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Molecular Restructuring of Water and Lipids upon the Interaction of DNA with Lipid Monolayers

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Abstract: Understanding the molecular mechanism of DNA/lipid interaction is critical in optimizing the use of lipid cofactors in gene therapy. Here, we address this question by employing label-free vibrational sum frequency (VSF) spectroscopy to study the interaction of DNA with lipid monolayers of the cationic lipids DPTAP(1,2-dipalmitoyl-3-trimethylammonium-propane) and diC14-amidine as well as the zwitterionic lipid DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) in the presence and absence of calcium. Our approach has the advantage both of allowing us to explicitly probe intermolecular interactions and of providing insight into the structure of water and lipids around DNA at the lipid interface. We find, by examination of the OD stretch of interfacial D_2O, that water structure differs markedly between systems containing DNA adsorbed to cationic and those that contain DNA adsorbed to zwitterionic lipid monolayers (in the presence or absence of Ca^{2+}). The spectral response of interfacial water in the cationic system is consistent with a highly structured, undercoordinated, structural 'type' of water. Further, by investigation of CH stretch modes of the diC14-amidine lipid tails, we demonstrate that the adsorption of DNA to this lipid leads to increased ordering of lipid tails.

Introduction

The ability to control the transfection of particular genes has enormous potential therapeutic benefits. One way to approach this challenge is to use genetically modified viruses, that is, to borrow evolution's solution to the biophysical challenge of moving genetic material from outside the cell, across the plasma membrane and into the nucleus. While extremely efficient, this way of approaching gene transfection is both specific to particular types of cells and has often had unintended toxic side effects.[1−5] For these reasons, a large amount of effort has been devoted over the past 20 years to the synthesis of cofactors that complex with DNA outside the cell and shepherd it toward the nucleus. Engineering these cofactors is challenging owing to the requirements of generality (suitable for many cell types), efficiency (require relatively small amounts of DNA), and nontoxicity.

To appreciate the challenge, it helps to consider the properties that such a cofactor should possess. The complex formed in solution should be stable outside the cell and should facilitate the interaction of polyanionic DNA with the outer leaflet of the negatively charged plasma membrane. Next, following cellular uptake, the complex should help stabilize DNA until diffusion brings it near the nucleus. Finally, the complex should be sufficiently unstable that, when relatively close to the nucleus, DNA will be released into the cytosol. Following the pioneering studies of Felgner and others 15−20 years ago, much of the work on this challenge has focused on using cationic lipids as a complexing agent.[6,7] Cationic lipids were chosen because they form either un- or slightly positively charged, stable aggregates (lipoplexes) with polyanionic DNA in solution. Lipoplexes thus meet the first requirement for synthetic cofactors by decreasing DNA's anionic character and thus decreasing the electrostatic barrier to DNA approaching the plasma membrane. Comparison of the results of in vivo studies of the transfection efficiency of such lipoplexes with in vitro structural studies of similar complexes has clarified that gene transfer across the plasma membrane happens mainly by endocytosis and that transfer efficiency out of an endosome is a function of the structure of the aggregate and the charge of the lipid headgroup.[8−11] In vitro studies of lipoplex structure, principally X-ray, neutron, and

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coarse grained computational studies, have clarified that structure can be sensitively tuned by varying the charge of the cationic lipid headgroup, changing the ratio of cationic to zwitterionic lipids (in lipoplexes composed of zwitterionic lipid + cationic lipid + DNA), modifying the lipid−DNA ratio and changing the valence and concentration of small ions also present.[12−27]

While in vivo transfection and imaging studies and in vitro structural studies are important in the optimization of gene transfection by synthetic vectors, the information such studies reveal is limited. For example, taken collectively, these approaches offer limited insight into such potentially important issues as the free energy of lipoplex formation or the detailed, molecular level interactions within a lipoplex or between lipoplex and endosome. To meet the former shortcoming a series of calorimetry,[28−31] pressure isotherm[18,32] and quartz crystal microbalance measurements have been performed.[31,33] Understanding how the molecular details of the DNA/lipid/interfacial water interaction influence lipoplex structure—understanding how molecular level information influences nanometer to micrometer scale structure—is, however, more complicated. In the computational realm, these sorts of studies are challenging because the problem is extremely multiscale: ideally, one would like insight into processes that occur on time scales of hundreds of femtoseconds to minutes and spatial scales of angstroms to millimeters.[24] In the experimental realm, finding approaches that allow both sufficient surface sensitivity and specificity is challenging. Several authors have addressed the sensitivity part of this equation, although not the surface specificity, by employing fluorescence techniques.[34−36] While such studies provide insight into membrane hydration and fluidity, these conclusions are both generally indirect (reached by empirical calibration of changes in the response of fluorophores with changes in their local molecular environment) and require the use of a fluorescent label that may potentially perturb this delicate system.

Information about the molecular level interactions in lipoplexes can potentially be directly gained in a label free manner from vibrational spectroscopy. In pursuit of this goal, several authors have conducted infrared absorbance measurements of various DNA/lipid systems.[17,18,37] This approach has been principally used to measure the amount of DNA at the surface through the quantification of absorbance of a DNA specific functional group. However, due to the lack of inherent surface sensitivity/specificity of infrared absorption, little attempt has been made to use changes in vibrational spectral response, of modes of either DNA, lipids, or interfacial water, to understand the complexation of DNA with lipids.

In this study, we overcome the surface specificity/sensitivity challenge by applying Vibrational Sum Frequency generation (VSF) spectroscopy to gain a molecular level picture of the interaction between DNA, lipids, and interfacial water. VSF is a second-order nonlinear optical technique in which pulsed visible and infrared laser beams are overlapped at an interface in space and time and the resulting emission at the sum of the two input frequencies monitored. The sum frequency process is, by its symmetry selection rules, interface specific (between bulk phases with inversion symmetry) and is a vibrational spectroscopy because emission can increase by many orders of magnitude when the energy of the infrared beam is resonant with a molecular vibration of an interfacial molecule. That is, VSF spectroscopy is an interface specific vibrational spectroscopy, well suited for the description of molecular structure at aqueous interfaces.[38−40] Previous work has demonstrated the applicability of VSF spectroscopy (and its nonresonant analog Second Harmonic Generation (SHG) spectroscopy) to the quantification of duplex formation and DNA conformation for small (less than 20 bp) largely monocomponent single and double stranded DNA, covalently attached to solid substrates,[41−47] as well as lipid−DNA interactions for much longer DNA strands

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at the water/lipid/air interface.[48] The present study shows that VSF spectroscopy can help elucidate molecular level interaction between lipids, interfacial water, and DNA strands of arbitrary composition.

