Published in final edited form as:

Anal Chem. 2011 August 1; 83(15): 5979–5988. doi:10.1021/ac2009614.

High-Throughput Screening of Drug-Lipid Membrane Interactions via Counter-Propagating Second Harmonic Generation Imaging

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Abstract

Here we report the use of counter-propagating second harmonic generation (SHG) to image the interactions between the local anesthetic tetracaine and a multi-component planar supported lipid bilayer array in a label-free manner. The lipid bilayer arrays, prepared using a 3D continuous flow microspotter, allow the effects of lipid phase and cholesterol content on tetracaine binding to be examined simultaneously. SHG images show that tetracaine has a higher binding affinity to liquid-crystalline phase lipids than to solid-gel phase lipids. The presence of 28 mol % cholesterol decreased the binding affinity of tetracaine to bilayers composed of the mixed chain lipid, 1-steroyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and the saturated lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) while having no effect on di-unsaturated 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). The maximum surface excess of tetracaine increases with the degree of unsaturation of the phospholipids and decreases with cholesterol in the lipid bilayers. The paper demonstrates that SHG imaging is a sensitive technique that can directly image and quantitatively measure the association of a drug to a multi-component lipid bilayer array, providing a high-throughput means to assess drug-membrane interactions.

INTRODUCTION

Most drugs on the market target proteins embedded within cell membranes.¹ As a result, any interaction between the drug and lipids surrounding the targeted proteins may influence the drug's activity, selectivity and toxicity.^{2,3} Consequently, several extensive studies have examined various interactions between drugs and lipid membranes including the effect of lipid membrane structure and composition on drug partitioning and the influence of the drug on membrane physical properties and structures. These studies typically utilize artificial model membranes such as liposomes or planar supported lipid bilayers (PSLBs).^{4–6} PSLBs provide an attractive platform for performing biological assays due to their resistance to nonspecific biomolecule adsorption and non-fouling nature. Furthermore, the use of PSLBs in measuring drug-membrane interactions offers several advantages over solution phase liposomes, including smaller sample solution volumes, ease of preparation, and, most importantly, the ability to be incorporated into high-throughput screening methods. For example, PSLBs can be patterned on surfaces to produce micro-patterned lipid bilayer arrays (MLBAs) which can be created by micro-contact printing,⁷ deep ultraviolet photolithography (UV),⁸ pre-patterned substrates,⁹ a combination of pre-patterned substrates with a robotic spotter systems,¹⁰ and 2D and 3D microfluidics.^{11,12} Among these techniques, 3D microfluidics has proven to be a promising method for making patterned

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PSLBs.¹² The microfluidic system delivers fluid to a discrete region on a substrate through a printhead with individually addressable microchannels within a polydimethylsiloxane (PDMS) substrate. This allows different lipid vesicle solutions to be introduced to the microchannels, thus forming an array of different PSLBs on a single substrate. The microfluidic system does not require the use of a pre-patterned substrate because the PSLBs are effectively “corralled” into discrete micro-sized domains by the residual PDMS deposited on the substrate from the printhead. Prevention of lipid spreading can also be enhanced by introducing bovine serum albumin or polyelectrolytes after the deposition step to bind to the residual hydrophobic PDMS.¹³

Several techniques have been successfully employed to detect drug-membrane interactions including UV-Vis,¹⁴ NMR,^{15,16} vibrational spectroscopies (IR, Raman),^{17,18} and fluorescence.^{19,20} However, most of these methods are not suitable to study MLBAs. Vibrational spectroscopic techniques require signal enhancement such as attenuated total internal reflection (ATR) IR²¹ or surface enhanced Raman scattering (SERS)²² if they are to be used for surface detection. Intrinsic fluorescence of drug molecules can be a useful probe if present, but self-quenching can influence the response at high concentrations of the drug.²³ In the absence of intrinsic fluorescence, the covalent attachment of a fluorescent label to the drug is possible, however such an alteration would severely change the drug's structure and more likely than not its biological activity.²⁴ The label-free technique of surface plasmon resonance has also been applied to detect drug-lipid membrane binding.^{25,26} In these studies, liposomes attached to a lipophilic functionalized gold surface were used as model membranes for drug binding.

We have recently shown that the surface specific non-linear optical spectroscopic technique ultraviolet-visible sum frequency generation (UV-Vis SFG) is an ultra-sensitive and powerful technique in the detection of drug-membrane interactions.²⁷ This method is based on the enhancement of the UV-Vis SFG intensity when the SFG frequency is resonant with an electronic transition of the molecule of interest. This technique allowed a direct measurement of the equilibrium association constant of ibuprofen, azithromycin, tolinaftate and tetracaine into a lipid membrane.²⁷ In addition, the drug concentrations in the membrane were accessible using UV-Vis SFG in combination with the bulk partition coefficients.²⁷ In the present work, the complimentary technique of counter-propagating second harmonic generation (SHG) was used to measure drug-membrane interactions. Counter-propagating SHG has previously been employed to measure the association of the peptide mellitin²⁸ and chiral molecules ((R)-(+)-1,1'-bi-2-naphthol (RBN) and (S)-(+)-1,1'-bi-2-naphthol (SBN)) into lipid bilayers.²⁹ It has also been successfully applied to image chiral RBN and SBN adsorbed to a patterned lipid bilayer.³⁰ In this study, SHG imaging is coupled with MLBAs to image drug-lipid membrane interactions directly for the first time in a high-throughput manner.

Counter-propagating SHG involves spatially and temporally overlapping two input beams at frequency ω from opposite directions at the sample of interest, yielding photons at twice the input frequency, 2ω . The theory of counter-propagating SHG has been described elsewhere.³¹ Briefly, the SHG intensity is proportional to the resonant portion of the second-order susceptibility tensor $\chi_{ijk}^{(2)}$, which is given by:

$$I_{SHG} \propto \left| \chi_{ijk}^{(2)} \right|_R^2 \propto \left| N \sum_{a,b,c} \frac{\langle a | \mu_i | c \rangle \langle a | \mu_j | b \rangle \langle b | \mu_k | c \rangle}{(2h\omega - E_{ca} - i\Gamma_{ca})(h\omega - E_{bc} - i\Gamma_{bc})} \right|^2 \quad (1)$$

where N is the surface density of molecules, h is Planck's constant, a , b and c represent the initial, intermediate and final states, respectively, μ is the Cartesian coordinate dipole

operator, and Γ represents the linewidth of the transition.³² The indices on $\chi_{ijk}^{(2)}$ denote the input (j,k) and output (i) fields which can assume any of the three Cartesian coordinates (x,y , or z). Examination of equation 1 shows that an increase in SHG signal will be observed when the incident, ω , or SHG, 2ω , frequency is in resonance with an electronic transition of the molecules at the interface. This can be used as an intrinsic probe to detect the presence of drug molecules in the lipid membrane if the drug has an electronic transition at the frequency of the incident or the SHG light.

