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Label-Free Detection of Drug-Membrane Association Using Ultraviolet−Visible Sum-Frequency Generation

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Drug−membrane interactions play a crucial role in the pharmacology and activity of drugs.[1,2] The equilibrium of a drug molecule between plasma and the cellular membrane has historically been modeled by bulk phase partitioning, usually between water and 1-octanol.[3] On a fundamental level, the thermodynamics of a lipid membrane, which is comprised of a bilayer of lipids ∼50 Å thick, cannot adequately be modeled by the bulk thermodynamic properties of a homogeneous liquid phase. In addition, the membrane, composed of various phospholipids and proteins, is best described as a finite interfacial region with a very high surface-to-volume ratio and a limited number of potential binding sites.[4] For this reason, solution phase liposome based assays have been developed to more accurately model and study drug−membrane association.[5]

Spectroscopic methods for detecting drug−membrane interactions such as UV−Vis absorbance spectroscopy,[6] fluorescence,[7] IR,[8] Raman,[9] and NMR[10] have all been utilized to measure drug interactions with solution phase vesicles. Such analyses require milliliter solution volumes, large quantities of analyte (i.e., drug) and lipid, and usually involve a separation step to remove unbound drug from the solution prior to analysis. These requirements are necessary due to the limited sensitivity of the spectroscopic methods employed.

The use of planar supported lipid bilayers (PSLBs) for measuring drug−membrane association has several advantages over solution phase vesicles, including the use of smaller solution volumes, elimination of the separation steps employed in liposome-based assays, and, in principle, a more rapid screening of interactions. However, to fully exploit the potential benefits of a PSLB assay, a detection method is needed with high sensitivity, a low detection limit, and the capability to measure drug−membrane interactions at the interfacial level without interference from solution phase species.

Several possible methods could be employed to measure drug−membrane interactions on surfaces. Surface enhancement vibrational techniques such as attenuated total reflection (ATR) IR and surface enhanced Raman scattering (SERS) are sensitive spectroscopic methods, but they suffer from spectral congestion and the inability to selectively isolate the resonances of the drug molecule from the surrounding lipid matrix. Fluorescence is an extremely sensitive method, capable of single molecule detection limits;[11] however, if the drug molecule lacks any intrinsic fluorescence, an extrinsic fluorescent tag must be covalently linked to the molecule for detection. Surface plasmon resonance (SPR) is another potential technique. However, SPR is not suitable for investigating drug−membrane interactions because the change in the local refractive index of a lipid bilayer ($n \sim 1.5$) is not altered significantly upon drug binding due to the similar refractive index of the drug and membrane. The inability of SPR to interrogate drug−membrane association is evidenced by the lack of any definitive literature example of a low molecular weight drug interaction with a PSLB being measured by SPR.

Here, the use of a novel deep-UV spectroscopic method, ultraviolet−visible sum-frequency generation (UV−Vis SFG), has been implemented to directly detect drug association to lipid membranes without the need for chemical modification. UV−Vis SFG is a surface sensitive technique which involves spatially and temporally overlapping a UV and visible laser source at the sample of interest, yielding photons at the sum of the two input frequencies. The UV−Vis SFG intensity is expressed as

$$I_{SFG} = \left(\tilde{f}_{sum} f_{UV} f_{vis} \chi^{(2)}\right)^2 \tag{1}$$

where f_{UV}, f_{vis}, and $\tilde{f}_{sum}$ are the geometric Fresnel coefficients for the ultraviolet (UV), visible (Vis), and sum-frequency light, respectively.[12] $\chi^{(2)}$ is the second-order nonlinear susceptibility which has a nonresonant (χ_{NR}) and resonant contribution (χ_R):

$$\left(\chi^{(2)}_{ijk}\right) \propto \chi_{NR} + N \sum_{a,b,c} \frac{\langle a|\mu_i|c\rangle \langle a|\mu_j|b\rangle \langle b|\mu_k|c\rangle}{(h\omega_{SFG} - E_{ca} - i\Gamma_{ca})(h\omega_{UV} - E_{ab} - i\Gamma_{ab})(h\omega_{vis} - E_{bc} - i\Gamma_{bc})} \tag{2}$$

In eq 2, N is the surface density of molecules; h is Planck's constant; E_{ca}, E_{ab}, and E_{bc} are the energies of the optical transitions in the deep UV, UV, and Vis, respectively; μ is the Cartesian coordinate dipole operator; and Γ represents the line width for the transitions. The indices on $\chi^{(2)}$ denote the input (j,k) and output (i) fields which can take on any of the three Cartesian coordinates (x,y,z). Examination of eq 2 shows that an increase in the UV−Vis SFG signal will be observed when the incident or SFG frequencies are resonant with electronic transitions of the molecules comprising the interface. When χ_R is large compared to χ_{NR}, which is the case when the incident and/or SFG wavelengths are in resonance with an optical transition, the nonresonant term can be neglected.[13]