While, as mentioned above, there is much evidence that lipoplex structure and transfection efficiency is a function of lipid headgroup charge density (for cationic lipids), not all cationic lipids are suitable for this application. In general, there is trade-off between increased transfection efficiency (with increasing concentration of lipid) and cellular toxicity (due, in part, to interaction between lipids and sulfated surface proteoglycan groups[10]). Indeed the cationic lipid employed in our previous study,[48] 1,1-dipalmitoyl-3-trimethylammonium propane (DPTAP), is known to be toxic to mammalian cells at concentrations well below that necessary for appreciable gene transfection. Here, we extend our prior work by additionally reporting on DNA interacting with (i) the cationic lipid diC14-amidine, for which a large body of work over the past 15 years testifies both to its low toxicity and its suitability for gene transfection;[49] and (ii) the nontoxic zwitterionic lipid dipalmitoylphosphatidylcholine (DPPC), both in the presence and absence of calcium (where the addition of calcium was investigated because a variety of prior studies have demonstrated that the interaction between DNA and DPPC becomes much more favorable in the presence of this ion[16−19,31,33,50]). We directly track the interaction of DNA with each lipid type by monitoring the change in the vibrational amplitude and line shape of the OD stretch (in interfacial D_2O or HOD) on adsorption of DNA. We find, in line with previous studies employing other techniques, that the interaction is predominantly electrostatic in nature: the cationic lipids interact with DNA similarly and this interaction differs from that of DNA and DPPC both in the presence and absence of calcium.

Further, because of our particular interest in diC14-amidine, we also quantify how DNA interaction with this monolayer leads to an ordering of the lipid tails (both by VSF spectroscopy and pressure/area isotherms). In a previous study, Benatti and coworkers have explored the effect of the adsorption of DNA on diC14-amidine bilayer phase as a function of temperature using electron spin resonance spectroscopy and spin labeled lipids. The results of this study suggest that the adsorption of DNA fluidizes gel phase diC14-amidine (below 23 °C) and rigidifies fluid bilayers (above 23 °C): adsorption of DNA acts to smooth the otherwise sharp gel/fluid phase transition with increasing temperature. Our VSF observations thus provide a molecular level view of the changes in lipid tail structure accompanying this diC14-amidine/DNA phase using a label-free experimental probe. Taken as a whole, these results suggest that the application of VSF spectroscopy to the study of lipoplex formation allow us to see aspects of lipoplex structure difficult to interrogate by other means and, therefore, is a useful tool for optimization of synthetic gene therapy.

Methods

Experimental Details. D_2O used in this study was obtained from Cambridge Isotope Laboratories (MA), 99.96% pure and was used as received. H_2O used in this study was distilled and then filtered using a Millipore unit to a final resistivity of 18.2 MΩ·cm. 1,2-Dipalmitoyl-3-trimethylammonium-propane (DPTAP) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (AL), the former as a chloride salt, and used as received. DiC14-amidine was synthesized using previously published procedures[49] (see Figure 1 for all lipid structures). λ-phage DNA (each molecule specified as 48 502 base pairs in length by the manufacturer) was purchased from Fermentas (Germany) and received in H_2O. For most of our experiments, we require DNA in D_2O. For this purpose, DNA was extracted from H_2O using a QIAEX II Gel Extraction Kit (Qiagen), then dissolved in TRIS buffered D_2O (10 mM TRIS-HCl, pD = 7). This procedure was repeated three times to ensure a low concentration of H_2O with a final DNA recovery of ≈80% (quantified by DNA absorbance at 260 nm). We did not confirm that this sample preparation procedure preserved DNA chain length.

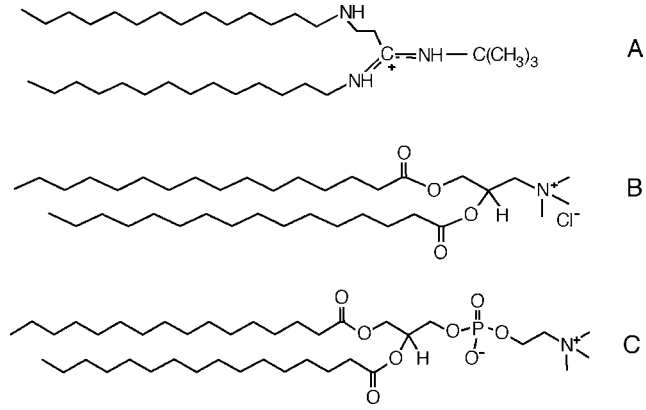

Figure 1. Molecular structure of (A) diC14-amidine; (B) DPTAP; and (C) DPPC.

Our VSF spectroscopy setup has been described in detail previously.[51] In brief, we employ a regeneratively amplified Ti:sapphire system (Legend, Coherent, Inc.) that is optimized for production of ≈100 fs pulses centered at 800 nm with a bandwidth of 12 nm; 1 mJ/pulse of this 800 nm light is used to generate tunable mid-IR pulses using a commercially available optical parametric amplifier and difference frequency generation unit (TOPAS, Light Conversion, Lithuania), while 0.5 mJ is spectrally narrowed using one or (for measurements focused on the CH stretch) two etalons. After IR generation and 800 nm spectral narrowing, both beams are passed through half wave plates and polarizers and impinge on the sample in a reflection geometry at 40° and 35° with respect to the surface normal (IR and visible, respectively). After the sample, all remaining 800 nm light is filtered out and the VSF signal is focused into a spectrograph (Acton Instruments) in which it is dispersed, via a grating, and focused onto an electron multiplied Charge Coupled Device (emCCD) camera (Newton, Andor). All spectra reported in this study were collected under the ssp polarization condition (s polarized SF, s polarized visible, p polarized IR). All measurements were conducted at 21.5 °C.

In this study, we are interested in both the spectral response in the OD and CH stretch region, 2100−2700 and 2800−3000 cm^{-1} of the IR frequency, respectively. Because the bandwidth of our IR source is less than the line width of the OD stretch vibration, we need to scan our IR frequency, by systematically changing the angles of the two nonlinear crystals in the TOPAS with respect to the incoming light. A consequence of this procedure is that the IR power is nonuniform over our desired frequency range. We correct for this nonuniformity by dividing all lipid/DNA spectra by a spectra over the same frequency range, measured from z-cut quartz. Our

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TOPAS/difference frequency generation unit is relatively inefficient at generating light above frequencies of 3400 cm^{-1}. It is for this reason that we examine the OD stretch of D_2O and HOD rather than the OH stretch of H_2O and HOD. Extensive comparisons by us and others find that the OD spectrum can be linearly scaled to quantitatively recover the OH spectrum: substituting heavy water for light does not influence molecular structure (to the degree visible in VSF spectra).

Some prior studies using VSF spectroscopy of the CH stretching frequency region of systems containing DNA attached end-on to solid surfaces have reported VSF active CH modes from DNA[43,44] while others have not.[42,45] Those studies in which DNA CH modes were observed are distinguished by the relative shortness and simplicity of their nucleobase sequence. Our DNA strands are 2000−5000× longer than those employed in these studies, are physisorbed to the interface (and thus might be expected to have less long-ranged order), and are chemically heterogeneous. These considerations suggest, as we indeed find, no VSF active CH modes of DNA itself in our experimental system.