The SHG process, under the electric-dipole approximation, is not allowed in the bulk of centrosymmetric medium but allowed at a surface or interfacial region between two centrosymmetric media due to the break in symmetry of the bulk phases, making SHG a surface specific technique. It should be noted that the electric-quadrupole contribution from the bulk medium can contribute to the overall SHG signal.^{33,34} However, its contribution can be neglected if the interfacial layer is highly symmetric³³ and/or the SHG frequency is in resonance with the electric-dipole allowed transitions of the interfacial layers.³⁴ In these cases, the SHG signal should be governed principally by the electric-dipole contribution from the interfacial layer. The local anesthetic tetracaine (extinction coefficient spectrum and molecular structure are shown in the Supporting information) was chosen as a model drug. In the study presented here, the SHG wavelength at 266 nm is in resonant with the $\pi \rightarrow \pi^*$ transition of the benzene ring of tetracaine, as a result, the SHG intensity is dominated by the dipole allowed process, described by equation 1.

The aim of the present work is to show that counter-propagating SHG imaging combined with MLBAs can be used a label-free technique to visualize tetracaine-membrane interactions in a high-throughput manner. Tetracaine's anesthetic activity mainly involves blocking Na^+ influx through Na^+ channels of the nerve axonal membranes.³⁵ It is still unclear if the anesthetic mechanism depends on the direct interaction between the drug and its target protein or on the passive interaction between the drug and the surrounding membrane.³⁶⁻³⁸ In an attempt to elucidate this mechanism, several extensive studies investigating tetracaine-membrane interactions have been conducted. Specifically, the effects of tetracaine on the structure and dynamics of phospholipids were studied by FTIR³⁹ and its effects on the phase behavior and thermodynamics of phospholipids were studied by differential scanning calorimetry.⁴⁰ The location of tetracaine in phospholipid vesicles has also been studied by fluorescence quenching and resonance energy transfer.⁴¹ Using the intrinsic fluorescence of tetracaine, the partitioning of tetracaine into lipid membranes was measured and found to depend on the physical state and composition of the lipids.²⁰ Additionally, the ionization state of tetracaine was reported to affect its interaction with lipid membranes. It was found that neutral tetracaine partitions more strongly and deeply into zwitterionic phospholipids than the protonated tetracaine.^{20,39} It is important to mention that these studies were performed using lipid vesicles, allowing only one lipid component to be studied in each experiment.

As a proof-of-principle investigation, we have examined the effects of the lipid phase state (solid-gel and liquid-crystalline) and membrane composition (saturated, unsaturated lipids and cholesterol content) on tetracaine binding using SHG microscopy. Using the MLBAs, we prepared multiple lipid membrane components with different phase states and cholesterol content on a single substrate allowing for simultaneous investigation of influence of these factors on tetracaine binding. The results were found to correlate with the literature reports, validating the SHG imaging technique as a label-free and high-throughput method to detect drug-membrane interactions. Moreover, the study of tetracaine binding to the unsaturated lipids, which has not been previously published, reveals the crucial role that the

phospholipid unsaturation plays in the binding affinity and surface concentration of tetracaine.

EXPERIMENTAL SECTION

Materials used in the study and preparation of MLBA are described in the Supporting Information.

SHG Imaging

Counter-propagating SHG imaging was used here for the detection of the tetracaine-lipid membrane interactions. The 2nd harmonic output (532 nm) of a Nd:YAG laser (Continuum, Surelite I, 20 Hz, 7ns) with an energy of 22 mJ/pulse was directed on the surface of a quartz prism under total internal reflection. A laser beam diameter of 3.5 mm was used. The reflected beam from the surface is reflected back upon itself using a 532 nm mirror. This results in two incident beams at the same frequency arriving at the surface from opposite directions, both with an incident angle of 67° to ensure total internal reflection, a schematic of the optical arrangement can be found elsewhere.³⁰ A slight displacement of the reflected beam is introduced in order to prevent the redirected beam from entering the laser cavity.

The resulting SHG is emitted at 266 nm along the surface normal and optically filtered to remove any scattered visible light prior to imaging. Imaging of the SHG signal was achieved using a modified Olympus microscope³⁰ with a 3× UV objective (Optics for Research). An image intensifier (Phototek) coupled to a CCD camera (Roper Scientific, 512 × 512 pixels) was used for image acquisition. An integration time of 1200 seconds was used to collect all SHG images. SHG images were analyzed and false color applied using the software package image J (<http://rsbweb.nih.gov/ij/index.html>). Each SHG image was first background corrected by subtracting the minimum pixel intensity from the image. The image was then flat-field corrected using the Image J macro available at the Integrated Microscopy Core Facility at the University of Chicago (http://digital.bsd.uchicago.edu/%5Cimagej_macros.html). The SHG images were normalized to allow for a direct comparison between the images recorded from different experiments. The normalization procedure was described in the Supporting Information.

Equilibrium Binding Affinity of Tetracaine

The SHG images were collected at various bulk concentrations of tetracaine, increasing from low to high. Each concentration was allowed to incubate for at least 30 minutes before imaging to insure tetracaine binding to the lipid bilayer had reached equilibrium. The SHG intensity for each lipid component was plotted versus tetracaine concentration. The equilibrium binding affinity was then extracted by fitting the data to either a Langmuir or Frumkin adsorption isotherm which was given in the Supporting information.

RESULTS AND DISCUSSION

MLBAs composed of eight different lipid components were used to examine the effect of lipid physical state and the presence of cholesterol on tetracaine binding in PBS buffer (100 mM NaCl and 50 mM Na₂HPO₄) at physiological pH 7.4. The pK_a of tetracaine in an aqueous solution is ~ 8.48²⁰ so the tetracaine used in this study is mostly positively charged at pH 7.4. Four primary lipid components, DOPC, SOPC, DMPC and DPPC, were examined with and without 28 mol % cholesterol. These different lipid compositions were deposited in a 5 column, 3 row array for a total of 15 individual spots as shown in Figure 1. In each column of the array, the primary lipid component was prepared in duplicate while the same lipid containing cholesterol was deposited once. The spot at 1C is the control where no lipid was deposited. The positions of the lipid bilayer spots were kept consistent throughout the

study. It should be noted that the two DOPC spots in column 1 (1A and 1B) were not used in the calculation. These two spots, labeled with a fluorescent dye rhodamine (Rh), were used to locate the position of the lipid bilayer spots in the array before imaging with the microscope.

Effect of lipid physical state on tetracaine binding

The effect of the lipid physical state on tetracaine binding was examined by comparing the relative adsorption of tetracaine to DOPC, SOPC, DMPC and DPPC bilayers which correspond to the lipid spots at the following positions in the array: 2A, 2B (DOPC, 18:1), 3A, 3B (SOPC, 18:0–18:1), 4A, 4D (DMPC, 14:0) and 5A, 5B (DPPC, 16:0), shown in Figure 1. The numbers in the parenthesis represent the number of carbons in the acyl chain followed by the number of double bonds in the acyl chain. The experiments examining the effect of lipid physical state were conducted at 18°C, 27°C and 46°C. At 18°C, DOPC and SOPC are in the liquid-crystalline (l_c) phase while DMPC and DPPC are in the solid-gel phase. When the temperature increases to 27°C, DOPC, SOPC and DMPC are in the l_c phase while DPPC is in the gel phase. At 46°C, all the lipids are in the l_c phase.

