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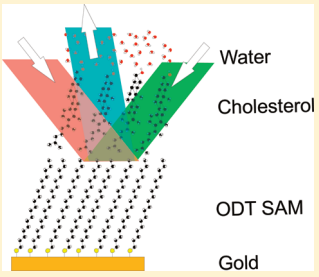
Structure of Mixed Phosphatidylethanolamine and Cholesterol Monolayers in a Supported Hybrid Bilayer Membrane Studied by Sum Frequency Generation Vibrational Spectroscopy

P. J. N. Kett, M. T. L. Casford,* and P. B. Davies

Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom

Supporting Information

ABSTRACT: The structure of hybrid bilayer membranes (HBMs) containing either a pure cholesterol or mixed cholesterol/dipalmitoylphosphatidylethanolamine (DPPE) proximal layer adsorbed onto an octadecanethiol (ODT) self-assembled monolayer (SAM) on a gold substrate have been investigated by sum frequency generation (SFG) spectroscopy. The HBMs were formed by the adsorption of either a pure cholesterol or mixed DPPE/cholesterol monolayer from the air/water interface of a Langmuir−Blodgett trough at surface pressures of 1, 20, or 40 mN·m^{-1}. SFG spectra were also recorded of HBMs where cholesterol was replaced by cholesterol-d_7, in which the terminal isopropyl group of the alkyl chain of cholesterol was isotopically labeled. In order to isolate the contribution to the SFG spectra from the cholesterol in the mixed cholesterol/phospholipid films, DPPE-*d* was used, in which the alkyl chains of the phospholipid were deuterated. The infrared spectra of solvent-cast cholesterol and cholesterol-d_7 films were recorded to aid with assignment of the SFG spectra of the HBMs. Features corresponding to methyl, methylene, and methine stretches of cholesterol were identified in the SFG spectra. Information on the polar orientation of SFG-active groups was obtained from the phases of the spectral features. The structure of the HBMs showed little dependence on the surface pressure at which they were formed. SFG spectra of HBMs with a mixed cholesterol/DPPE proximal layer were very similar to the spectra of HBMs with a pure cholesterol proximal layer, although the features in the spectra were more intense than anticipated for a film with half the number of cholesterol molecules, indicating that DPPE did have some effect on the orientation of cholesterol molecules in the film.



INTRODUCTION

Supported lipid membranes are frequently used as simple model systems for cell membranes.[1] They provide a template for studying static and dynamic structural changes as the molecular composition or temperature is altered. Hybrid bilayer membranes (HBMs) are a class of supported lipid membranes that comprise a distal monolayer chemically bound to the supporting substrate and a physisorbed or chemisorbed phospholipid proximal monolayer.[2−6] The structure of either monolayer can be investigated by making them chemically or isotopically distinguishable from each other.

In the present study, the structure of cholesterol and mixed cholesterol/phosphatidylethanolamine (DPPE) monolayers forming the proximal leaflet of an HBM is investigated by sum frequency generation (SFG) vibrational spectroscopy, an interface-specific technique. The distal component of the bilayer is a self-assembled monolayer of octadecanethiol (ODT) covalently bound to a planar gold substrate. The structure of either monolayer in the HBM can be selectively probed by SFG by suitable isotopic labeling (H/D) of the relevant layer.

SFG spectroscopy provides a powerful method for probing lipid monolayer film structure at the air/solution, solid/solution, and solid/air interfaces. In the SFG technique, two pulsed laser beams, one at a fixed frequency in the visible region and the other tunable in the infrared, are combined temporally and spatially at the interface under study. As a result of the nonlinear optical phenomenon of SFG, light is emitted from the interface with a frequency at the sum of the two input frequencies.[7] The vibrational spectrum of the molecules adsorbed at the interface is recorded by measuring changes in the SFG beam intensity as the infrared laser frequency is tuned across the relevant frequency range, in the present case across the C−H vibrational stretching region between 2800 and 3000 cm^{-1}. The molecules comprising the interfacial layer must have an overall net polarization for their vibrational modes to be SFG-active. Consequently the vibrational modes of isotropically ordered molecules are SFG-inactive. The SFG selection rule requires vibrational modes to be both infrared- and Raman-active, and therefore as a result of the rule of mutual exclusion the vibrational modes of molecules that have an overall center of symmetry or of functional groups that are locally centrosymmetric are SFG-inactive.

SFG has previously been used to study the interactions within mixed cholesterol and dipalmitoylphosphatidylcholine (DPPC) monolayers and bilayers at the air/solution interface and within supported lipid membranes. Using SFG, Levy and Briggman[6] have shown that the incorporation of cholesterol into a phospholipid

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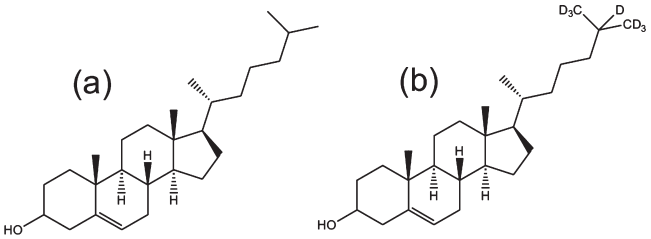

Figure 1. Structures of (a) cholesterol and (b) cholesterol-d_7.

layer increases the phase-transition temperature of a DPPC HBM. Bonn et al.[8] and Ohe et al.[9] have shown that introducing cholesterol into a loosely packed monolayer of DPPC at the air/water interface increases the conformational and orientational ordering of the phospholipid alkyl chains. They demonstrated that this effect was least pronounced for monolayers at high surface pressures where the DPPC monolayer was already well ordered. The distribution of cholesterol and distearoylphosphatidylcholine (DSPC) between the proximal and distal leaflets of a lipid bilayer on silica and sapphire supports has been shown by Liu and Conboy[10] to be dependent on both the temperature and composition of the lipid bilayer.