Lipid stock solutions were prepared in chloroform. Monolayers were prepared by adding these stock solutions dropwise over a subphase containing D_2O and tris buffer in the presence and absence of DNA. The optical analyses were performed on samples prepared in home-built Teflon troughs. Surface pressure in these troughs was quantified using a commercially available tensiometer (Kibron, Finland). High resolution pressure (π)/area isotherms were measured on a commercially available trough with moving barriers (Micro Trough X, Kibron, Finland).

Data Analysis. Ultimately, we wish to compare the spectral amplitudes and line shapes of various vibrational modes as a function of DNA concentration in the subphase. Because VSF is a coherent spectroscopy, and our data contains multiple resonances that may interfere, it can be difficult to infer spectral amplitudes from inspection of the raw data. Following prior authors, we surmount this problem by modeling the sum frequency response as a collection of Lorentzians with an additional nonresonant contribution (in which $\chi^{(2)}$ is the second order susceptibility, I_v is the intensity of the incident visible field, I_{ir} is the intensity of the incident infrared field, A_{nr} is the nonresonant amplitude, ϕ_{nr} is the nonresonant phase, A_n is the amplitude of vibrational mode n, ω_n is the center frequency of vibrational mode n, ω_{ir} is the frequency of the infrared field and Γ_n is the homogeneous line width of vibrational mode n)[52]

$$\begin{aligned} I_{sf} &\propto |\chi^{(2)}|^2 I_v I_{ir} \\ &\propto |\chi^{(2)}_{nr} + \chi^{(2)}_{r}|^2 I_v I_{ir} \\ I_{sf} &\propto \left| A_{nr} e^{i\phi_{nr}} + \sum_n \frac{A_n}{\omega_n - \omega_{ir} - i\Gamma_n} \right|^2 I_v I_{ir} \end{aligned} \quad (1)$$

In what follows, we are principally interested in comparison of the extracted spectral amplitudes divided by the line widths: the A_n/Γ_n. To facilitate comparison to measured sum frequency intensities, we plot $(A_n/\Gamma_n)^2$. We extract these quantities by fitting the data using eq 1 and an implementation of the Levenberg−Marquardt algorithm in the commercially available analysis and graphing program Igor Pro (Wavemetrics, OR). As will be clarified below, we are principally interested in using this data fitting to extract qualitative trends in spectral amplitudes as a function of DNA concentration. For this reason, we made no attempts to exhaustively probe the phase space of model parameters in data analysis.

Results and Discussion

Spectral Response of Interfacial Water. VSF spectra from 2100−3100 cm^{-1} for DPTAP, diC14-amidine, and DPPC monolayers with increasing concentrations of DNA in the subphase are shown in Figure 2. Cursory inspection of this data show several features. Focusing on Figure 2 panel A and moving

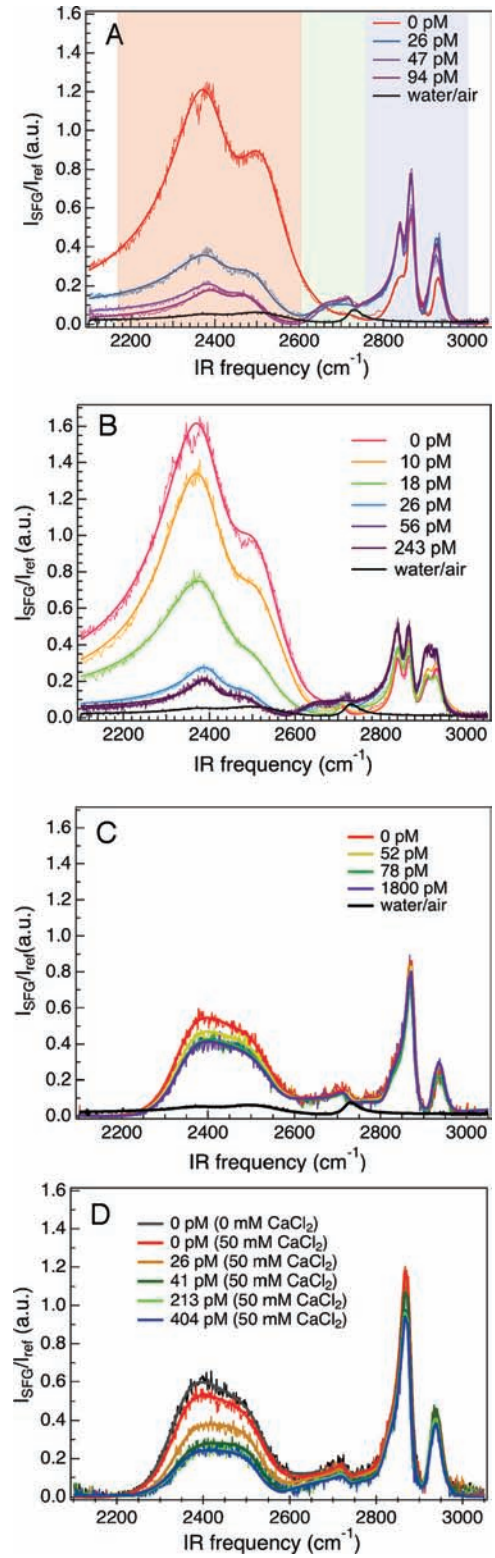

Figure 2. VSF spectra as a function of DNA concentration for the OD stretch region, 2100−2700 cm^{-1}, and the CH stretch region, 2850−2975 cm^{-1}. Cationic lipid monolayers are at lipid densities of 52 Å ^{2}/molecule, zwitterionic lipid monolayers at 47 Å ^{2}/molecule. Panel A is VSF spectra underneath a monolayer of DPTAP, panel B underneath a monolayer of diC14-amidine, panel C underneath a monolayer of DPPC, and panel D underneath a DPPC monolayer with an additional 50 mM of $CaCl_2$. Subphases in all experiments are phosphate buffered. The individual spectra in each panel are identical systems with different concentrations of λ−DNA phage. The air−phosphate buffered D_2O spectra for this frequency window are shown in each panel for comparison. Solid curves are fit to the data using eq 1.

from low frequency to high, it is clear that there is a large double peaked structure that decreases abruptly in amplitude with the addition of DNA (transparent red rectangle); a small, higher frequency peak (centered at ≈2675 cm^{-1}) that increases with the addition of DNA (transparent green rectangle); and several narrower peaks in the frequency range 2800−2950 cm^{-1} (these peaks are the result of CH stretching vibrations, are shown in a transparent blue rectangle and are discussed further below).

Twenty years of experimental and computational work following the pioneering efforts of the Shen group[38−40] have clarified that the large, double peaked structure is the OD stretch of interfacial, hydrogen bonded water (see reviews by Richmond, Gopalakrishnan et al., Ostroverkhov and Shen and Allen et al. and references therein[53−56]). Recently, we have shown that these two peaks do not result from two distinct structural types of interfacial water: they are a single peak with an apparent dip due to a Fermi resonance of the OD stretch with the 0 → 2 transition of the D_2O bend.[57−59] As will be discussed further below, this Fermi Resonance can be "switched off"—the resulting spectra have only a single peak—by probing the OH or OD stretch modes in interfacial HOD. Armed with such HOD spectra, a semiquantitative interpretation of interfacial water structure as possessing a continuum of different states becomes possible.

