface excess as function of bulk concentration shown in (d). (e) Fluorescence from the MLBA as a function of surface excess with respect to DOPS concentration. The symbols are the same as those represented in (c). Scale bar represents 400 μm.

decrease at 510 nm which corresponds to an increased concentration of monomers and decreased occurrence of dimers supporting the shift in equilibrium toward the monomers. When MC540 dimerizes its quantum efficiency will be drastically reduced in comparison to its monomeric form which explained the changes in the absorbance observed by Ross et al. [42]. This hypothesis was also later confirmed by fluorescence polarization experiments on hemispherical bilayer membranes performed by Dragsten and Webb [43].This interpretation is also consistent with absorbance studies of MC540 binding to increasingly charged liposomes performed by Mateašik et al. [14]. They found that the absorbance maxima of MC540 monomers decreased as the surface charge increased with a change in the monomer–dimer equilibrium toward a decreased occurrence of monomers. There was also an accompanied blue shift in the monomer adsorption peak from 568 to 560 nm when 20% of the negatively charged lipid analog was incorporated into the liposomes. This is indicative of MC540 monomers residing in a slightly more hydrophilic environment. A new absorbance was also observed at 530 nm which was suggested to be the formation of aggregates on the charged membrane. The results presented in Fig. 4 are consistent with these previous observations.

4. Conclusion

MLBAs were used for small molecule–membrane binding experiments to rapidly study the effect different membrane parameters have on MC540 binding. It has been shown that MC540 can distinguish between gel and liquid phase membranes, with measured MC540 fluorescence intensity being twice as great in bilayer spots containing lipids above their T_m. In the presence of cholesterol, there was a significant decrease in the fluorescence intensity in the saturated DMPC, DPPC and DSPC lipid membranes while cholesterol had a minimal impact on MC540 binding to the unsaturated (DOPC) and mixed chain (SOPC and POPC) lipids. The MC540 fluorescence obtained from the MLBAs for the MC540 binding experiments correlated extremely well to experiments using solution phase liposome. When DOPC membranes were doped with the negatively charged DOPS lipids, MC540 fluorescence was attenuated due to a shift in the monomer–dimer ratio. The reproducibility of the binding experiment with the MLBAs was very good with on average <9% relative error for the three separate experiments. The MLBAs presented here provide a promising platform for small molecule–membrane studies with important applications in areas such as pharmaceutical drug discovery and delivery. For example, coupling lipid bilayer arrays with a label-free imaging technique will provide a high-throughput noninvasive method for investigating how a particular drug interacts with specific membrane components, assisting in the discovery of potential targets for drug binding.

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