To demonstrate the ability of UV−Vis SFG to detect drug association to a membrane, four drugs were examined: ibuprofen, azithromycin, tolnaftate, and tetracaine. The structures of these compounds and their associated UV−Vis spectra are shown in Figure 1. These drugs were chosen as they belong to four important classes of known membrane associated drug compounds; nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, antifungals, and anesthetics, respectively.[14−17] Ibuprofen, tolnaftate, and tetracaine all contain a π-conjugated ring system in their structures, resulting in strong electronic transitions in the UV (Figure 1). Azithromycin was chosen as it possesses only a single carbonyl bond, giving rise to a small absorbance at 213 nm ($\varepsilon_{213} = 988\ M^{-1}\ cm^{-1}$), which makes it a significantly weaker absorber compared to the other drugs examined. Unlike UV−Vis or fluorescence, UV−Vis SFG is not dependent on direct electronic excitation or emission as it is a coherent scattering process. As a result, problems associated with photodegradation or photobleaching are considerably reduced, especially when the electronic transitions being probed

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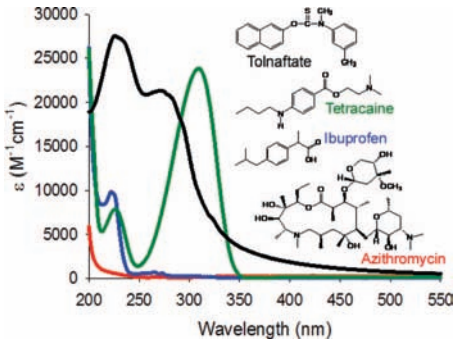

Figure 1. UV−Vis spectra and molecular structures of ibuprofen, azithromycin, tetracaine, and tolnaftate.

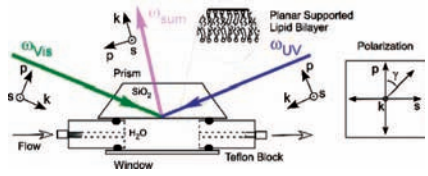

Figure 2. UV−Vis SFG cell showing the geometric arrangement of the UV, vis, and sum-frequency beams. Insert: representation of the polarization state of the incident and output fields denoted as the angle γ with respect to the propagation direction (k).

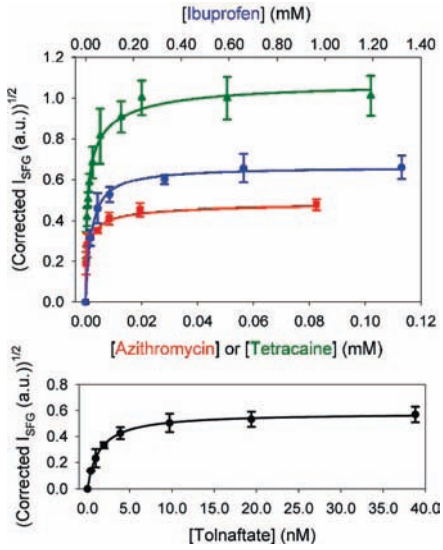

Figure 3. UV−Vis SFG adsorption isotherms for: (top) ibuprofen (blue), azithromycin (red), tetracaine (green); (bottom) tolnaftate. The solid lines are the fits to the data using the Frumkin isotherm (tetracaine and azithromycin) and Langmuir isotherm (ibuprofen and tolnaftate).

are near the sum-frequency wavelength, as direct electronic excitation from the pump lasers is eliminated.

The UV−Vis SFG experiments were performed by combining the UV (355 nm, mixed-polarization, $\gamma = 45°$) and Vis (532 nm, $\gamma = 45°$) outputs of a Nd:YAG laser both spatially and temporally on a 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) PSLB which was deposited on a fused silica trapezoidal prism, Figure 2. Increasing concentrations of a drug in phosphate buffered saline (PBS) pH 7.5 were equilibrated above the membrane surface, and the s-polarized UV−Vis SFG intensity was recorded. The resulting adsorption isotherms for the studied drugs are shown in Figure 3. To allow for a direct comparison of the system response for the various drug molecules, the SFG intensities were corrected for changes in collection efficiency, so that a relative comparison of