Moving to higher frequencies, there is a small peak at 2675 cm^{-1}. This peak does not appear at the water/lipid interface in the absence of DNA and there are no known resonances of DNA that appear in this frequency window. Much prior work on the OD (OH) stretch of interfacial water at the air/water interface (where the aqueous phase may contain acid, bases, or salts) has shown a large, double peaked feature at lower frequencies and a much narrower peak at higher frequencies (2735 cm^{-1}).[53,54,56,60,61] On the basis of OD stretch modes of isolated water molecules in vacuum and high level calculation, it is clear that the peak centered at 2735 cm^{-1} is the free OD: a D_2O molecule that straddles the air/water interface in such a manner that one OD is sticking into the vapor phase (see the air/water spectra in Figure 2). Because our observed peak is both at lower frequency than this free OD and only appears in the presence of DNA, it can most easily be explained either by water directly, yet weakly, hydrogen bonded to DNA or the adsorption of DNA creating a structural type of water not present in its absence (e.g., a hydrophobic pocket). Constraints on this assignment are discussed further below.

We can make our discussion of these spectral features easier by considering intensities (i.e., $(A_n/\Gamma_n)^2$) extracted from the data in Figure 2. The results of this approach for the low frequency, large OD peak (spectral feature within the transparent red rectangle in Figure 2A) are shown in Figure 3 (for all systems OD intensity is normalized by the signal in the absence of DNA). These results quantify our qualitative impressions from Figure 2: increasing the bulk concentration of DNA from 0 to 40 pM results in a loss of more than 80% of OD intensity for DNA adsorbed to the cationic lipids DPTAP or diC14-amidine. Increasing DNA concentration underneath the zwitterionic lipid DPPC, results in a decrease in OD intensity from 0 to 80 pM DNA but with a total loss of only 20% of the initial value. Finally, in our fourth system, increasing concentration of DNA beneath a DPPC monolayer in the presence of 50 mM of $CaCl_2$ generates a spectral response that is intermediate between these two extremes: with increasing concentration of DNA, the intensity of the low frequency OD stretch peak decreases by ≈70%. The small peak at ≈2675 cm^{-1} is clear only for systems in which DNA is adsorbed to DPTAP and diC14-amidine (see Figure 2A,B). If we use eq 1 to quantify the intensity of this peak, we obtain the results shown in Figure 4. For both lipids, the trend is the same: a rapid increase in intensity that saturates at ≈40 pM bulk concentration of DNA with a slight drop in amplitude thereafter. While we discuss further the detailed assignment of this peak below, we here note that OH groups in DNA strands are only present at the end of the chain: for each DNA chain, there are only two terminal OH groups. The resulting density of DNA OH groups amounts to one per

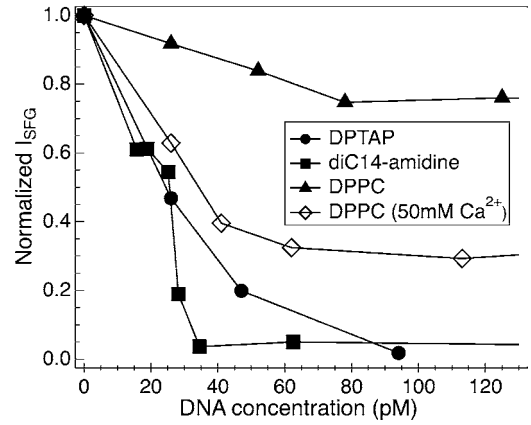

Figure 3. Fitted hydrogen bonded OD stretch intensities (i.e., $(A_n/\Gamma_n)^2$) from data in Figure 2. The hydrogen bonded OD stretch peak is highlighted in the transparent red rectangle in Figure 2A. These results clearly show that, while OD intensity underneath all lipid monolayers decreases with increasing DNA concentration, the change underneath the charged lipids is much larger.

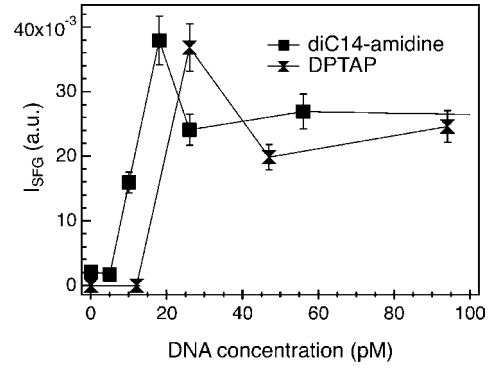

Figure 4. Fitted spectral intensities for 'weak' OD stretch peak for the spectra shown in Figure 2, panels A and B. These results clearly indicate that the intensity of the 'weak' OD stretch peak plateaus above ≈30 pM bulk concentration of DNA.

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$\approx$15 000 nm^2; sufficiently small that the density of DNA OH groups is too low to be responsible for any of the observed spectral changes.

In principle, each vibrational mode in our data contains three observables: the spectral amplitude, line shape, and the center frequency of the underlying mode. The intensities shown in Figures 3 and 4 are influenced by the first two factors, essentially we are observing total integrated spectral intensity, but do not address the third. For the DNA/DPPC and DNA/DPPC/Ca^{2+} systems, inspection of Figure 2, panels C and D, suggests this manner of extracting information from the spectrum is justified: there is no apparent shift in center frequency. For the DNA/DPTAP and DNA/diC14-amidine systems, the situation is more complex. For example, for the DNA/DPTAP system, it is apparent that the shoulder at $\sim$2500 cm^{-1} increases in intensity relative to the main peak at $\sim$2400 cm^{-1} with increasing DNA concentration. Whether this apparent shift in frequency of the underlying mode corresponds to an actual shift in frequency is discussed in more detail below.

We are thus left with three principle observations of the OD stretch frequency region of these systems. (i) The integrated spectral intensity for the low frequency peak decreases with the addition of DNA. The intensity of the spectral response correlates with electrostatics: interfacial water beneath cationic lipid monolayers is much more sensitive to the adsorption of polyanionic DNA than that beneath zwitterionic monolayers. Water beneath zwitterionic monolayers in the presence of Ca^{2+} (thought to make the zwitterionic monolayer nominally cationic[16]) has an intermediate sensitivity. (ii) Beneath cationic monolayers, the center frequency of this OD stretch mode may shift with the addition of DNA (as it decreases amplitude); beneath zwitterionic monolayers it does not. (iii) An OD stretch peak appears at higher frequencies, 2675 cm^{-1}, only in the presence of DNA beneath cationic monolayers. To understand the significance of these observations for molecular structure, we need to consider the factors that can influence the VSF spectral response in more detail.