In the present work we have used SFG to study the structure of pure cholesterol and mixed cholesterol/DPPE monolayers physisorbed from the air/water interface of a Langmuir−Blodgett (LB) trough onto an ODT monolayer at three different surface pressures: 1, 20, and 40 mN·m^{-1}. Examining the SFG spectra of a series of monolayers adsorbed at different surface pressures allows for the effect of packing density on the orientation of the cholesterol within the lipid layer to be investigated. Spectra were recorded of both cholesterol and the isotopologue cholesterol-d_7, in which the isopropyl group of cholesterol was deuterated (Figure 1). The SFG spectra of pure cholesterol or cholesterol-d_7 monolayers were recorded so that the spectral features could be assigned without any possible interference from the phospholipid molecules. It also meant that any changes to the spectra upon addition of DPPE could be observed. The mixed cholesterol/DPPE monolayers contained an equimolar concentration of cholesterol and lipid. This is a higher cholesterol concentration than is typically found in a biological cell membrane; however, at lower cholesterol concentrations there is phase separation into micrometer-sized cholesterol-rich and DPPE-rich domains.[11] The analysis of SFG spectra of HBMs containing lower concentrations of cholesterol would be complicated by contributions to the spectra from cholesterol-rich and cholesterol-poor domains, as these micrometer-sized domains are typically much smaller than the area sampled by the SFG technique. The focus of this investigation is therefore on HBMs containing equimolar concentrations of cholesterol and DPPE, when only a single, homogeneous phase is present.

EXPERIMENTAL SECTION

Chloroform (≥99.9%) and cholesterol (≥99%) were purchased from Aldrich. Cholesterol-25,26,26,26,27,27,27-d_7 was purchased from CK Gas Products (≥98%), and 1,2-dipalmitoyl-d_{62}-*sn*-glycero-3-phosphatidylethanolamine (DPPE-*d*) was purchased from Avanti Polar Lipids (Alabaster, AL). A sample of ODT-*d* was custom-synthesized by Dr. Robert Thomas of the University of Oxford. The cholesterol and DPPE were used as received. The silicon wafers, glassware, and stainless steel liquid cell were cleaned before use by placing them in a detergent solution (Decon 90) overnight, rinsing 20 times in ultrapure water (resistivity >18.2 MΩ·cm), leaving them overnight in concentrated nitric acid, and finally rinsing again in ultrapure water. The O-rings and fused silica prism were cleaned in detergent solution and rinsed in ultrapure water.

The substrates were formed by thermal evaporation of a 200 nm gold layer onto precleaned silicon wafers, which had been primed with a thin layer of chromium (∼10 nm). The gold surfaces were rendered hydrophobic by placing them into methanolic solutions of ODT-*d* (concentration ∼millimolar) for a minimum of 12 h.

The HBMs were prepared by the transfer of a single layer of phospholipid and/or cholesterol from the air/water interface of a Langmuir−Blodgett (LB) trough (Nima 611, Coventry, U.K.) by a modified Langmuir−Schaefer (LS) method as described in detail in ref 5. A custom-built stainless steel liquid cell with a fused silica equilateral prism attached was submerged into the ultrapure water subphase (resistivity >18.2 MΩ·cm) of the LB trough prior to film preparation. The DPPE and cholesterol were dissolved separately in chloroform (1 mg/mL) and the DPPE was gently warmed to approximately 30 °C to ensure the phospholipid had completely dissolved. The DPPE and cholesterol were premixed before being spread on the water surface of the LB trough and left for 30 min to allow the solvent to evaporate. The barriers of the trough were then compressed to the required surface pressure. An ODT-covered gold substrate was held parallel to the water surface on the dipping mechanism of the LB trough by an aspirator pump before being lowered onto the trough surface at a rate of 1 mm/min and contacted on the surface for 30 s. Instead of following the conventional LS procedure and withdrawing the sample back into air, the substrate was slowly pushed through the monolayer into the water subphase. Once the sample was fully submerged, the remaining phospholipid and/or cholesterol was drawn off the water surface. The sample was then slowly lowered into the awaiting liquid cell until there was an approximately micrometer-thick layer of water between the sample surface and the prism. Once the sample had been pushed through the air/water interface, it remained submerged in the pure water subphase such that it was never exposed to air. All the spectra reported here were recorded under water. The back plate of the liquid cell was screwed down to seal the cell. The liquid cell containing the sample was removed from the LB trough, and the sum frequency spectrum was recorded. The samples were formed and the spectra were recorded at 20 °C, which is well below the gel−liquid and lamellar-inverted hexagonal phase-transition temperatures for DPPE in aqueous solution.[12,13]

The SFG spectra were recorded on the Cambridge nanosecond spectrometer, details of which can be found elsewhere.[14] The infrared (11.5 Hz, 10 ns, 1.8 mJ/pulse at 2900 cm^{-1}) and visible (532 nm, 0.8 mJ/pulse, resolution <1 cm^{-1}) laser beams were aligned in a counterpropagating geometry, such that their angles of incidence at the gold/water interface were 60° and 65° respectively. The beams were focused through the fused silica prism to generate spot sizes of ∼1 mm^2. Spectra were recorded in the C−H (2800−3000 cm^{-1}) stretching region in both the PPP (SFG, visible, infrared) and SSP polarization combinations. At least 20 scans were averaged to produce the final spectra. The spectra were normalized and then modeled by use of a least-squares Levenberg−Marquardt algorithm to fit the resonance profiles to a Lorentzian description of the second-order

susceptibilities (see Supporting Information).[15] The spectra were recorded at least twice to ensure reproducibility.

The P-polarized attenuated total reflection (ATR) spectra of cholesterol and cholesterol-d_7 were recorded on a Perkin-Elmer Spectrum 100 FT-IR spectrometer fitted with a liquid nitrogen-cooled mercury−cadmium−telluride (MCT) detector, by casting films from chloroform onto a germanium ATR crystal. At least 32 scans were averaged to produce the final spectra.

RESULTS AND DISCUSSION

ATR Infrared Spectra of Cholesterol and Cholesterol-d_7 Films. To aid the assignment of resonances in the SFG spectra, the infrared spectra of cholesterol and cholesterol-d_7 solvent-cast films were recorded in the C−H stretching region (Figure 2 and Table 1). The five prominent bands at 2850, 2868, 2882, 2901, and 2932 cm^{-1} shown in Figure 2 are at almost identical frequencies to the bands in the infrared spectrum of thiocholesterol recorded by Yang et al.[17] and the Raman spectrum of anhydrous cholesterol recorded by Faiman[16] (Table 1). Additionally there are weak shoulders in the ATR spectra at approximately 2827 and 2983 cm^{-1} and a much more pronounced shoulder at ~2960 cm^{-1}. There is also evidence of a weak feature at 3040 cm^{-1} (not shown in Figure 2). In order to identify differences in the intensities of individual features in the ATR spectra in Figure 2, the spectra were deconvoluted into a set of Gaussian curves (see Supporting Information, Figure S1) with their centers at the frequencies of the peaks and shoulders listed above and in Table 1. The deconvolution showed that the intensities of the bands at 2850, 2882, and 2932 cm^{-1} along with the shoulders at 2827 and 2983 cm^{-1} were essentially identical in the spectra of both compounds. In contrast, the bands at 2868 and 2901 cm^{-1} along with the shoulder at ~2960 cm^{-1} were all weaker in the spectrum of cholesterol-d_7.