Table 1. Measured Equilibrium Association Constant K_a, $\sqrt{I_{SFG}^{MAX}}$, and g Values for Ibuprofen, Azithromycin, Tetracaine, and Tolnaftate

drug	K_a (M^{-1})	$\sqrt{I_{SFG}^{MAX}}$ (a.u.)	g
ibuprofen	$(4.37 \pm 1.65) \times 10^4$	0.67 ± 0.01	
tetracaine	$(5.44 \pm 0.96) \times 10^6$	1.08 ± 0.03	-3.09 ± 0.45
azithromycin	$(1.25 \pm 0.09) \times 10^8$	0.49 ± 0.05	-6.51 ± 2.19
tolnaftate	$(7.00 \pm 0.26) \times 10^8$	0.58 ± 0.02	

Table 2. Partition Constants, Normalized Surface Densities, and Limits of Detection (LOD) for the Drugs Studied

drug	P_i	Γ^{max} (molc/cm²)	LOD (pg/cm²)
ibuprofen	64.6	$(9.22 \pm 0.14) \times 10^{11}$	46.9 ± 4.7
tetracaine	128.8	$(5.31 \pm 0.15) \times 10^{10}$	1.3 ± 0.1
azithromycin	131.8[a]	$(1.58 \pm 0.16) \times 10^{10}$	3.6 ± 0.3
tolnaftate	147 910.8[a]	$(1.00 \pm 0.03) \times 10^{13}$	1306.8 ± 52.8

[a] See SI.

intensities from experiment to experiment and between drugs is possible (Supporting Information, SI).

For these resonant UV−Vis SFG experiments, the surface density of the drug is proportional to the square root of the SFG intensity. To extract the equilibrium drug−membrane association constants from the UV−Vis SFG data, the adsorption isotherms were fit to the Langmuir model or Frumkin model (eq 3) which accounts for repulsive interactions of the ionized drug molecules:[18]

$$\exp\left(g \cdot \frac{\sqrt{I_{SFG}}}{\sqrt{I_{SFG}^{MAX}}}\right) \cdot [\text{drug}] K_a = \frac{\sqrt{I_{SFG}}}{\sqrt{I_{SFG}^{MAX} - \sqrt{I_{SFG}}}} \qquad (3)$$

where [drug] is the bulk concentration of drug, K_a is the equilibrium association constant, I_{SFG} is the SFG intensity, I_{SFG}^{MAX} is the maximum SFG intensity at surface saturation, and g is a constant used to account for repulsive/attractive interactions of the adsorbate. When $g = 0$, the Frumkin isotherm becomes the Langmuir isotherm. The results from a nonlinear least-squares regression of the data in Figure 3, to eq 3, are listed in Table 1.

The affinity constants for the drugs were found to increase in the order ibuprofen < tetracaine < azithromycin < tolnaftate. This order agrees well with the partition coefficients of the drugs into liposomes (listed in Table 2).[19] The negative values of g for tetracaine and azithromycin illustrate the repulsive interaction between the charged drug molecules. At pH 7.5, tetracaine and azithromycin have one and two positive charges, respectively. Ibuprofen possesses one negative charge; however the Langmuir model provides a better fit to the adsorption data, presumably due to the electrostatic shielding from the supporting electrolyte. It has been reported that ionized ibuprofen (weak acid) locates closer to the membrane−water interface making it more accessible to the solvent while ionized tetracaine and azithromycin (weak bases) associate strongly into the hydrophobic tails of the lipid membrane.[20] For the supporting electrolyte to shield the electrostatic interaction between the ionized tetracaine and azithomycin, ions from the bulk have to move deeper into the membrane, which is not energetically favorable.[20] The screening of the charge on ibuprofen has been verified by performing the adsorption experiment using a lower PBS salt concentration (15 mM, see SI for results). Under these conditions, the electrostatic screening between ibuprofen molecules is reduced and a negative g value of -2.36 ± 0.67 was obtained. The absence of any electrostatic repulsion is clearly seen for tolnaftate, which is the only neutral drug in this study and is best fit with a Langmuir isotherm ($g = 0$). For every drug, the validity of using a Frumkin isotherm or Langmuir isotherm was verified by an F-test.