There are five factors that may cause changes in OD stretch spectral response with increasing DNA concentration. (i) The DC interfacial field may change due to the interaction of the lipid with polyanionic DNA (where the initial field is the result of the charged lipid headgroup (DPTAP and diC14-amidine) and the dipole of the zwitterionic headgroup (DPPC) and the field change accompanying DNA adsorption is expected only to modulate spectral amplitude). (ii) The creation of new structural types of water (water hydrogen bonded to new acceptors or donors) which are expected to alter the OD stretch center frequency and line shape and, the density of other types of interfacial water remaining equal, leads to an increase in spectral amplitude. (iii) The adsorbed DNA can displace interfacial water leading to a reduction in the number of interfacial water molecules (where such displacement will cause only a decrease in spectral amplitude if DNA displaces all 'types' of water to an equal extent). (iv) The adsorption of DNA may create additional noncentrosymmetric regions (i.e., the lipid−water interface may be changed to lipid−water−DNA−water interfaces). This would lead to a reduction of spectral amplitude if water molecules adsorbed on the opposite side of DNA helices destructively interfere. (v) Adsorbed DNA may cause interfacial water molecules to reorient in such a manner that their OD stretch amplitude (as apparent in our experimental geometry) strongly decreases.

Effect (iv) suggests that the adsorption of DNA creates a large new population of interfacial water. Inspection of DNA structure suggests such water would be most likely hydrogen bonded to phosphates that festoon the outside of the double helix. Extensive infrared and simulation studies of hydrated bilayers suggest, however, that phosphate is a better hydrogen bond acceptor than bulk water: increasing amounts of VSF active water adsorbed to DNA should lead to a red shift in the center frequency of the OD stretch band.[62,63] As we see a shift to higher frequencies of this band for systems containing cationic lipids and no shift for systems containing DPPC, it seems improbable that this effect contributes significantly and it is not discussed further. Polarization analysis of the VSF spectral response of interfacial water suggests the sort of decrease in amplitude of the hydrogen bonded OD stretch intensity we see (red feature in panel A of Figure 2) would require a reorientation of water molecules in excess of 60° (with respect to the surface normal).[60] As to the best of our knowledge no independent evidence for such extreme reorientation exists for this or any other aqueous interface, we also do not consider effect (v) further. In what follows, we consider effects (i)−(iii) in more detail.

Much prior work has demonstrated that sum frequency intensities decrease with decreasing surface potentials either as a result of the decreasing surface field orienting interfacial liquid molecules less strongly, a decreasing $\chi^{(3)}$ contribution to the sum frequency signal or both.[64−66] McLoughlin et al. have shown that adsorption of DNA to the cationic lipid DODAB (dioctadecyldimethylammonium bromide) leads to a decrease in surface potential of $\approx$0.3 V while adsorption of DNA to the zwitterionic lipid DSPC (distearoyl-*sn*-glycero-3-phosphocholine) in the presence of Ca^{2+} (the change in surface potential for the bare DSPC monolayer in the presence of DNA was not measured) leads to a decrease in surface potential of less than 0.1 V. If we assume that DPTAP and diC14-amidine behave similarly to DODAB and DPPC similarly to DSPC, the change in spectral response with DNA adsorption appears to map qualitatively to the potential decrease: DNA adsorbed to a cationic lipid leads to a larger surface potential decrease than that adsorbed to a zwitterionic and the former system shows a larger decrease in hydrogen bonded OH stretch intensity than the latter.

These qualitative trends in surface potential and OD stretch intensities are also consistent with much prior X-ray, simulation, and thermodynamic work. DNA is known to adsorb relatively strongly (with a concomitant relatively large change in surface potential) and have a relatively high maximum adsorbed density, to lipid monolayers or bilayers containing cationic lipids. It is for systems containing cationic monolayers that we see the strongest spectroscopic response of interfacial water to the presence of DNA. On the other hand, DNA is known to adsorb weakly (if at all) to monolayers or bilayers containing only zwitterionic lipids;[19,21,31,33,67] underneath the zwitterionic lipid DPPC, we see a weak spectral response of interfacial water to

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increasing concentration of DNA. Finally, systems containing zwitterionic lipids and small cations of di- or higher valence are known to have both an intermediate adsorption energy of DNA, an intermediate maximum adsorbed density, and an intermediate change in surface potential on adsorption.[17−19,31,67] For our experiments with DPPC/Ca^{2+} and increasing concentrations of DNA, the spectral response of interfacial water is clearly intermediate.

It thus appears that the reduction of the VSF water signal for DNA adsorbed to lipid monolayers is qualitatively accounted for by the decrease in surface potential. We have previously presented a quantitative description of DNA adsorption leading to decrease in VSF spectral amplitude solely through modulation of the interfacial field of cationic lipids using a simple surface complexation + Gouy−Chapman (SCGC) model.[48] The logic of this model (the particular formulation we employed is valid only for the case of DNA adsorption to cationic lipids) is that the influence of increasing bulk concentration of DNA on the VSF OD stretch intensity underneath a cationic monolayer is to decrease the effective surface charge which then decreases the surface potential (in a manner quantifiable by the Gouy−Chapman equation). This approach worked surprisingly well given that it does not explicitly account for an entropic contribution to the free energy of adsorption of DNA nor does it allow for changes in interfacial water structure as bulk DNA concentration changes.

For application of the SCGC model to be appropriate, interfacial water structure cannot change as a function of interfacial concentration of DNA. If any such changes were to occur, we would expect them to cause a shift in the center frequency of the OD stretch of interfacial water. Similar experiments to those reported here, adding NaCl to an aqueous subphase beneath a DPTAP monolayer,[48] and adding various ions to an aqueous phase beneath CaF_2[68] and Alumina[69] show no changes in the center frequency of the interfacial water bond stretch: adding ions appears only to damp the OD(OH) stretch of interfacial $D_2O(H_2O)$. Similar to those systems, no shift in center frequency of the OD stretch in D_2O for the DPPC and DPPC/Ca^{2+} systems with increasing DNA concentration is apparent (see Figure 2 panels C and D). Hence, the observed variations of the water response upon interaction with DNA for the DPPC and DPPC/Ca^{2+} systems are consistent with changes in the DC interfacial field due to the interaction of the lipid with the polyanionic DNA.

However, as mentioned above, the situation for the systems DPTAP/DNA (Figure 2A) or diC14-amidine/DNA (Figure 2B) is more opaque. For these systems, the spectral response clearly changes significantly but, by inspection of the data in Figure 2, it is difficult to tell whether these changes can be purely explained by changes in line shape or spectral amplitude. As we have discussed previously, extracting changes in center frequency in this situation for interfacial water is difficult because of effects related to intra- and possibly intermolecular coupling.[57−59] This intramolecular coupling makes it difficult to see, both by inspection, and by fitting the data with the line shape model in eq 1, whether the center frequency or width of the underlying OD(OH) stretch mode changes with addition of DNA.[57,58] This problem is a general one for the study of interfacial water using VSF spectroscopy. As we have noted previously, and demonstrated for several different lipid monolayers on water and the silica/water interface, this complication can be overcome by examining the OD stretch of HOD rather than D_2O.[59] The results of this approach, for the diC14-amidine system, are shown in Figure 5. From these HOD data, it is apparent that the center frequency of the HOD resonance shifts toward higher frequencies by ≈80 cm^{-1}, with increasing bulk DNA concentration.