The effect of the lipid physical state was first investigated at 18°C. The SHG images of tetracaine binding to DOPC, SOPC, DMPC and DPPC bilayer spots at this temperature are represented in Figure 1. As the concentration is increased, more tetracaine associates into the lipid bilayers causing the intensity at each lipid bilayer spot to increase. The control spot (1C) remains unchanged over the concentration range confirming the specific binding of tetracaine to the lipid bilayers. The binding curves of tetracaine to DOPC, SOPC, DMPC and DPPC bilayers are shown in Figure 2. The data presented in the figure are the average of four bilayer spots from two independent arrays (two spots in each array). These data were best fit by the Langmuir isotherm model and the extracted equilibrium binding affinities are given in Table 1. We can assume the formation of a monolayer of tetracaine in the lipid bilayer based on several factors. First, it has been previously proposed that tetracaine is present mainly in the outer leaflet of the lipid bilayer where the positive dimethylammonium group of tetracaine lies close to the negative phosphate group of the lipid interfacial region.⁴² This proposed bilayer location is supported by a NMR study showing that no flip-flop of tetracaine across the lipid bilayer occurs.⁴² In addition, if tetracaine is present in both the inner and outer leaflets, the dipole moments of tetracaine in the two leaflets would cancel out, resulting in no net change in the SHG signal. In this study, we observed an increase in the SHG intensity with increasing tetracaine concentration up to 3.2 mM, suggesting that tetracaine binds as a monolayer in one leaflet of the bilayer. However, at concentrations above 3.2 mM, the intensity began to decrease which could be attributed to the binding of tetracaine into the both leaflets of the bilayers. Although the decrease in the SHG intensity can be ascribed to the dipole moment cancellation of tetracaine, it is possible that the decrease in the intensity is due to the disruption of the lipid bilayer caused by high concentration of tetracaine.⁴³ Nonetheless, the binding data exhibit one plateau at saturation as opposed to multiple plateaus which indicates that an individual adsorbed tetracaine layer is formed in the lipid bilayer. Collectively, this information allows us to infer that the measured SHG signal in these experiments is from a monolayer of tetracaine inserting into one leaflet of the lipid bilayer.

Tetracaine binding to DMPC and DPPC bilayer spots at 18°C does not reach saturation over the concentration range used in this study, as seen in Figure 2, and we were not able to obtain the binding affinity of tetracaine to gel phase DMPC and DPPC. Although we cannot quantitatively assess tetracaine binding we can qualitatively observe that the binding affinity of tetracaine to DMPC and DPPC at this temperature would be the lowest of the lipids in this study.

The data in Table 1 shows that the binding affinity, K_a , of tetracaine at 18°C to the lipids in this study increases in the order DPPC $\approx$ DMPC < SOPC $\approx$ DOPC. At 18°C, DOPC and SOPC are in the l_c phase while DMPC and DPPC are in the gel phase. The higher K_a of tetracaine to DOPC and SOPC suggests that tetracaine associates into the l_c phase lipids to a greater extent than into the gel phase lipids DMPC and DPPC. This is due to the fact that lipids in the l_c phase are more loosely packed ($\sim 60\text{--}75 \text{ \AA}^2/\text{molecule}^{44}$) which allows tetracaine to partition into the bilayer more easily than the tightly packed solid-gel phase ($\sim 45\text{--}50 \text{ \AA}^2/\text{molecule}^{44}$). The association behavior of tetracaine reported here is consistent with literature reports that tetracaine prefers to incorporate into more into l_c DMPC^{20,41} and DPPC²⁰ liposomes over those lipids in the gel phase. This same trend has been observed for other local anesthetic drugs, dibucaine, bupivacaine and lidocaine.⁴⁵

To further examine the impact of lipid phase on tetracaine binding, the same experiment was performed at a higher temperature, 27°C. The SHG images of tetracaine binding to the MLBA and the binding curves at this temperature are shown in Figures 1 and 2, respectively. The binding data at 27°C presented in the Figure 2 are the average of six bilayer spots from three independent arrays. Upon increasing the temperature from 18°C to 27°C, DMPC goes from the gel phase to the l_c phase which allows tetracaine binding to DMPC to significantly increases. It should be noted that tetracaine binding to DPPC bilayer spots at 27°C still does not reach saturation (Figure 2) and we can deduce that the binding affinity of tetracaine to DPPC at this temperature would be the lowest relative to the other lipids in the study. The K_a of tetracaine to the DOPC, SOPC, DMPC and DPPC at 27°C is listed in Table 1 and increases in the order DPPC < DPMC < DOPC $\approx$ SOPC.

Similarly, when the temperature is increased to 46°C and DPPC enters the fluid phase there is an increase in tetracaine binding as illustrated in Figures 1 and 2. The K_a of tetracaine to DOPC, SOPC, DMPC and DPPC at 46°C is listed in Table 1. The K_a increases in the order DPPC < DMPC < DOPC $\approx$ SOPC. Interestingly, the K_a of tetracaine binding to the unsaturated lipids (DOPC and SOPC) is much greater than for the saturated lipids (DMPC and DPPC) when all the lipids are in the fluid phase. This could be related to the difference in the molecular packing of the unsaturated and saturated lipids. The *cis* double bond in the unsaturated lipids results in a larger area per molecule ($\sim 75 \text{ \AA}^2/\text{molecule}$ for l_c phase DOPC⁴⁴) as compared to the saturated lipids ($\sim 67 \text{ \AA}^2/\text{molecule}$ for l_c DPPC⁴⁴). Accordingly, the more loosely packed unsaturated lipids may allow more tetracaine binding. Surprisingly, very little work regarding tetracaine or other local anesthetics-lipid membrane interactions has been reported in literature using unsaturated lipids despite the fact that the lipids in cellular membranes are predominantly unsaturated.⁴⁶ Instead, saturated DMPC and DPPC are commonly used in tetracaine and other local anesthetic membrane studies.^{16,20,47}

A significant reduction in tetracaine binding to DOPC and SOPC was observed when the temperature increases from 18°C to 27°C and 46°C. The decrease in drug partitioning into fluid lipids at elevated temperatures was also reported by Wright *et al.* who found teniposide, an anticancer agent, partitioning into DOPC gradually decreases with increasing temperature above the lipid's phase transition temperature.⁴⁸ Liu and coworkers reported the effect of temperature on the decrease in the partition coefficients of beta blockers such as propranolol, alprenolol, and pindolol.⁴⁹ Additionally, a decrease in the partitioning of dopamine antagonists into fluid lipid bilayers with increasing temperature was obtained by Sarmento *et al.*⁵⁰ A possible explanation is that the water solubility of hydrophobic drugs increases with temperature resulting in the decrease in the membrane partitioning.^{48,49} Another reason could be related to the conformational change in the lipid head group induced by temperature.^{48,50,51} As the temperature increases, the positively charged amine of the phosphocholine head group normally staying parallel to the lipid bilayer surface moves deeper into the hydrophobic core of the bilayer,⁵¹ where tetracaine is located. The

electrostatic repulsion between the amine group and protonated tetracaine could repel tetracaine from the lipid bilayer.