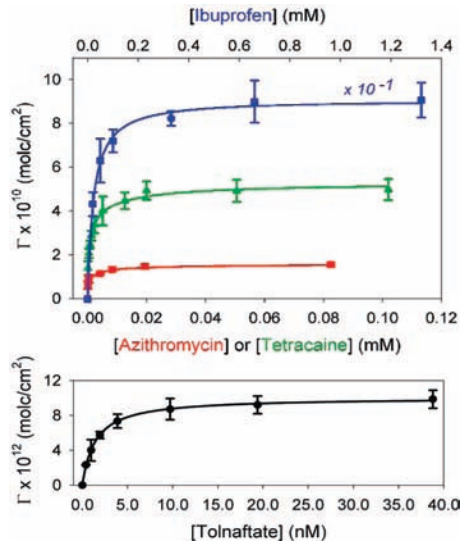

Figure 4. Surface excesses Γ for: (Top) ibuprofen (blue), azithromycin (red), tetracaine (green); (Bottom) tolnaftate. Solid lines are the fits to the data using the Frumkin isotherm (tetracaine and azithromycin) and Langmuir isotherm (ibuprofen and tolnaftate).

In addition to retrieving information on the thermodynamics of drug−membrane association, UV−Vis SFG can also be used to obtain information on the surface excess of the drug (Γ_{max}) in the membrane. As with other spectroscopic methods, UV−Vis SFG needs to be calibrated. This could be achieved by using lipid membranes which have fixed and known concentrations of drug in the membrane and measuring the UV−Vis SFG response. As drug−membrane association is a dynamic process, it is not possible to create such standards easily. However, data do exist, in the form of partition coefficients for the drugs in liposome−membrane systems or octanol−water partitioning data,[21] which can be used to calibrate the UV−Vis SFG data. In the linear region of the binding isotherms, at low surface density, the partitioning of the drug in the membrane is identical to that in a solution phase liposome as there is effectively no competition for binding sites.[22] Using the linear regions of the isotherms shown in Figure 3, the UV−Vis SFG intensity was calibrated for each drug. The liposome−water partition coefficients were used to determine the membrane concentration from the bulk aqueous concentration of the drug, [membrane] $= P_i \cdot$ [aqueous]. The surface concentration in molecules (molc)/cm^2 was then determined by assuming an effective thickness of the DOPC bilayer of 50 Å.

The calculated surface excess (in molecules/cm^2) is plotted as a function of bulk concentration in Figure 4. Although the affinity constants for the drug are in the order ibuprofen < tetracaine < azithromycin < tolnaftate, the same cannot be said of the saturation concentration of the drugs in the membranes. Azithromycin and tetracaine have very similar surface saturation values (((1.58−5.31) ± 0.16) × 10^{10} molc/cm^2) while ibuprofen and tolnafate are considerably larger ((9.22 ± 0.14) × 10^{11} and (1.00 ± 0.03) × 10^{13} molc/cm^2). The large difference in surface converges is a reflection of the repulsive interactions between drug molecules. Azithromycin and tetracaine have the lowest surface converge and largest g values, while ibuprofen and tolnaftate which have shown no repulsive term ($g = 0$) have the highest saturation levels.

The absence of a direct link between K_a and Γ_{max} is not surprising, as the factors determining these two quantities are not necessarily correlated. K_a is not dependent on the absolute number of binding sites, but rather is a reflection of the fraction of surface occupancy at a specific bulk concentration; which is why knowledge of the affinity constant alone is not sufficient to determine the absolute surface density. A quantitative determination of drug saturation in the membrane is possible when both bulk equilibrium measurements and UV−Vis SFG are used in combination.

A quantitative assessment of the limit of detection (LOD) of UV−Vis SFG can also be made, based on the results presented in Table 2. Using the spectroscopic sensitivity determined from the calibration of the UV−Vis SFG intensity and the standard deviation in the measured signal, the LODs for the three drugs examined were calculated and are listed in Table 2. The lowest calculated LOD is 1.3 ± 0.1 pg/cm^2 for tetracaine with the highest observed for tolnaftate at 1.31 ± 0.05 ng/cm^2. These LODs are far superior to the only other surface specific and label-free method, SPR, and are comparable to fluorescence measurements but without a label being needed and with reduced photodegradation. The impressive LOD and the surface specificity of UV−Vis SFG illustrate the potential of the method for measuring low molecular weight drug−membrane interactions.

We have demonstrated here that UV−Vis SFG is an ultrasensitive and powerful technique to directly detect drug−membrane association without chemical modification. The equilibrium association constants of ibuprofen, azithromycin, tolnaftate, and tetracaine into a lipid membrane have been measured and shown to increase with the drugs' hydrophobicity. A quantitative assessment of membrane concentrations is achievable using bulk distribution coefficients and UV−Vis SFG measurements. These findings suggest that UV−Vis SFG is a valuable alternative in measuring the association of drugs to the membranes.

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Supporting Information Available: Detailed experimental description. This material is available free of charge via the Internet at http://pubs.acs.org.

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