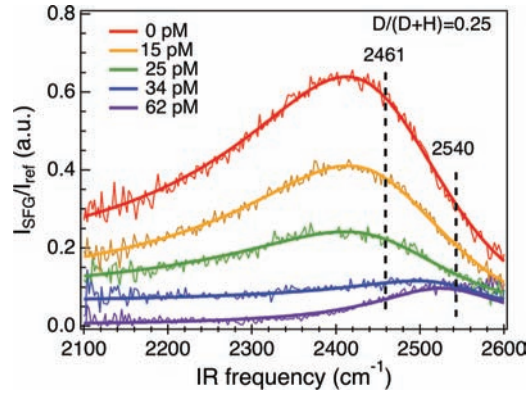

Figure 5. Spectra of the OD stretch frequency window of HOD in H_2O given a phosphate buffered subphase containing increasing concentration of DNA beneath a diC14-amidine monolayer. OD stretch spectra of HOD have only a single peak structure and clearly show that the resonance shifts toward higher frequencies as it decreases in amplitude with increasing DNA concentration.

The observation of a shifting OD stretch resonance frequency testifies to a change in the structure of interfacial water with adsorption of DNA. Hence, the decrease in the VSF intensity of interfacial water for the cationic lipids cannot be solely accounted for by the decrease in surface potential, in contrast to our previous conclusion.[48] That is, it is apparent that the interfacial water reorganizes upon addition of DNA for the cationic lipids. The fact that the HOD spectrum shows a shift to higher frequencies on increasing DNA concentration reveals that the average hydrogen-bond of interfacial water decreases in strength. Average hydrogen bonding strengths that are weaker than those experienced by water in bulk have been previously observed in, for example, undercoordinated water contained within reverse micelles.[70,71]

We are thus left with the observation that, with increasing concentrations of DNA adsorbed to cationic lipids, the low frequency OD stretch peak (transparent red rectangle in Figure 2) shifts to higher frequencies, while with increasing concentration of DNA adsorbed to zwitterionic lipids no significant shift in center frequency of this mode is observed. Prior observations by us and others of the adsorption of smaller ions to various interfaces show, similar to the zwitterionic lipid/DNA case, no shift in center frequency.[48,68,69] As the molecular footprint of individual negatively charged nucleobases is of order the lipid headgroup size (and very large relative to anions such as Cl^-) and the adsorption energy of DNA chains much larger than that of small ions, it is perhaps unsurprising that the adsorption of the polymeric DNA evidently perturbs interfacial water structure while that of monomeric ions does not.

To understand why DNA adsorbed to cationic lipid monolayers alters interfacial water structure but DNA adsorbed to

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zwitterionic lipids does not is more subtle. Prior X-ray scattering and diffraction studies have clarified that DNA binding to cationic lipids is stoichiometric: for bicomponent (one cationic lipid, one zwitterionic lipid, and DNA) lipoplexes the amount of adsorbed DNA is observed to linearly increase (over a wide range) with increasing relative cationic lipid concentration,[12,13,15,72] while for monocomponent cationic lipid monolayers, adsorbed DNA density linearly increases with increasing total lipid density.[73] The implication of these observations is that one might write a chemically realistic formula describing the binding of individual DNA bases to individual cationic lipid molecules. More quantitatively, X-ray reflection studies of DNA adsorbed to monolayers of the cationic lipid methyltrioctadecylammonium bromide and the zwitterionic lipid 1,2-dimyristoyl-phosphatidylethanolamine in the presence of Mg^{2+} suggest that, at lipid densities similar to our experiments, interchain spacings on the cationic monolayer reach ≈30 Å while those on the zwitterionic monolayer are ≈42 Å.[17,18,73] Studies of lipoplexes of DNA and cationic lipids in the presence of the divalent cations Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+} suggest still smaller interchain spacings may be possible above a critical ion concentration (as a result of 2D ion condensation), but this phenomena has not been explored for monolayers.[15] Finally, in agreement with these experimental studies, simple thermodynamic arguments suggest that each DNA molecule adsorbed to a cationic lipid monolayer will lay flat (see supporting information of Wurpel et al.[48]). Taken as a whole, this prior work paints a picture of DNA adsorbed to cationic lipids at relatively high surface coverage in a manner that is stoichiometric with respect to lipid environment and provides a unique interfacial environment for small cations. Given that this interfacial environment is highly structured with respect to DNA, lipids, and small cations, we wish to pose the question, what is the effect of this order on the spectral response of the final principal interfacial chemical species, water?

As water hydrating lipid head groups are known to experience a highly heterogeneous hydrogen bonding environment,[58,59,63,74−78] we expect the adsorption of DNA to cationic lipids to effect the spectral response of interfacial water in two ways. First, a decrease in the number of water molecules present at the interface: DNA displaces water. Because water experiences a heterogeneous hydrogen bonding environment near lipids, and because DNA is unlikely to displace all of these types of interfacial water with equal efficiency, this dewatering would be expected (as we observe) to lead to a shift in the center frequency of the underlying mode while it decreases in amplitude. Second, because the DNA cationic lipid complex is known to be stoichiometric, DNA adsorbed density on cationic lipids is known to be high, and the adsorbed DNA is known to create a unique ionic environment, we expect that we should see a type of interfacial water not present in the absence of DNA.

The expectation that there should be an additional structural type of water present only in the presence of DNA adsorbed to cationic lipids suggests that there should be an OD mode that appears only in the presence of DNA adsorbed to cationic lipids. As discussed earlier, the 2675 cm^{-1} peak can be most easily understood to be such a mode. While absent in infrared measurements of bulk water, multiple, well separated resonances for interfacial water have been seen in a variety of different systems. By far the most common example is the free and hydrogen bonded OD resonances that appear at air/aqueous interfaces (see the reviews by Richmond, Gopalakrishnan et al. Allen et al., and references therein[53,54,56]). Other systems in which multiple, well separated interfacial water resonances appear at frequencies lower than that of the free OD (OH) are less common but have been observed in VSF studies of some simple surfactant and phospholipid Langmuir monolayers.[61,79] Similarly, split OH stretch modes have been observed in infrared absorbance studies of partially hydrated Nafion membranes and polymeric systems.[80,81] For the Langmuir monolayer and Nafion membrane studies, the origin of the higher frequency mode was attributed to water molecules that existed in relatively hydrophobic microenvironments. For the hydrated polymeric materials, these peaks were attributed to water hydrogen bonded to relatively nonpolar ester oxygens.[81]

Inspired by this previous work, it seems clear that two molecular scenarios, or some combination thereof, can explain the appearance of the 2675 cm^{-1} peak. First, one can imagine water molecules relatively isolated from bulk water that donate a hydrogen bond to a weaker acceptor than other water molecules. Second, a collective effect resulting from geometrical considerations, where confinement makes it impossible for some subpopulation of water molecules to satisfy their full hydrogen bond quota. While it is challenging to determine which of these two scenarios, or some combination, is more likely for the DNA/cationic lipid system, it is worth emphasizing that neither VSF studies of water beneath monolayers of phospholipids, IR studies of the solvation of simple sugars, nor IR studies of partially hydrated bilayer stacks show such separate water bond stretch vibrations.[63,75−78,82] Taken together, these studies suggest the appearance of this 2675 cm^{-1} can be most easily explained by, in the presence of a DNA, the appearance of an undercoordinated, confined water species similar to that previously proposed for divalent cations in such systems.[15] Note that the fact that we see this mode both for DNA adsorbed on DPTAP (which has relatively nonpolar ester oxygens) and also for diC14-amidine (which does not) clarifies that this peak is unlikely to be the result of water hydrogen bonded to this relatively nonpolar acceptor.