A quantitative comparison can be made between our measured binding affinities of tetracaine and the values reported in previous studies. Using UV-Vis SFG spectroscopy, we recently measured an affinity of $5.1 \pm 0.1 \times 10^4$ for tetracaine binding to a DOPC bilayer (at room temperature).^{27,52} In this work, we obtained a slightly smaller value ($1.3 \pm 0.1 \times 10^4$) for tetracaine associated into a DOPC bilayer at 27°C. Additionally, the adsorption models used to fit the data in the two studies are different. The Frumkin model fit best to the data in the previous study with a g value of -1.51 demonstrating the electrostatic repulsion between the charged tetracaine molecules. However, we have found that the Langmuir model was the statistically better fit to the data obtained in the current study. The difference between the Frumkin and Langmuir models is most pronounced at surface coverage below saturation. At low surface coverage, i.e. at low bulk tetracaine concentrations, the low signal/noise ratio in the SHG imaging data, is not sufficient for the nonlinear regression to identify the Frumkin behavior in the isotherm. On the other hand, the higher signal/noise ratio in the UV-Vis SFG experiment allows the Frumkin behavior in the isotherm to be statistically discerned as determined from a f -test analysis of the two models. There are several factors that contribute to the lower signal/noise ratio in the SHG imaging data. The most significant difference between the two experiments is the illuminated sample area. For the UV-Vis SFG experiments a continuous bilayer was examined with an illumination area of approximately 18 mm^2 compared to the 0.16 mm^2 dimensions of the lipid spots in the current study. As the SHG signal is proportional to the surface density squared, the two-order of magnitude decrease in the surface area of the lipid bilayer spots compared to the UV-SFG study will decrease the SHG signal by four orders of magnitude. Additionally, the sensitivity of the photocathode of the solar blind photomultiplier tube ($\sim 60 \text{ mA/W}$) used to collect the UV-Vis SFG signals is twice that of the image intensifier ($\sim 30 \text{ mA/W}$) used in the SHG imaging; thus, the measured SHG intensity is further reduced by a factor of two. In another study, Zhang *et al.* obtained a partition coefficient of $(1.18 \pm 0.2) \times 10^4$ for DMPC (at 30°C) and $(1.31 \pm 0.13) \times 10^4$ for DPPC (at 45°C) at pH 5.5 (tetracaine is completely protonated).²⁰ Here we measured a binding affinity of $(0.53 \pm 0.07) \times 10^4 \text{ M}^{-1}$ and $(0.24 \pm 0.08) \times 10^4 \text{ M}^{-1}$ for fluid DMPC (at 27°C) and DPPC (at 46°C), respectively. The difference between these values can be related to the models used to describe the interactions between tetracaine and lipid membrane. Zhang and coworkers employed a partitioning equilibrium model to study tetracaine and lipid membrane interaction with an unlimited number of binding sites in the membrane. The data presented here were described by the Langmuir model which has been proposed to be the more suitable model to characterize tetracaine-lipid membrane interaction.⁴¹ Additionally, the 3D structure of the lipid vesicles used in Zhang's study could be more flexible to accommodate more tetracaine molecules as compared to 2D structure of the planar supported lipid bilayers used here.

In addition to retrieving the binding affinity of tetracaine to lipid membranes, the maximum surface excess Γ_{max} of tetracaine in the membrane is assessable with the use of the SHG binding curves and the bulk partition coefficient of tetracaine as described previously.²⁷ Knowledge of the surface excess of the drug in the membrane is essential in determining the bioavailability of the drug to the targeted protein embedded in the membrane. It is important to point out that this information cannot be determined solely from the partition coefficient data. The calculated Γ_{max} of tetracaine in DOPC, SOPC, DMPC and DPPC bilayers at 18°C, 27°C and 46°C is given in Table 1. In general, the Γ_{max} of tetracaine in the unsaturated lipid bilayers (DOPC and SOPC) is greater than in the saturated lipids bilayers (DMPC and DPPC). This is consistent with the higher binding affinity of tetracaine to the unsaturated lipids as compared to the saturated lipids. This behavior illustrates the correlation between the presences of the double bonds in the lipid acyl chains and the subsequent increase in the

area per lipid molecule resulting in more tetracaine association. The effect of temperature on the Γ_{max} of tetracaine, however, is not significant. As the temperature increases from 18°C to 27°C and 46°C, the Γ_{max} of tetracaine in DOPC and SOPC does not significantly change, in contrast to the considerable reduction in the binding affinity of tetracaine to these lipids. This suggests that the molecular packing of the lipid membrane primarily governs the amount of tetracaine adsorbed at saturation. When the lipids are in the fluid phase, the maximum surface excess is unaffected by temperature.

Effect of cholesterol on tetracaine binding

The effect of 28 mol % cholesterol on tetracaine binding was investigated using the lipid bilayer spots at the following positions in the array: 2C (DOPC + CHO), 3C (SOPC + CHO), 4C (DMPC + CHO) and 5C (DPPC + CHO) at 18°C, 27°C and 46°C (Figure 1). The binding curves for these lipid compositions are shown in Figure 2. The data at 18°C represent the average of two spots from two separate arrays while the data at 27°C and 46°C are the average of three spots from three arrays. The binding affinities at 18°C, 27°C and 46°C are given in Tables 1. It should be noted that the binding affinities of tetracaine to DMPC in the presence of cholesterol at 18°C and DPPC in the presence of cholesterol at the temperature range used in the study are not available because the binding does not reach saturation as seen in Figure 2. However, the SHG response from the binding of tetracaine to DMPC in the presence of cholesterol is lower than that of the pure lipid at 18°C. Similarly, the SHG response from the binding of tetracaine to DPPC bilayer containing cholesterol is lower than that of the pure lipid at both 27°C and 46°C. Accordingly, we can infer that cholesterol reduces the binding of tetracaine into DMPC bilayer at 18°C and DPPC bilayer at both 27°C and 46°C.