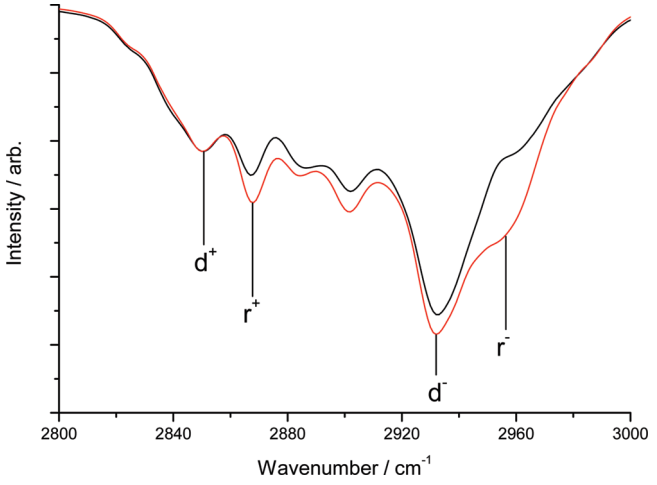

Figure 2. IR spectra of solvent-cast cholesterol (red) and cholesterol-d_7 (black) films on a germanium ATR crystal normalized to the cholesterol peak at 2850 cm^{-1}.

On the basis of the infrared spectra of linear alkanes, the bands at 2850 and 2932 cm^{-1} in the ATR spectra can be assigned to the symmetric (d^+), and asymmetric methylene stretches (d^-), respectively, of cholesterol.[18] d^+_{FR} is likely to be contributing to the band at 2882 cm^{-1} as this resonance is typically around 2890 cm^{-1} in the infrared spectra of alkanes.[18] The shoulder at 2960 cm^{-1} in the ATR spectra of cholesterol, which shows significant changes in the spectrum of cholesterol-d_7, can be assigned to the asymmetric methyl stretch (r^-). It should be noted that, in order to accurately fit the ATR spectra, the r^- resonance was placed at 2955 cm^{-1} in the spectrum of cholesterol and at 2961 cm^{-1} in cholesterol-d_7. The fitted positions of all the other bands were the same in both spectra to within 1 cm^{-1}. The band at 2868 cm^{-1}, the intensity of which is reduced in the spectrum of cholesterol-d_7, can be unambiguously assigned to the methyl symmetric stretch (r^+). The corresponding r^+_{FR} band, usually observed at around 2930 cm^{-1} in the infrared spectra of linear alkanes, is likely to be contributing to the band assigned above to the d^- resonance.[18]

The band at 2901 cm^{-1} is weaker in the ATR spectrum of cholesterol-d_7 than in the spectrum of cholesterol. In previous Raman spectroscopic studies of cholesterol analogues, a feature around this frequency has been assigned to an asymmetric methylene stretch or alternatively to an angular methyl stretching mode of groups bonded directly to the sterol ring system.[16,19,20] Yang et al.[17] tentatively assigned a band at 2903 cm^{-1} in the transmission spectrum of thiocholesterol to a methine stretch

Table 1. Frequencies and Assignments of Infrared and Raman Spectra of Cholesterol and Cholesterol Analogues in the C−H Stretching Region

infrared spectra				Raman spectra					
cholesterol[a]		thiocholesterol[17]		anhydrous cholesterol[16]		cholesteryl chloride[19]		cholesteryl formate[20]	
frequency/cm^{-1}	assignment	frequency/cm^{-1}	assignment	frequency/cm^{-1}	assignment	frequency/cm^{-1}	assignment	frequency/cm^{-1}	assignment
2827[b]	d^+	2824				2828	d	2821, 2832	d^+
2850	d^+	2848	d^+	2850	d^+	2850	d^+	2841, 2853	d^+
2868	r^+	2867	r^+	2868	r^+	2861	r^+	2864, 2881	r^+
2882	d^+_{FR}			2886		2891	R_3C-H	2896	d^-
2901	R_3C-H	2903	R_3C-H	2902−2910	d^-	2905	r (ring CH_3 group)	2910	d^-
2932	d^-	2932	d^-	2936	d^+, r	2930	d^-	2934	d^-
2960[b]	r^-	2961	r^-	2950	r^+	2960	r^-	2959	r^-
2983[b]		2976						2971	r^-
3040	=C−H	3032	=C−H	3050	=C−H	>3000	=C−H	3029	=C−H

[a] This work, Figure 2. [b] Appears as a shoulder on the main peaks.

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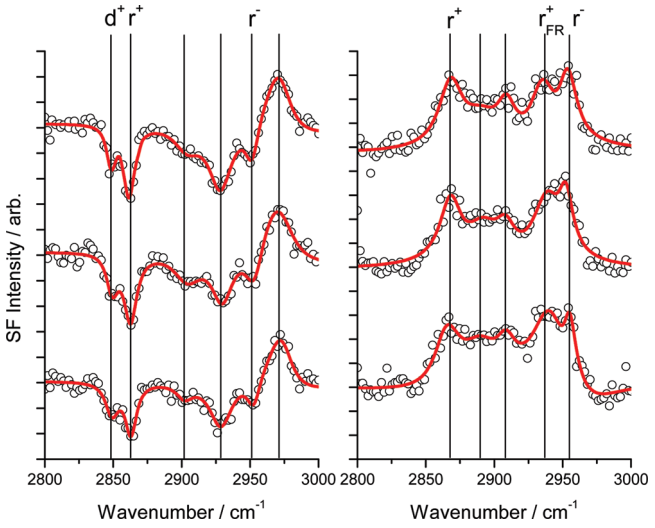

Figure 3. SFG spectra of HBMs comprising monolayers of cholesterol on ODT-*d* recorded in PPP (left-hand spectra) and SSP (right-hand spectra) polarization combinations. The spectra, from bottom to top, correspond to films adsorbed at surface pressures of 1, 20, and 40 mN·m^{-1}. Assignments of the peaks are discussed in the text.