While DNA is known to adsorb to cationic lipid monolayers in a stoichiometric manner, much previous work has shown that this is not the case for DNA adsorbed to the zwitterionic lipid DPPC.[16−19,21] In this system, DNA adsorbs very weakly in the absence of Ca^{2+} or some other multivalent ion, and in the presence of such an ion does not absorb stoichiometrically: in the presence of excess DNA and Ca^{2+} in bulk, the surface density of adsorbed DNA is observed to be independent of lipid

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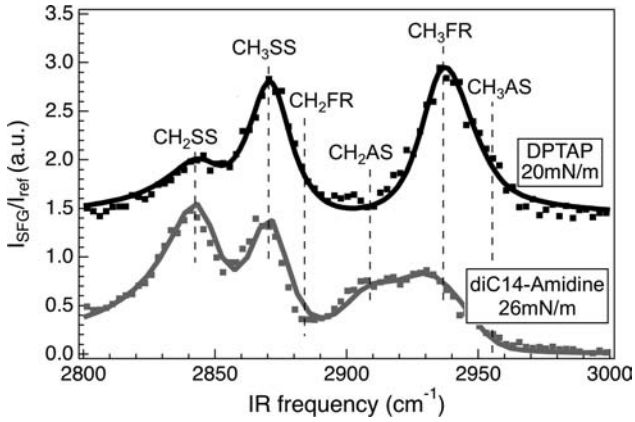

Figure 6. SFG spectra (dots) of diC14-amdine at 26 mN/m (gray) and DPTAP (black) at 20 mN/m. The vertical lines indicate the positions of methylene symmetric stretch (CH_2SS), methyl symmetric stretch (CH_3SS), methylene asymmetric stretch-Fermi resonance (CH_2FR), methylene asymmetric stretch (CH_2AS), methyl symmetric stretch-Fermi resonance (CH_3FR) and methyl asymmetric stretch (CH_3AS). The DPTAP spectrum is offset for clarity. The solid curves are fits to the data using a Lorentzian model.

density.[18,31,33] This absence of specificity in adsorption has been argued to be the result of relatively high Ca^{2+} mobility in the monolayer (leading to each DNA strand feeling a mean field of adsorbed calcium). The weak adsorption of DNA to DPPC in the absence of Ca^{2+} and its nonspecific adsorption in the presence of this ion are consistent both with the lack of shift in the low frequency OD stretch peak as it decreases in amplitude and the absence of the peak at 2675 cm^{-1}. Both of these observations are most easily understood as the result of a structured, stoichiometric DNA/lipid complex that also creates structured, relatively confined interfacial water, neither of which exist for systems containing DNA and DPPC.

Spectral Response of CH Modes of Lipid Tails. If we return to the data shown in Figure 2, it is clear that there are also several peaks in the 2800−2950 cm^{-1} region (transparent blue rectangle). Prior studies employing VSF spectroscopy of monolayers of lipids or simple surfactants have established, building on several decades of IR absorbance and spontaneous Raman measurements, that this spectral region contains six CH stretch modes: the CH_2 symmetric stretch, the CH_3 symmetric stretch, Fermi resonance of the CH_2 symmetric stretch and bend overtone, the CH_2 asymmetric stretch, the CH_3 asymmetric stretch and a Fermi Resonance of the CH_3 symmetric stretch and bend overtone.[52,74,83−86] Because the appearance of these six peaks in interfacial spectra is a function both of the cross section of each mode, spectral resolution of the VSF setup, and the structure of the monolayer, in many systems only some fraction of them is apparent. This is illustrated for DPTAP and diC14-amidine monolayers (in the absence of DNA) in Figure 6.

Much prior work by us and others has shown the possibility of using changes in the CH stretch spectra (e.g., as a function of surface pressure or cholesterol concentration) as a probe of

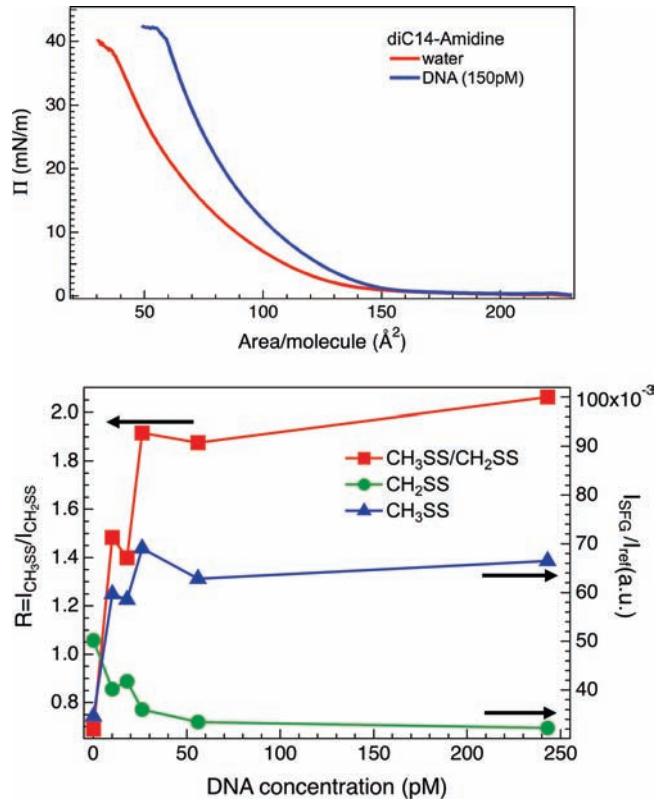

Figure 7. (Upper panel) $\pi-$Area isotherms for a diC14-amidine monolayer above a phosphate buffered subphase in the absence (red line) and in the presence (blue line, 150 pm) of DNA. For a given lipid density, the addition of DNA to the subphase results in an increase in surface pressure, or equivalently, a condensation of lipid molecules. (Lower panel) VSF intensity of CH_2SS and CH_3SS resonances (triangle and circle, right axis) and their ratio R (squares, left axis) of diC14-amidine monolayer on DNA solution with various concentrations. The solid lines are guides to the eye.

local ordering of lipid tails in monolayers.[87−90] In particular, our metric of lipid tail order is the ratio of the intensity of a CH_3 mode to that of a CH_2 mode. Such a ratio is a probe of order because at low lipid densities terminal methyl groups tend to be randomly oriented with respect to one another and thus, on average, give little VSF signal. In contrast, at high lipid densities, tails are known to pack in a compact, all trans arrangement. In such a configuration, terminal CH_3's point in a similar direction (and thus give strong VSF signals) while individual CH_2 groups have inversion symmetry with their neighbors (thus reducing the VSF signal in methylene related modes). We are here interested, in particular, in the effect of DNA adsorption on the ordering of diC14-amidine tails. Following this prior work,[87−90] we address this topic by fitting the raw data in the CH stretch frequency region, extracting the relevant CH intensities and determining the ratio of CH_3SS/CH_2SS as well as measuring compression isotherms in the presence and absence of DNA for the diC14-amidine monolayer. The results of these experiments are shown in Figure 7.