The incorporation of 28 mol % cholesterol into the lipid bilayers does not have any effect on tetracaine binding to DOPC but does decrease tetracaine binding to SOPC, DMPC and DPPC in the temperature range used here. In particular, cholesterol incorporated into SOPC bilayers decreases the K_a of tetracaine by 68% at 18°C, 40% at 27°C and 10% at 46°C. In DMPC, the K_a of tetracaine in the presence of cholesterol is reduced by 42% and 60% at 27°C and 46°C, respectively. The reduction in tetracaine binding caused by cholesterol has been previously reported.^{16,20} Auger and coworkers found that the addition of 30 mol % cholesterol into fluid DMPC decreases the partition coefficient of tetracaine in both the neutral and charged forms.¹⁶ Zhang *et al.* observed a decrease on the partition coefficient of tetracaine to DPPC bilayer below and above its phase transition temperature with 28 mol % cholesterol in the bilayer.²⁰ The same effect has also been observed for other small molecules partitioning into lipid membranes.^{48,49,53}

The addition of 28 mol % cholesterol, in the temperature range used in this study (18°C – 46°C), puts DOPC + CHO and SOPC + CHO in a liquid ordered phase (l_o),^{54,55} which exhibits the same fluidity as the l_c phase but possesses highly ordered acyl chains similar to those in the condensed phase.⁵⁶ On the other hand, for the binary mixtures of DMPC + CHO and DPPC + CHO, there is a phase coexistence between a solid ordered (s_o) and liquid ordered (l_o) phases below the T_m of the lipid and a liquid disordered (l_d) phase coexists with a liquid ordered (l_o) phase above the T_m .^{57–59} It has been reported that phase segregation occurs between the s_o cholesterol-rich domains and the l_o phospholipid-rich domains, with a domain size in the range of ~ 18 nm^{60,61} to few μm .⁶² Although the l_o and l_d domains do coexist in a binary mixture of phospholipid and cholesterol, there are still unanswered questions regarding phase separation between the l_o phase and l_d phase.^{63–65} The nm- μm domain size in the s_o - l_o phase coexistence region is too small to be visualized in this study as the resolution of the SHG images is limited by the image intensifier to ~14 μm .

The decrease in tetracaine binding caused by the incorporation of cholesterol into the lipid bilayers could be related to the increase in the lipid packing density/decrease in the area per phospholipid induced by cholesterol.⁶⁶⁻⁶⁸ This condensing effect of cholesterol has been previously reported for several lipid + CHO binary mixtures which exhibit a lower area per molecule compared to the average molecular areas of the lipid and cholesterol alone.^{62,69,70} Specifically, Kim and coworker obtained a maximum decrease in the average area of DPPC in the $s_o - l_o$ phase coexistence region in a binary mixture of DPPC and 30 mol % cholesterol at 20°C.⁶⁶ Hung *et al.* observed a reduction in the area per molecule of DMPC when the lipid is in the $l_o - l_d$ phase coexistence region for DPPC and 30 mol % cholesterol at 30°C.⁷¹ Most likely, the more densely packed lipid bilayers created by the presence of cholesterol results in less tetracaine partitioning into the lipid bilayers. In addition, cholesterol is known to reside in the hydrophobic core of the lipid bilayer with the hydroxyl group staying close to the lipid ester carbonyl head group.⁷² Evidence from a NMR study showed that tetracaine is located closer to the interfacial region of DMPC containing 30 mol % cholesterol in the $l_o - l_d$ phases as compared to pure DMPC (l_c).¹⁶ This indicates that cholesterol occupies space between the lipids and could possibly exclude tetracaine from the lipid bilayer resulting in a decrease in tetracaine binding.

One of the models which have been proposed to explain the condensing of cholesterol on the surrounding lipid matrix is the formation of a stoichiometric cholesterol and lipid complex.^{62,73} In the condensed complex, the lipid acyl chains are more ordered as the interaction between cholesterol and the acyl chains facilitates the extension of the acyl chains adjacent to cholesterol indicated by an increase in the membrane thickness,^{69,74} and a decrease in the number of *gauche* rotamers along the acyl chains.⁷⁵ The ordering effect is much more significant on saturated lipids than unsaturated lipids as the proximity between the acyl chains and cholesterol facilitate the interaction between cholesterol and the lipids. In the unsaturated lipid DOPC, the *cis* double bond configuration between C-9 and C-10 forms a bend of 30 degree in the aliphatic chains leading to a conformational mismatch in contact with cholesterol.⁷⁶ Therefore, the ordering effect of cholesterol on DOPC is much less than that on saturated lipids (DMPC, DPPC).^{76,77} This explains why the effect of cholesterol on tetracaine binding to DOPC is insignificant as compared to DMPC. This behavior is further supported by a simulation study performed by Martinez-Seara and coworkers that the ordering effect caused by cholesterol is minimized for DOPC but maximized for fully saturated 1,2-distearoyl-*sn*-glycero-3-phosphocholine DSPC (18:0, same chain length with DOPC).⁷⁸ In the case of the mixed-chain SOPC lipid which contains a saturated *sn*-1 chain and a monounsaturated *sn*-2 chain, an intermediate behavior between the fully saturated lipid (DMPC) and di-unsaturated lipid DOPC was observed in the present study. This observation is supported by the calculation from a recent molecular dynamics work showed that the increase in the lipid bilayer thickness caused by the incorporation of 20 mol % cholesterol is greatest for DSPC, moderate for SOPC and lowest for DOPC as compared to the pure lipid systems.⁷⁹ This study also demonstrated that cholesterol increases the order parameter S , which is used to quantify the ordering of the lipid acyl chain, in the order of DOPC < SOPC < DSPC.⁷⁹

A reduction in the Γ_{max} of tetracaine upon the addition of 28 mol % cholesterol was observed for all the lipids, as listed in Table 1. However, this reduction is not as significant as the decrease in the K_d . In the presence of cholesterol, the Γ_{max} of tetracaine in the unsaturated lipids DOPC and SOPC is much higher than that in the saturated lipid DMPC confirming the important role of the lipid molecular packing in the membrane being the predominate governor of the maximum amount of tetracaine adsorbed to the lipid bilayer.

Thermodynamics of tetracaine binding to MLBAs

In addition to providing information about the binding affinity and maximum surface excess of tetracaine in lipid bilayers, the data presented here can also be used to obtain the thermodynamics of the binding process. The van't Hoff plot shown in Figure 3 was used to determine the enthalpy (slope) and entropy (intercept) for tetracaine binding to the lipid bilayers. These values are given in Table 2 in addition to the calculated free energy. As seen in Table 2, the binding of tetracaine to pure DOPC and SOPC as well as DOPC containing 28 mol % cholesterol (DOPC + CHO) is associated with a large negative change in enthalpy (ΔH) with values ranging from -32 to -34 kJ/mol. This can be attributed to the van der Waals interactions of the nonpolar portion of the drug with the hydrophobic region of the lipid bilayer where tetracaine inserts relative to the aqueous solution phase species.^{80,81} The change in entropy (ΔS) is much smaller (-26 to -33 J/mol K) most likely due to the competing processes of desolvation of tetracaine upon insertion into the membrane (increase in S) and the ordering of water at the interface of the lipid bilayer (decrease in S) due to the presence of the positive charge on tetracaine. It has been reported that the water molecules at the air/water interface become more ordered, i.e. more hydrogen bonded with icelike structure in the presence of charged surfactants at the interface.⁸² The charged surfactants create a large electrostatic field which induces the alignment of the water at the interface.⁸² Similarly, the presence of the protonated tetracaine in the lipid bilayer could increase the surface potential, and thus facilitates the ordering of the water at the interface. The significant contribution from ΔH to the free energy ($\Delta G \sim -23$ kJ/mol) indicates that the binding of tetracaine to the lipid bilayers is largely enthalpy-driven. This finding is consistent with previous studies which show that H dominates the partitioning of small molecules into the lipid membranes.^{80,81,83-85}