(that is, a C−H stretch of the R_3C−H group where R denotes any functional group aside from a hydrogen atom). A definitive assignment of this band is not possible although it is most likely that there is a significant contribution to this feature from the methine stretch on the grounds that one of the methine groups in the molecule is deuterated in cholesterol-d_7 and that this mode is typically around 2900 cm^{-1} in infrared spectra.[21]

The remaining unassigned features in the ATR spectra of the two cholesterol compounds are the weak shoulders at 2827 and 2983 cm^{-1} along with the weak feature at 3040 cm^{-1}. The latter, which is observed at the same frequency in the spectra of cholesterol and cholesterol-d_7, can be assigned to the olefinic C−H stretch of cholesterol. The assignment of the other features is not as straightforward, although it should be noted that, in the infrared spectrum of thiocholesterol recorded by Yang et al.,[17] there were similar features at 2824 and 2976 cm^{-1}. Bresson et al.[20] observed a peak in the Raman spectrum of cholesteryl formate at 2832 cm^{-1} that they assigned to a d^+ resonance. Similarly, Bulkin and Krishnan[19] recorded a peak at 2828 cm^{-1} in the Raman spectrum of cholesteryl chloride that they assigned to an unspecified d resonance. These results would imply that the shoulder at 2827 cm^{-1} in the ATR spectra should be assigned to a d^+ resonance, although it is not clear which methylene groups are responsible for this shoulder and why it is so much lower in frequency than a typical d^+ band. No peaks in the Raman spectrum of cholesterol or other cholesterol analogues have been reported as high as the shoulder at 2983 cm^{-1} in the ATR spectra in Figure 2. However, an unassigned peak at 2990 cm^{-1} has been observed in the SFG spectrum of a cholesterol monolayer on sapphire.[10]

Measured SFG Spectra of Cholesterol and Cholesterol-d_7 HBMs. Figure 3 presents SFG spectra in the C−H stretching region, in both PPP and SSP polarizations, of HBMs comprising cholesterol monolayers deposited at film pressures of 1, 20, and 40 mN·m^{-1} onto ODT-*d* monolayer substrates. Spectra were recorded between 2800 and 3100 cm^{-1}, although only the region between 2800 and 3000 cm^{-1} is presented, as no spectral features were observed above 3000 cm^{-1}. The PPP and SSP spectra provide complementary information on the structure of the cholesterol component of the bilayer. The SSP spectrum probes only a single component of the resonant nonlinear susceptibility, $\chi^{(2)}_{yyz}$, and so can be used to determine the absolute polar orientation of a functional group. On a gold surface and by use of a counterpropagating beam geometry, peaks in the SSP spectrum arise from functional groups that have a net polar orientation toward the sample surface, while dips are from functional groups oriented away from the surface. On the other hand, PPP spectra probe four independent components of the nonlinear susceptibility. However, the most significant contributions to $\chi^{(2)}_{PPP}$ are from $\chi^{(2)}_{zzz}$ and $\chi^{(2)}_{xxz}$, both of which probe vibrational transition moments parallel to the surface normal. Thus the phase of the resonant susceptibility of C−H resonances in a PPP spectrum is usually a significant indication of polar orientation. Dips in the PPP spectrum are indicative of groups oriented toward the surface, and peaks are indicative of groups oriented away from the surface and along the surface normal.

The PPP spectra of the cholesterol HBMs can be modeled with a series of dips at 2849, 2863, 2904, 2929, and 2953 cm^{-1} along with a peak at 2973 cm^{-1} (Table 2). The spectra were recorded on at least two occasions, and the dips and peaks that have been modeled are those that consistently appeared in the spectra. ODT-*d* has no SFG-active modes in the C−H stretching region and therefore these spectral features must be due to vibrational modes of cholesterol (see Supporting Information, Figure S2). Aside from the dip at 2904 cm^{-1} that displays some variability in position and intensity between the spectra, the positions of the peaks and dips show no significant dependence on film formation surface pressure. The intensities of individual features in the spectra increase very slightly with the surface pressure at which the film was formed. The intensities of the resonances did not increase systematically with surface pressure (i.e., some increased more than others), but on average they did increase by approximately a third when the 1 and 40 mN·m^{-1} spectra are compared. This increase can largely be explained by the fact that more molecules are being probed at higher surface pressures, although small changes in the structure of the film upon adsorption to the ODT-*d* film cannot be entirely ruled out (see Supporting Information).

The more intense features of the SSP spectra can be modeled with three peaks at 2867, 2937, and 2953 cm^{-1}, although the latter two peaks are incompletely resolved. A further weaker peak can be modeled at 2906 cm^{-1} (which appears to correspond to the dip at 2904 cm^{-1} in the PPP spectrum). A much weaker peak can be modeled at 2888 cm^{-1} but only in HBMs formed at lower surface compressions. The positions of all these peaks do not appear to vary significantly with film pressure, although there are some nonsystematic changes in their relative intensities. The peaks in the SSP spectra are on average approximately a third more intense in the 40 mN·m^{-1} film than in the 1 mN·m^{-1} film (see Supporting Information). This is in agreement with the PPP spectra, in which it was shown that such an increase can largely be attributed to more molecules being probed at higher surface pressures. The changes in intensity of the individual features in the SSP spectra are more variable, which in part may be due to the difficulties associated with modeling SSP spectra. For gold surfaces in the SSP polarization combination, the nonresonant susceptibility is much smaller than in the PPP polarization combination, making the SFG signals noticeably weaker and the signal-to-noise ratios much smaller. This makes it more difficult to accurately model fine details in the spectra as

Table 2. Frequencies and Assignments of SFG Spectra of Monolayers of Cholesterol and Cholesterol-d_7 in the C−H Stretching Region

cholesterol monolayer on ODT-*d* SAM[a]		cholesterol monolayer on ODT-*d* SAM[b]		cholesterol monolayer at the air/water interface[b,9]		cholesterol-d_7 monolayer on sapphire[b,10]	cholesterol monolayer on silica[b,10]	
frequency/ cm^{-1}	assignment	frequency/ cm^{-1}	assignment	frequency/ cm^{-1}	assignment	frequency/ cm^{-1}	frequency/ cm^{-1}	assignment
				~2820		2828	~2826	
2849	d^+			2850	d^+	2843	2856	d^+
						2866		
2859[c]	r^+ (sterol CH_3 groups)	2860[c]	r^+ (sterol CH_3 groups)				2876[c]	r^+
2863[d]	r^+ (isopropyl group)	2867[d]	r^+ (isopropyl group)	2878	r^+	2878	2880[d]	r^+
		2888						
2904	d^+_{FR} and R_3C-H	2906		~2910		2910	2912	d^-
2929	r^+_{FR} and d^-	2937	r^+_{FR}	2940	r^+_{FR}	2939	~2949	
2953[d]	r^-	2953	r^-	2960	r^-	2955	2964	
2973						2990		