The compression isotherms shown in Figure 7 (upper panel) clearly show that the effect of DNA on diC14-amidine mono-

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layers, over a wide range of densities, is to increase surface pressure, or equivalently, decrease surface tension. That is, on addition of DNA, the free energy of surface creation always becomes more favorable. Without further information, however, these experiments provide little insight into the molecular underpinnings of this change in surface thermodynamics. VSF spectra of the CH region of diC14-amidine monolayers as a function of DNA concentration, and the resulting CH_3SS/CH_2 SS ratios, provide such insight. As is clearly shown in Figure 7 (lower panel), adding DNA to the subphase, even at bulk concentrations below 40 pM, causes a strong increase in lipid tail order. Benatti and co-workers have previously argued, in interpreting the results of an electron spin resonance study, that adsorbed DNA fluidizes the gel phase diC14-amidine bilayer (below 23 °C) and rigidifies the fluid phase bilayer (above 23 °C) effectively smoothing a thermal phase transition that is sharp in the absence of DNA.[91] Both our optical and pressure isotherm measurements were conducted slightly below the melting temperature. Our compression isotherms suggest that the effect of DNA is to increase interaction between individual chains on the surface (decrease surface tension) while the optical measurements suggest this increase in chain interaction leads to greater ordering of lipid tails. Intriguingly, these results are consistent with a scenario in which the addition of DNA appears to create lipids that both interact more strongly than in the absence of DNA, have more ordered tails than in the absence of DNA and enhanced chain mobility. This kind of decoupling of lipid chain mobility and lipid chain order is similar to that observed previously for mixtures of cholesterol and DPPC as a function of temperature.[92]

Summary and Conclusions

Understanding of the detailed, molecular-level, interactions that underlie DNA/lipid association in lipoplexes has the potential to radically improve gene therapy. While a number of investigators have demonstrated the applicability of structure probing techniques (principally X-ray and neutron reflection and diffraction) to these systems, spectroscopic studies, particularly those that directly probe intermolecular interactions, have been much less frequent. The lack of application of these techniques to study lipoplex formation can be partially understood as a result of the difficulty in applying spectroscopic techniques that are bulk sensitive to describe phenomena that are inherently interfacial in nature. Here, we circumvent this problem by application of vibrational sum frequency spectroscopy to study lipid/DNA association. We study the complexation of DNA with lipid head groups indirectly, using the OD stretch of interfacial heavy water molecules as a reporter, and the effect of adsorbed DNA on monolayer structure directly, using the CH stretch modes of the cationic lipid diC14-amidines tail.

We find the most significant differences in spectroscopic response correlate with changes in electrostatics: the amplitude and line shape of the OD stretch of interfacial D_2O beneath cationic lipids is both much more sensitive than that of OD amplitude and line shape underneath the zwitterionic lipid DPPC to increasing concentration of DNA. For the DPPC/DNA/Ca^{2+} system, a response intermediate between that of cationic lipid/DNA and DPPC/DNA is observed, in agreement with prior work that has proposed the effect of Ca^{2+} is to partially neutralize the DPPC phosphate group, making zwitterionic DPPC somewhat positive and thus enabling lipid/DNA binding.[16−19,21]

More detailed examination of the OD stretch response to increasing DNA concentrations in these four systems reveals that, for the cationic lipids, but not for zwitterionic lipids, an additional, high frequency OD peak appears only in the presence of DNA. The presence of this peak can be most easily explained as resulting from an undercoordinated water species. For such a species to be visible in VSF spectra, it must be microscopically ordered (i.e., break inversion symmetry on the molecular length scale). X-ray studies make clear that cationic lipid/DNA systems are well ordered with defined and small interchain spacing of adsorbed DNA.[12,13,15,72] Our water results are consistent with such an ordered environment. In contrast to X-ray studies of DNA/cationic lipid systems, studies of DNA/zwitterionic lipid systems show little adsorption of DNA in the absence of divalent cations and nonspecific adsorption of DNA in the presence of Ca^{2+} with lower interchain ordering.[16−18] The observation that no 2675 cm^{-1} peak is apparent for such systems is thus consistent with the X-ray results here as well.

For the zwitterionic lipids, inspection of spectra collected over a D_2O subphase makes clear that there is no change in center frequency with increasing concentration of DNA, just a decrease in spectral amplitude. For the OD stretch beneath the cationic lipids, the response does change, but this change is difficult to quantify for D_2O in part because of the apparent double peaked nature of this resonance. We surmount this problem by focusing on the change in OD stretch spectrum of HOD with increasing concentration of DNA beneath diC14-amidine. These results make clear that as DNA is added the resonance both decreases in amplitude and shifts to higher frequencies. This observed blue shift is consistent with our conclusions from the emergence of the 2675 cm^{-1} peak for these systems: that the low frequency OD stretch peak reveals information about a structured form of interfacial water that happens only in the presence of DNA.

Finally, we also examined the manner in which DNA adsorption modifies lipid order and packing, for diC14-amidine, by measuring compression isotherms in the presence and absence of DNA and examining the evolution of the CH stretch frequency region as a function of DNA concentration. The π /area experiments demonstrated that, over all lipid densities, the presence of DNA leads to a decrease in surface tension of the lipid monolayer. The spectra of the CH frequency range showed that the effect of adding DNA was to order the lipid tails (on average the diC14-amidine tails are closer to an all trans conformation in the presence of DNA than in its absence). This structural information complements that obtainable by other means (e.g., electron spin resonance) and, taken with those previous studies, presents an intriguing picture in which lipid tail order and chain mobility are decoupled at the temperature of our experiment.

Collectively, our results here both largely confirm expectations from many prior X-ray and neutron studies and provide additional information on the role of interfacial water in DNA/lipid interaction inaccessible with those tools. More generally, these results highlight the manner in which, in the study of DNA/lipid interaction, VSF spectroscopy complements more commonly employed structural probes. We believe the results presented here just scratch the surface of possible applications of VSF spectroscopy to this important system. For example, future application of VSF spectroscopy to DNA/lipid systems should be able to provide definitive evidence for the splitting of DNA duplexes thought to happen at certain cationic lipid

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monolayers (currently inferred from thicknesses of adsorbed DNA measured in X-ray experiments[93]). Further, in more complicated experiments, one might imagine, as recently demonstrated by some of us for simpler systems,[94] probing energy transfer between interfacial water and lipid functional groups in the presence and absence of DNA to gain further insight into how waters local environment changes under these conditions.

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