Interestingly, the thermodynamics of tetracaine binding to SOPC + CHO are entirely different. The enthalpic contribution is small (-4.6 kJ/mol) while the entropy increases and becomes positive (~ 60 J/mol K). A similar decrease in the enthalpic contribution was reported by Rowe *et al.* for alcohols partitioning into lipid bilayers in the presence of cholesterol, which they attributed to the disruption of the lipid packing by the alcohol.⁸⁶ As previously discussed, cholesterol increases the lipid packing density and order of the SOPC acyl chains greater than those of DOPC, and thus it is likely that tetracaine disrupts the more densely packed SOPC + CHO to a greater extent than DOPC + CHO, causing a larger reduction in the van der Waals interactions between the lipid molecules. This results in a decrease in enthalpy when tetracaine binds to SOPC in the presence of cholesterol. The relatively large increase in entropy observed for tetracaine binding to SOPC + CHO may be explained by a disordering of the lipid acyl chains induced by tetracaine.¹⁶ The ordering of the lipid acyl chains by cholesterol is much more pronounced in SOPC relative to DOPC due to the close proximity of cholesterol to the saturated *sn-1* chains of SOPC. Consequently, when tetracaine binds to the lipids the better ordered SOPC + CHO undergoes a larger entropic increase as the lipid transforms from a relatively ordered state into a disordered one. This larger entropy contribution indicates that the binding of tetracaine to SOPC + CHO is entropy-driven.

We could not obtain the enthalpy and entropy for tetracaine binding to DMPC and DMPC + CHO as the binding affinities at 18°C were not available. However, as previously mentioned we can infer that the binding affinities of tetracaine to DMPC and DMPC + CHO at 18°C would be lower than at 27°C based on the data in Figure 2. This is consistent with an entropy-driven binding process as observed with SOPC + CHO. It is important to note that the previously discussed unsaturated lipids do not go through a phase transition over the temperature range presented here. The introduction of this phase change to DMPC and DMPC + CHO complicates the interpretation of the thermodynamic behavior beyond the scope of the present study.

CONCLUSION

We have demonstrated the use of counter-propagating SHG microscopy for the detection of drug-membrane interactions. The use of a MLBA allowed for the effects of lipid physical state and cholesterol content on tetracaine binding to be investigated simultaneously. The results show that tetracaine binds more strongly into lipid bilayers in the liquid-crystalline phase as compared to the solid-gel phase. Cholesterol reduces the binding affinity of tetracaine to saturated DMPC and DPPC and mixed chain (saturated *sn-1* and monounsaturated *sn-2*) SOPC while having no effect on di-unsaturated DOPC. In addition, the maximum surface excess of tetracaine in the unsaturated lipids is greater than that in the saturated lipids. Furthermore, the binding of tetracaine into DOPC and SOPC bilayers is governed by enthalpic contributions while the entropy dictates the tetracaine binding to DMPC. The presence of cholesterol does not change the thermodynamics of tetracaine binding to DOPC but makes the binding of tetracaine to SOPC become entropy-driven. The study presented here illustrates that SHG imaging can be used to directly image and quantitatively measure the association of a drug molecule into multi-component lipid microarrays without using any extrinsic label. More interestingly, the drug concentration in the membrane is accessible with the use of SHG data and the knowledge of the drug partition coefficient. The signal/noise ratio in the SHG imaging experiments can be improved by using a SHG wavelength in better resonance with the electronic transitions of tetracaine and/or using a more sensitive detector for image acquisition. In addition, the acquisition time can be speeded up with use of a high repetition rate laser. This study opens up further opportunities for the use of SHG imaging in high-through put screening applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Wasatch Microfluidics, Prof. Bruce Gale at the Mechanical Engineering Department, University of Utah for supplying the CFM and Ms. Kathryn Smith for assistance with preparing the MLBAs by the CFM. Thanks also go to the reviewer of the manuscript who suggested the use of a Van't Hoff plot for the thermodynamic analysis of the binding data. The authors also thank the National Institutes of Health (R01-GM068120) for support. Any opinions, findings, conclusions, or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Institutes of Health.

References

- Overington JPA-LB, Hopkins AL. Nature Rev Drug Dis. 2006; 5:993–996.
- Seydel JK. Methods and Principles in Medicinal Chemistry. 2002; 15:217–289.
- Triggle, DJ. Drug-Membrane Interactions. Analysis, Drug Distribution, Modeling. Methods and Principles in Medicinal Chemistry. Seydel, JK.; Wiese, M., editors. Vol. 15. Vol. 46. 2003.
- Chapman D. Langmuir. 1993; 9:39–45.
- Brian AA, McConnell HM. Proc Natl Acad Sci U S A. 1984; 81:6159–6163. [PubMed: 6333027]
- Sackmann E. Science (Washington, D C). 1996; 271:43–48.
- Kung LA, Kam L, Hovis JS, Boxer SG. Langmuir. 2000; 16:6773–6776.
- Yee CK, Amweg ML, Parikh AN. Adv Mater (Weinheim, Ger). 2004; 16:1184–1189.
- Cremer PS, Yang T. J Am Chem Soc. 1999; 121:8130–8131.
- Yamazaki V, Sirenko O, Schafer Robert J, Nguyen L, Gutschmann T, Brade L, Groves Jay T. BMC Biotechnol. 2005; 5:18–28. [PubMed: 15960850]
- Yang T, Jung S-y, Mao H, Cremer PS. Anal Chem. 2001; 73:165–169. [PubMed: 11199961]
- Smith KA, Gale BK, Conboy JC. Anal Chem (Washington, DC, U S). 2008; 80:7980–7987.