[a] Recorded in the PPP polarization combination. [b] Recorded in the SSP polarization combination. [c] Resolvable only in spectra of cholesterol-d_7. [d] Absent from spectra of cholesterol-d_7.

exemplified by the feature at 2906 cm^{-1}. Nevertheless, the SSP spectra allow a comparison to be made with the cholesterol spectra of Ohe et al.[9] and Liu and Conboy,[10] obtained in the same polarization combination at the air/water and air/silica interfaces, respectively (see Table 2). Ohe et al.[9] reported bands of pure cholesterol monolayers at 2850, 2878, 2940, and 2960 cm^{-1}. Weaker unassigned features in their spectra are also evident at ~2820 and ~2910 cm^{-1} at all three surface pressures they used. Bands in the spectra recorded by Liu and Conboy[10] occur at ~2826, 2856, 2880, 2912, 2949, and 2964 cm^{-1}. Our spectra recorded with lower signal-to-noise ratios are in satisfactory agreement with both these studies, although the peak at ~2820/2826 cm^{-1} is too weak to detect in our spectra. Furthermore, the band at 2867 cm^{-1} in our spectra falls between two resolved bands in the spectra of Ohe et al.[9] (2850 and 2878 cm^{-1}) and Liu and Conboy[10] (2856 and 2880 cm^{-1}), which suggests that in our spectrum it is an amalgamation of both these bands.

The PPP and SSP SFG spectra of monolayers of cholesterol-d_7 adsorbed onto a ODT-*d* SAM at 1, 20, and 40 $mN \cdot m^{-1}$ are shown in Figure 4, along with the corresponding spectra of cholesterol at 20 $mN \cdot m^{-1}$ (taken from Figure 3) for comparison purposes. There are some notable differences between the spectra of cholesterol-d_7 and cholesterol. In the PPP spectra, the first difference is that the dip at 2863 cm^{-1} is much weaker and is shifted down in frequency by 4 cm^{-1} to 2859 cm^{-1}. It is now much less intense than the neighboring dip just below 2849 cm^{-1}, which has the same frequency and intensity in the spectra of both compounds. The second difference is that the dip at 2929 cm^{-1} is less intense in cholesterol-d_7 than in cholesterol. Third, the dip at 2953 cm^{-1} is significantly weaker in the spectra of cholesterol-d_7 and is effectively absent.

The SSP spectra of cholesterol and cholesterol-d_7 are more similar to each other than the corresponding PPP spectra. The most notable difference between them is the reduction in intensity and red shift, by 7 cm^{-1}, of the peak centered at 2867 cm^{-1} in the spectra of cholesterol. Liu and Conboy[10] noted a significant decrease in intensity of the peak at 2880 cm^{-1} and a small shift (from 2880 to 2876 cm^{-1}) in its position in cholesterol-d_7 on silica. This supports the hypothesis above that the peak at 2867 cm^{-1} in our spectrum of cholesterol is actually an amalgamation of the two resolved peaks reported by Ohe et al.[9] and by Liu and Conboy.[10]

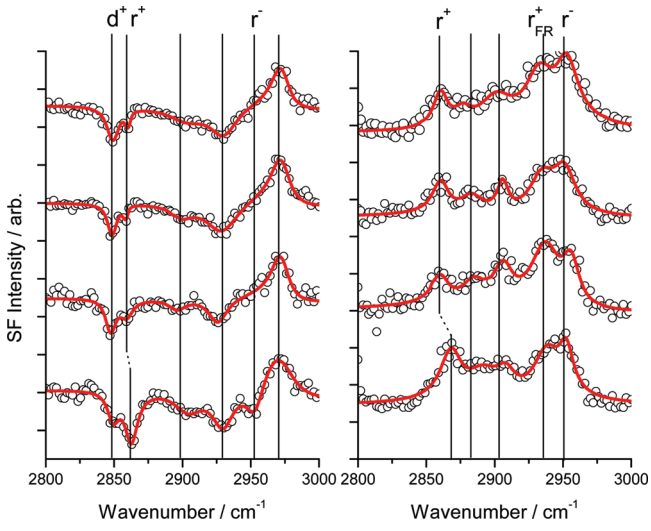

Figure 4. SFG spectra of HBMs comprising monolayers of cholesterol-d_7 on ODT-*d* in PPP (left-hand spectra) and SSP (right-hand spectra) polarization combinations. The lowermost spectra are of 20 $mN \cdot m^{-1}$ cholesterol films reproduced from Figure 3 for comparison purposes. The remaining spectra, from bottom to top, correspond to cholesterol-d_7 films adsorbed at surface pressures of 1, 20, and 40 $mN \cdot m^{-1}$.

Assignment of SFG Spectra of Cholesterol. If the SFG spectra of phospholipids and linear alkanes are used as a guide, the dip at 2859 cm^{-1} in the PPP spectra of cholesterol-d_7 (Figure 4) can be assigned to the r^+ resonance of the three nondeuterated methyl groups of the molecule.[5,7] Similarly, the dip at 2863 cm^{-1} in the PPP SFG spectra of cholesterol can be assigned to the r^+ resonance of the isopropyl group. It must also have some contribution from the symmetric stretch of the other methyl groups of the molecule. The SFG spectra indicate that the r^+ resonance of the methyl groups either close to or directly on the sterol ring system are ~4 cm^{-1} lower in frequency than the

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r^+ resonance of the isopropyl group. There was no evidence for different symmetric methyl stretching frequencies in the ATR spectra of cholesterol, as the r^+ resonance was identified at 2868 cm^{-1} in the spectra of both cholesterol compounds. However, in the Raman spectrum of cholesteryl formate recorded by Bresson et al.,[20] two different methyl symmetric stretches were identified at 2864 and 2881 cm^{-1}. It should be noted, though, that the difference in frequency between these two r^+ resonances is much greater than the ~4 cm^{-1} in the SFG spectra of the cholesterol HBMs. When Liu and Conboy[10] recorded the SFG spectra of monolayers of cholesterol and cholesterol-d_7 on silica in the SSP polarization combination, they also noted a 4 cm^{-1} difference in position of the r^+ resonance in spectra of the two compounds. However, the r^+ resonances in their spectra were 15 cm^{-1} higher in frequency than in the PPP spectra of cholesterol HBMs presented in Figures 3 and 4. Similarly, the r^+ resonance in the SSP SFG spectra of a monolayer of cholesterol at the air/water interface recorded by Ohe et al.[9] was also higher in frequency at 2878 cm^{-1}.

The broad feature at 2929 cm^{-1} in the PPP spectra of cholesterol and cholesterol-d_7 can be assigned to the r^+_{FR} of all five cholesterol methyl groups. Evidence for this is the decrease in intensity of this dip in the SFG spectra of cholesterol-d_7 where two of the methyl groups have been deuterated.

In the PPP spectra, the dip at 2953 cm^{-1}, which is virtually absent in the spectra of cholesterol-d_7, can be assigned to the r^- resonance. This is in a similar position to the feature at 2960 cm^{-1} that Ohe et al.[9] assigned to the r^- resonance in the SSP spectra of cholesterol at the air/water interface. Likewise, the same resonance has been observed at 2960 cm^{-1} in both the infrared spectrum of cholesterol (Figure 2) and Raman spectra of cholesteryl chloride and cholesteryl formate.[19,20]

The dip at 2849 cm^{-1} in the PPP spectra of cholesterol and cholesterol-d_7 can be assigned to the d^+ resonance.[7] This is supported by the assignment of a band at 2850 cm^{-1} in the infrared spectrum to the symmetric methylene stretching mode (Figure 2). The position of this resonance is in good agreement with the SFG spectra of cholesterol in the SSP polarization combination obtained by Liu and Conboy[10] (2856 cm^{-1}) and Ohe et al.[9] (2850 cm^{-1}). The d^+_{FR} resonance is typically a very broad feature in the PPP SFG spectra of molecules adsorbed on hydrophobic gold substrates, usually appearing somewhere between 2890 and 2930 cm^{-1}.[7] It is therefore to be expected that the dip at 2904 cm^{-1} in the PPP spectra of both cholesterol and cholesterol-d_7 contains a contribution from this resonance. There is also likely to be a contribution to the dip at 2904 cm^{-1} from the methine CH stretching mode. It has been observed at 2902 cm^{-1} in both the SFG spectra of leucine (an amino acid containing the isopropyl moiety), the infrared transmission spectrum of thiocholesterol, and the ATR spectra of cholesterol (Figure 2).[22] In cholesterol-d_7, one of the methine groups is deuterated, and thus this feature ought to be weaker than in the spectrum of cholesterol. Unfortunately, the dip at 2904 cm^{-1} in Figure 3 shows significant variation in intensity and position between the spectra of cholesterol films adsorbed at different surface pressures, making it difficult to confirm whether or not the intensity of this feature is isotope-dependent.

The d^- resonance is usually very weak or not observed at all in SFG spectra due to the considerable differences in the position of this mode in linear infrared and Raman spectra.[7] However, in contrast, the d^- resonance of cholesterol may be SFG-active as this mode has been observed at similar positions in the infrared and Raman spectra. It occurs at 2932 cm^{-1} in the linear infrared spectrum (Figure 2) and at 2930 and 2934 cm^{-1} in the Raman spectra of cholesteryl chloride and cholesteryl formate, respectively. The dip in the PPP spectra at 2929 cm^{-1}, which has been assigned above to the r^+_{FR}, may therefore also contain a contribution from the d^- resonance.

The remaining unassigned feature in the PPP spectra is the peak at 2973 cm^{-1}. This feature occurs consistently at the same position in the PPP spectra of both cholesterol and cholesterol-d_7. It is noticeably absent from the SSP spectra (Figure 3) and, significantly, in the SSP spectra recorded by Liu and Conboy[10] and by Ohe et al.[9] Its absence from the SSP spectrum would suggest that it arises from a vibrational mode that has its transition moment orientated parallel to the metal surface. The shape of this feature in the PPP spectra is clearly influenced by the presence or absence of the dip at 2953 cm^{-1} from the r^- mode. This peak is tentatively assigned to a CH vibrational mode of the sterol ring system. The same feature appeared as a dip in the PPP SFG spectrum of a self-assembled monolayer of thiocholesterol on gold in air (see Supporting Information, Figure S3). The change in phase of this feature relative to cholesterol is due to thiocholesterol and cholesterol having different polar orientations. A feature appearing at 2970 cm^{-1} in the PPP SFG spectrum of thiocholesterol indicates that the same feature in the spectrum of cholesterol cannot be due to either an O−H stretch of the hydroxyl group of cholesterol nor an O−H stretch of ordered water in the vicinity of the HBM. Likewise, this feature being out of phase with all the other resonances in the SFG spectrum cannot be a result of interference between a C−H stretching mode of cholesterol and an O−H stretch of cholesterol or water. Identifying precisely which vibrational mode or modes give rise to this feature in the PPP spectra and understanding why it appears to be out of phase with all of the other resonances is the focus of an ongoing investigation.

There was no evidence in the PPP SFG spectra of the olefinic C−H stretch of cholesterol. The spectra were recorded up to 3100 cm^{-1}, but no spectral features above 3000 cm^{-1} could be modeled. In the ATR spectrum of cholesterol, the olefinic C−H stretch at 3040 cm^{-1} was markedly less intense than any of the other bands in the spectrum. It was, for example, only $^1/_{15}$ as intense as the band assigned to the d^+ resonance. In the Raman spectrum of cholesterol recorded by Faiman,[16] the olefinic C−H band was also very weak. The fact that there was no feature in the SFG spectra corresponding to this vibrational mode can be attributed to its inherently weak infrared and Raman transition moments.

The peak in the SSP spectra of cholesterol at 2867 cm^{-1} can be assigned to the r^+ resonance of the isopropyl group of cholesterol. Likewise, the peak at 2860 cm^{-1} in the SSP spectra in Figure 4 can be assigned to the three protonated methyl groups of cholesterol-d_7.[7] As with the PPP spectra, these features are somewhat lower in frequency than the corresponding resonances in the SFG spectra of cholesterol and cholesterol-d_7 that have been recorded previously. The peak at 2937 cm^{-1} can be assigned to the corresponding r^+_{FR}. The neighboring peak at 2953 cm^{-1}, which is more prominent in the spectrum of cholesterol than cholesterol-d_7, can be assigned to the r^- resonance on the basis of its proximity to the position of the dip in the PPP spectra assigned above to this resonance. The poorly resolved peaks at 2888 and 2906 cm^{-1} are likely to contain contributions from the d^+_{FR} and methine resonances. The d^+ resonance, which is at 2849 cm^{-1} in the PPP spectra of cholesterol, cannot be resolved in the SSP spectra.