13. Burrige KA, Figa MA, Wong JY. *Langmuir*. 2004; 20:10252–10259. [PubMed: 15518521]
14. Custodio JB, Almeida LM, Madeira VM. *Biochem Biophys Res Commun*. 1991; 176:1079–1085. [PubMed: 2039491]
15. Boulanger Y, Schreier S, Smith IC. *Biochemistry*. 1981; 20:6824–6830. [PubMed: 6895602]
16. Auger M, Jarrell HC, Smith ICP. *Biochemistry*. 1988; 27:4660–4667. [PubMed: 3167009]
17. Auger M, Smith ICP, Mantsch HH, Wong PTT. *Biochemistry*. 1990; 29:2008–2015. [PubMed: 2328235]
18. Fox CB, Horton RA, Harris JM. *Anal Chem*. 2006; 78:4918–4924. [PubMed: 16841911]
19. Barghouthi S, Eftink MR. *Biophys Chem*. 1993; 46:13–19. [PubMed: 8443333]
20. Zhang J, Hadlock T, Gent A, Strichartz GR. *Biophys J*. 2007; 92:3988–4001.
21. Arkin IT, Russ WP, Lebendiker M, Schuldiner S. *Biochemistry*. 1996; 35:7233–7238. [PubMed: 8679552]
22. Aoki PHB, Alessio P, De Saja JA, Constantino CJL. *J Raman Spectrosc*. 2010; 41:40–48.
23. Haugland, RP. *Handbook of Fluorescent Probes and Research Chemicals*. 6. Molecular Probes Inc; Eugene, OR: 1996.
24. Heithier H, Ward LD, Cantrill RC, Klein HW, Im MJ, Pollak G, Freeman B, Schiltz E, Peters R, Helmreich EJ. *Biochim Biophys Acta*. 1988; 971:298–306. [PubMed: 2844291]
25. Abdiche YN, Myszkowski DG. *Anal Biochem*. 2004; 328:233–243. [PubMed: 15113702]
26. Nussio Matthew R, Sykes Matthew J, Miners John O, Shapter Joseph G. *Chem Med Chem*. 2007; 2:366–373. [PubMed: 17191292]
27. Nguyen TT, Rembert K, Conboy JC. *J Am Chem Soc*. 2009; 131:1401–1403. [PubMed: 19140762]
28. Kriech MA, Conboy JC. *J Am Chem Soc*. 2003; 125:1148–1149. [PubMed: 12553799]
29. Kriech MA, Conboy JC. *J Opt Soc Am B*. 2004; 21:1013–1022.
30. Kriech MA, Conboy JC. *J Am Chem Soc*. 2005; 127:2834–2835. [PubMed: 15740102]
31. Conboy JC, Kriech MA. *Anal Chim Acta*. 2003; 496:143–153.
32. Shen, YR. *The Principles of Nonlinear Optics*. Wiley; New York: 1984.
33. Shen YR. *Appl Phys B: Lasers Opt*. 1999; 68:295–300.
34. Guyot-Sionnest P, Shen YR. *Phys Rev B Condens Matter*. 1987; 35:4420–4426. [PubMed: 9941996]
35. Roth SH. *Annu Rev Pharmacol Toxicol*. 1979; 19:159–178. [PubMed: 378102]
36. Lee AG. *Nature*. 1976; 262:545–548. [PubMed: 958412]
37. Seeman P. *Pharmacol Rev*. 1972; 24:583–655. [PubMed: 4565956]
38. Ueda I, Kamaya H. *Anesth Analg (N Y)*. 1984; 63:929–945.
39. Auger M, Jarrell HC, Smith IC, Siminovich DJ, Mantsch HH, Wong PT. *Biochemistry*. 1988; 27:6086–6093. [PubMed: 3191108]
40. Boettner M, Winter R. *Biophys J*. 1993; 65:2041–2046. [PubMed: 8298033]
41. Hutterer R, Kramer K, Schneider FW, Hof M. *Chem Phys Lipids*. 1997; 90:11–23.
42. Yeagle PL, Hutton WC, Martin RB. *Biochim Biophys Acta, Biomembr*. 1977; 465:173–178.
43. Kitagawa N, Oda M, Totoki T. *Anesthesiology*. 2004; 100:962–967. [PubMed: 15087634]
44. Marsh, D. *Handbook of Lipid Bilayers*. CRC Press; 1990.
45. Hata T, Sakamoto T, Matsuki H, Kaneshina S. *Colloids Surf, B*. 2001; 22:77–84.
46. Gennis, RB. *Biomembranes: Molecular Structure and Function*. Springer-Verlag; New York: 1989.
47. Barghouthi SA, Puri RK, Eftink MR. *Biophys Chem*. 1993; 46:1–11. [PubMed: 8443332]
48. Wright SE, White JC, Huang L. *Biochim Biophys Acta, Biomembr*. 1990; 1021:105–113.
49. Liu XY, Yang Q, Kamo N, Miyake J. *J Chromatogr, A*. 2001; 913:123–131. [PubMed: 11355804]
50. Sarmento AB, Pedroso de Lima MC, Oliveira CR. *J Pharm Pharmacol*. 1993; 45:601–605. [PubMed: 8105052]
51. Dill KA, Stigter D. *Biochemistry*. 1988; 27:3446–3453. [PubMed: 3390444]
52. Nguyen TT, Rembert K, Conboy JC. *J Am Chem Soc*. 2011; 133:6096–6097.

53. Wisniewska A, Wolnicka-Glubisz A. *Biophys Chem.* 2004; 111:43–52. [PubMed: 15450374]
54. Polozov IV, Gawrisch K. *Biophys J.* 2006; 90:2051–2061. [PubMed: 16387785]
55. Davis JH, Clair JJ, Juhasz J. *Biophys J.* 2009; 96:521–539. [PubMed: 19167302]
56. Ipsen JH, Karlstroem G, Mouritsen OG, Wennerstroem H, Zuckermann MJ. *Biochim Biophys Acta, Biomembr.* 1987; 905:162–172.
57. Vist MR, Davis JH. *Biochemistry.* 1990; 29:451–464. [PubMed: 2302384]
58. Sankaram MB, Thompson TE. *Proc Natl Acad Sci U S A.* 1991; 88:8686–8690. [PubMed: 1656453]
59. Almeida PFF, Vaz WLC, Thompson TE. *Biochemistry.* 1992; 31:6739–6747. [PubMed: 1637810]
60. Huang TH, Lee CWB, Das Gupta SK, Blume A, Griffin RG. *Biochemistry.* 1993; 32:13277–13287. [PubMed: 8241184]
61. Marsh D. *Biochim Biophys Acta, Biomembr.* 2010; 1798:688–699.
62. McConnell HM, Radhakrishnan A. *Biochim Biophys Acta, Biomembr.* 2003; 1610:159–173.
63. Veatch SL, Keller SL. *Biophys J.* 2003; 85:3074–3083. [PubMed: 14581208]
64. Krivanek R, Okoro L, Winter R. *Biophys J.* 2008; 94:3538–3548. [PubMed: 18199673]
65. Almeida PF. *Biophys J.* 2011; 100:420–429. [PubMed: 21244838]
66. Kim K, Kim C, Byun Y. *Langmuir.* 2001; 17:5066–5070.
67. Sabatini K, Mattila J-P, Kinnunen PKJ. *Biophys J.* 2008; 95:2340–2355. [PubMed: 18515391]
68. Dynarowicz-Latka P, Hac-Wydro K. *Colloids Surf, B.* 2004; 37:21–25.
69. de Meyer F, Smit B. *Proc Natl Acad Sci U S A.* 2009; 106:3654–3658. [PubMed: 19225105]
70. Radhakrishnan A, McConnell HM. *J Phys Chem B.* 2002; 106:4755–4762.
71. Hung W-C, Lee M-T, Chen F-Y, Huang HW. *Biophys J.* 2007; 92:3960–3967. [PubMed: 17369407]
72. Worcester DL, Franks NP. *J Mol Biol.* 1976; 100:359–378. [PubMed: 943549]
73. Radhakrishnan A, McConnell HM. *Biophys J.* 1999; 77:1507–1517. [PubMed: 10465761]
74. Pan J, Mills TT, Tristram-Nagle S, Nagle JF. *Phys Rev Lett.* 2008; 100:198103/198101–198103/198104. [PubMed: 18518492]
75. Rog T, Pasenkiewicz-Gierula M, Vattulainen I, Karttunen M. *Biophys J.* 2007; 92:3346–3357. [PubMed: 17293396]
76. Pasenkiewicz-Gierula M, Subczynski WK, Kusumi A. *Biochemistry.* 1990; 29:4059–4069. [PubMed: 2163271]
77. Kusumi A, Subczynski WK, Pasenkiewicz-Gierula M, Hyde JS, Merkle H. *Biochim Biophys Acta, Biomembr.* 1986; 854:307–317.
78. Martinez-Seara H, Rog T, Pasenkiewicz-Gierula M, Vattulainen I, Karttunen M, Reigada R. *Biophys J.* 2008; 95:3295–3305. [PubMed: 18621818]
79. Martinez-Seara H, Rog T, Karttunen M, Vattulainen I, Reigada R. *J Phys Chem B.* 2009; 113:8347–8356. [PubMed: 19469492]
80. Seelig J, Ganz P. *Biochemistry.* 1991; 30:9354–9359. [PubMed: 1832558]
81. Ikonen M, Murtomaeki L, Kontturi K. *Colloids Surf, B.* 2010; 78:275–282.
82. Gragson DE, Richmond GL. *J Am Chem Soc.* 1998; 120:366–375.
83. Wenk MR, Fahr A, Reszka R, Seelig J. *J Pharm Sci.* 1996; 85:228–231. [PubMed: 8683453]
84. Matos C, Lima JLC, Reis S, Lopes A, Bastos M. *Biophys J.* 2004; 86:946–954. [PubMed: 14747330]
85. Baeuerle HD, Seelig J. *Biochemistry.* 1991; 30:7203–7211. [PubMed: 1830218]
86. Rowe ES, Zhang F, Leung TW, Parr JS, Guy PT. *Biochemistry.* 1998; 37:2430–2440. [PubMed: 9485391]