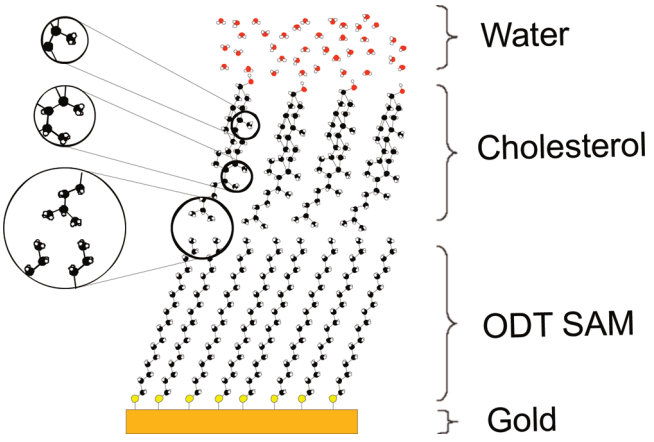

Figure 5. Schematic showing the orientation of cholesterol in HBMs. The methyl groups of the sterol ring and the isopropyl group are shown with a net polar orientation toward the ODT SAM and gold surface.

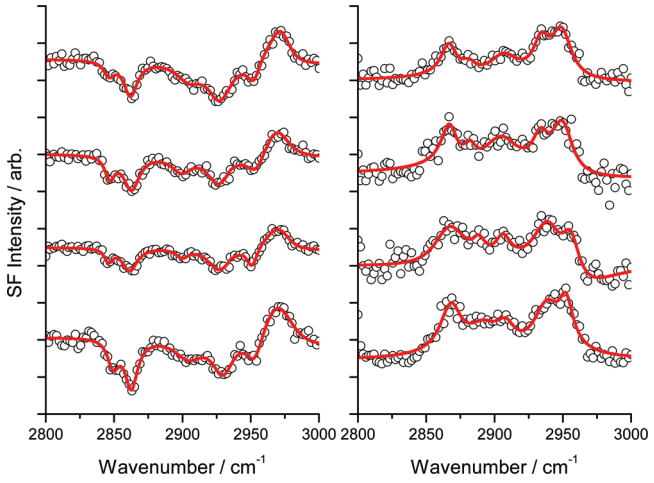

Figure 6. SFG spectra of HBMs comprising a monolayer of DPPE-*d* and cholesterol in a 1:1 molar ratio in PPP (left-hand spectra) and SSP (right-hand spectra) polarization combinations. For comparison purposes, the lowermost spectra are of a pure cholesterol monolayer adsorbed at 20 mN·m^{-1} reproduced from Figure 3. The remaining spectra, from bottom to top, correspond to mixed DPPE-*d*/cholesterol layers adsorbed at 1, 20, and 40 mN·m^{-1}.

Structural Interpretations of SFG Spectra of Cholesterol HBMs. SFG spectra recorded in the SSP polarization combination of HBMs containing a pure cholesterol proximal layer show many similarities to those that have been previously recorded (Table 2). Discrepancies between the positions and relative intensities of the features in our HBM spectra and those recorded by Liu and Conboy[10] and Ohe et al.[9] can largely be attributed to the different surfaces on which the cholesterol was adsorbed and whether the cholesterol monolayer was in contact with air or water. In this study, SFG spectra were recorded for cholesterol HBMs on a metal surface. This allows the phases of the resonances and hence the polar orientation of the SFG-active groups to be determined directly from the spectra. A comparison of the PPP spectra in Figures 3 and 4 indicates that the net contribution of the isopropyl group of cholesterol is a series of methyl resonances at 2863, 2929, and 2953 cm^{-1}. The r resonances appearing as dips in the PPP spectra are a strong indication that the isopropyl group of cholesterol is oriented toward the ODT SAM and gold surface. Further conformation of this comes from the SSP spectra, where the r^{+} resonance of the same functional group appears as a peak. The r resonances of cholesterol-d_7 are also all dips in the PPP spectra and peaks in the SSP spectra, indicating that the three protonated methyl groups of cholesterol-d_7 have the same net polar orientation as the isopropyl group and are also pointing toward the ODT SAM and gold surface. The d resonances of cholesterol appear as dips in the PPP spectra, indicating that the SFG-active methylene groups also have a net polar orientation toward the ODT SAM. Confirmation of this orientation cannot be obtained from the SSP spectra as the d resonances are not sufficiently well-resolved for the phase of the resonances to be determined unambiguously. There was no evidence above 3000 cm^{-1} of the olefinic C−H stretch and therefore this vibrational mode cannot be used to help determine the orientation of the cholesterol sterol ring system. Based upon the information on the orientation of cholesterol molecules that could be determined from the spectra in Figures 3 and 4, a possible structure of the cholesterol HBM is presented in Figure 5. The diagram shows the cholesterol molecules packed closely together, as would be anticipated for a monolayer adsorbed in the liquid condensed phase, the isopropyl group oriented toward and in close proximity to the ODT SAM, and the sterol ring system aligned predominantly along the surface normal, although tilted slightly such that the methyl groups are pointing toward the metal surface. It was not possible to determine the orientation of the cholesterol hydroxyl group from the SFG spectra that have been recorded. However, as it is the only hydrophilic part of the molecule, it is logical to assume that it will be interacting with the water layer that is in contact with the HBM.

SFG Spectra of Mixed Cholesterol/Lipid HBMs. Figure 6 shows the PPP and SSP SFG spectra of HBM films formed from 1:1 cholesterol/DPPE-*d* mixtures at the same surface pressures as presented in Figure 3. The DPPE-*d* molecule has no SFG-active modes in the C−H stretching region and thus only the cholesterol molecules contribute to the spectra.[5] There are some differences with the spectra for the pure cholesterol monolayers shown in Figure 3. The positions of the peaks and dips in the spectra of pure cholesterol and mixed cholesterol/DPPE-*d* monolayers are very similar for both polarization combinations, although they are noticeably weaker in the spectra of the mixed HBMs. The amount by which the features decrease in intensity is somewhat variable, although on average they are half as intense in mixed DPPE-*d*/cholesterol films as they are in pure cholesterol monolayers (see Supporting Information). This reduction in intensity is less than would be anticipated purely on the basis of the lower number of cholesterol molecules in the mixed films. The intensity of a feature in an SFG spectrum is proportional to the square of the number of molecules that are contributing to it, and thus the resonances in the mixed film should be a quarter as intense as those in the spectrum of the pure cholesterol monolayer, as the film contains half the number of cholesterol molecules. The fact that the reduction in intensity of the resonances cannot be solely attributed to there being fewer cholesterol molecules in the film would imply that the cholesterol molecules adopt a slightly different orientation in the pure cholesterol and mixed DPPE-*d*/cholesterol films. Due to the large number of different methyl, methylene, and methine groups that contribute to the features in the SFG spectrum, it is difficult to specifically identify exactly how the orientation of the

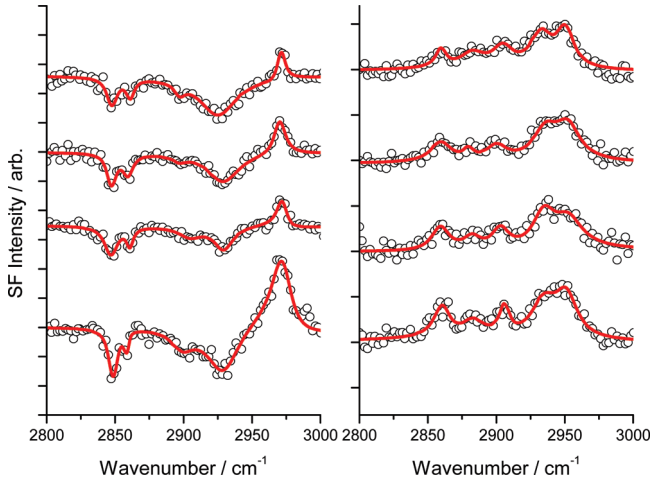

Figure 7. SFG spectra of HBMs comprising a monolayer of DPPE-*d* and cholesterol-d_7 in a 1:1 molar ratio in PPP (left-hand spectra) and SSP (right-hand spectra) polarization combinations. For comparison purposes, the lowermost spectra are of a pure cholesterol-d_7 monolayer adsorbed at 20 mN·m^{-1} reproduced from Figure 4. The remaining spectra, from bottom to top, correspond to mixed DPPE-*d*/cholesterol-d_7 layers adsorbed at 1, 20, and 40 mN·m^{-1}.

cholesterol molecule is altered by interaction with the DPPE. It can be assumed, though, that the higher than anticipated spectral intensity in the mixed films cannot be attributed to the formation of cholesterol-rich domains, as previous studies have shown that only a single phospholipid/cholesterol phase is present at this cholesterol concentration.[11]

Notably, the phases of the resonances in spectra of pure cholesterol and mixed cholesterol/DPPE films are the same, implying that the polar orientation of the cholesterol is unchanged from that depicted in Figure 5. Previous theoretical and experimental investigations of model membranes of phospholipid bilayers containing cholesterol have suggested a similar orientation for the cholesterol.[23] They have shown that cholesterol is embedded within the lipid layer, with the hydroxyl group interacting either with the headgroup of a neighboring phospholipid or with the water layer that is in contact with the membrane. Furthermore, they have suggested that the sterol ring system is buried within the phospholipid alkyl chains and, according to molecular dynamics simulations of DMPC and DPPC bilayers, is tilted away from the surface normal by anywhere between 10° and 30°, with the exact tilt angle dependent on the phospholipid, cholesterol concentration, and temperature of the membrane.[24−26]

The SSP and PPP spectra of DPPE-*d*/cholesterol-d_7 monolayers in the C−H stretching region at 1, 20, and 40 mN·m^{-1} shown in Figure 7 are very similar to the spectra of the pure cholesterol-d_7 monolayers. There are no significant differences in the relative intensities, positions, or phases of the features in the spectra, although as with the DPPE-*d*/cholesterol spectra in Figure 6 the peaks and dips are weaker due to the reduction in the surface density of cholesterol molecules. As with the DPPE-*d*/cholesterol films, the reduction in intensity of the features in DPPE-*d*/cholesterol-d_7 spectra were not as large as would be expected solely on the basis of the lower number of cholesterol molecules in the film (see Supporting Information). Any change in the orientation of the cholesterol molecules by DPPE is likely to be relatively small due to the relatively low affinity that DPPE has for cholesterol, caused by the very strong electrostatic and hydrogen-bonding interhead group interactions between DPPE molecules, resulting in lipid−lipid interactions being favored over cholesterol−lipid interactions.[23]

CONCLUSION

SFG spectra have been recorded in the SSP and PPP polarization combinations of HBMs composed of either cholesterol, cholesterol-d_7, or a 1:1 mixture of DPPE-*d* and cholesterol, adsorbed at surface pressures of 1, 20, and 40 mN·m^{-1} from the air/water interface of an LB trough onto an ODT-coated gold substrate. Features in the SSP and PPP spectra were assigned to the methine, methylene, and methyl stretches of cholesterol. Small differences between the spectra of cholesterol and cholesterol-d_7 arose from contributions from the isopropyl group of cholesterol.

The detection of SFG spectra of cholesterol indicated that the molecules adopted a net orientational order in the monolayer. The SFG spectra were very similar at each of the three surfaces pressures at which the cholesterol monolayer was formed, indicating that the polar orientation of the pure cholesterol monolayer was not significantly dependent on the packing density of the LB film molecules over the range of surface pressures investigated. The r resonances of cholesterol and cholesterol-d_7 appearing as dips in the PPP spectra and peaks in the SSP spectra indicated that both the isopropyl group and the three protonated methyl groups of cholesterol-d_7 had a net polar orientation toward the ODT SAM and gold surface. Likewise, as the d resonances were dips in the PPP spectra, this implied that the SFG-active methylene groups had a net orientation toward the metal surface.

The spectra of HBMs in which the proximal layer contained a 1:1 mixture of DPPE-*d* and cholesterol (or cholesterol-d_7) were very similar to the spectra of the HBMs with a pure cholesterol monolayer. Features in the SSP and PPP spectra of the mixed and pure proximal layers were at identical frequencies, although based upon the intensities of the features in the spectra, there were indications that the DPPE had some effect on the orientation of the cholesterol molecules in the film. The phases of the features were the same in the different HBMs, indicating that the net polar orientation of the SFG-active groups of cholesterol was not altered by the presence of DPPE. It was not experimentally possible to obtain complementary information on the effect of cholesterol on the orientation and alkyl chain conformation of DPPE.

ASSOCIATED CONTENT

Supporting Information. Additional text and three figures, describing SFG fitting routine, deconvolution of ATR spectra of solvent-cast cholesterol and cholesterol-d_7 films, PPP and SSP SFG spectra of ODT-*d* in the C−H stretching region, and SFG spectra of thiocholesterol on gold recorded in air in SSP and PPP polarization combinations; and eight tables listing peak positions, widths, and amplitudes that were used to fit SFG spectra. This material is available free of charge via the Internet at http://pubs.acs.org/

AUTHOR INFORMATION

Corresponding Author
*E-mail mtlc2@cam.ac.uk.

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