Figure 1.

The normalized SHG images of tetracaine binding to a multi-component lipid bilayer array which contains the following lipid compositions: DOPC (1A, 1B, 2A, 2B); no lipids, control spot (1C) labeled by the white box; DOPC + 28 mol % cholesterol (2C); SOPC (3A, 3B); SOPC + 28 mol % cholesterol (3C); DMPC (4A, 4B); DMPC + 28 mol % (4C); DPPC (5A, 5B); DPPC + 28 mol % cholesterol (5C). Each image represents a different bulk tetracaine concentration: 0 mM (a); 0.05 mM (b); 0.11 mM (c); 0.21 mM (d); 0.42 mM (e); 0.83 mM (f); 1.59 mM (g), and 3.32 mM (h). The images were collected at 18°C (top), 27°C (middle) and 46°C (bottom). Each bilayer patch is approximately 400 μm $\times$ 400 μm .

Figure 2.

The normalized SHG intensities from the images in Figure 1 for the following lipid compositions: DOPC (filled circle), DOPC + 28 mol % cholesterol (open circles) (a); SOPC (filled square), SOPC + 28 mol % cholesterol (open square) (b); DMPC (filled triangle), DMPC + 28 mol % cholesterol (open triangle) (c); DPPC (filled diamond), DPPC + 28 mol % cholesterol (open diamond) (d). The data represent averages for all temperatures: 18°C (top), 27°C (middle) and 46°C (bottom). The lines are fits to the Langmuir adsorption isotherm model for the pure lipids (solid) and membranes containing cholesterol (dash). The error bars represent the standard deviations obtained by averaging two (at 18°C) and three (at 27°C and 46°C) independent bilayer arrays with two spots for the pure lipids and one spot for the lipids containing cholesterol.

Figure 3. Van't Hoff plot for tetracaine binding to various lipid components: DOPC (red filled circle), DOPC + 28 mol % cholesterol (red open circle), SIPC (blue filled square), SIPC + 28 mol % cholesterol (blue open square), DMPC (green filled triangle), DMPC + 28 mol % cholesterol (green open triangle). The lines are the linear fits for the pure lipids (solid) and lipids containing cholesterol (dash).

Table 1

Measured binding affinity (K_a) and maximum surface excess (Γ_{max}) of tetracaine to lipid bilayers of various compositions.

Lipid bilayer	$K_a \times 10^4$ (M ⁻¹)				$\Gamma_{max} \times 10^{12}$ (mole/cm ²)			
	18°C	27°C	46°C	18°C	27°C	46°C	18°C	46°C
DOPC	2.9 ± 0.2	1.3 ± 0.1	0.78 ± 0.08	9.68 ± 0.08	8.63 ± 0.08	8.71 ± 0.08	8.71 ± 0.08	8.71 ± 0.08
DOPC + CHO	2.7 ± 0.5	1.2 ± 0.1	0.76 ± 0.08	9.60 ± 0.16	7.02 ± 0.08	8.23 ± 0.16	8.23 ± 0.16	8.23 ± 0.16
SOPC	2.8 ± 0.5	1.4 ± 0.1	0.83 ± 0.04	10.0 ± 0.16	8.23 ± 0.08	9.11 ± 0.08	9.11 ± 0.08	9.11 ± 0.08
SOPC + CHO	0.89 ± 0.25	0.82 ± 0.06	0.75 ± 0.11	8.39 ± 0.25	7.82 ± 0.16	7.34 ± 0.32	7.34 ± 0.32	7.34 ± 0.32
DMPC	N.A.	0.53 ± 0.07	0.55 ± 0.08	N.A.	6.37 ± 0.16	5.56 ± 0.24	5.56 ± 0.24	5.56 ± 0.24
DMPC + CHO	N.A.	0.31 ± 0.05	0.22 ± 0.05	N.A.	4.60 ± 0.24	5.24 ± 0.40	5.24 ± 0.40	5.24 ± 0.40
DPPC	N.A.	N.A.	0.24 ± 0.08	N.A.	N.A.	6.69 ± 0.73	6.69 ± 0.73	6.69 ± 0.73
DPPC + CHO	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

Table 2

Free energy ΔG , enthalpy ΔH and entropy ΔS of tetracaine binding to lipid bilayers of various compositions.

Lipid bilayer	ΔG^* (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol K)
DOPC	-23.6 ± 0.19	-34.2 ± 10.9	-33.4 ± 36.0
DOPC + CHO	-23.4 ± 0.21	-32.8 ± 14.4	-29.3 ± 47.5
SOPC	-23.8 ± 0.18	-31.9 ± 10.5	-25.6 ± 34.7
SOPC + CHO	-22.5 ± 0.18	-4.58 ± 0.89	59.7 ± 2.94

* determined at 27°C