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Tethered bilayer lipid membranes studied by simultaneous attenuated total reflectance infrared spectroscopy and electrochemical impedance spectroscopy

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Abstract

The formation of tethered lipid bilayer membranes (tBLMs) from unilamellar vesicles of egg yolk phosphatidylcholine (EggPC) on mixed self-assembled monolayers (SAMs) from varying ratios of 6-mercaptohexanol and EO₃Cholesteryl on gold has been monitored by simultaneous attenuated total reflectance fourier transform infrared (ATR–FTIR) spectroscopy and electrochemical impedance spectroscopy (EIS). The influence of the lipid orientation (and hence the anisotropy) of lipids on a gold film on the dichroic ratio was studied by simulations of spectra with a matrix method for anisotropic layers. It is shown that for certain tilt angles of the dielectric tensor of the adsorbed anisotropic layer dispersive and negative absorption bands are possible. The experimental data indicates that the structure of the assemblies obtained varies with varying SAM composition. On SAMs with a high content of EO₃Cholesteryl, tBLMs with reduced fluidity are formed. For SAMs with high content of 6-mercaptohexanol, the results are consistent with the adsorption of flattened vesicles, while spherical vesicles have been found in a small range of surface compositions. The kinetics of the adsorption process is consistent with the assumption of spherical vesicles as long-living intermediates for surfaces of high 6-mercaptohexanol content. No long-living spherical vesicles have been detected for surfaces with large fraction of EO₃Cholesteryl tethers. The observed differences between the surfaces suggest that for the formation of tBLMs (unlike supported BLMs) no critical surface coverage of vesicles is needed prior to lipid bilayer formation.

Introduction

Tethered bilayer lipid membranes (tBLMs) have become important model systems for biological membranes.^{1–4} Their properties make them an interesting platform for biosensor applications.¹ Especially appealing is the fact that, with the right tethering system, the presence of an ion reservoir on both sides of the lipid bilayer can be achieved.^{5,6} This opens up the possibility of incorporating membrane constituents like peptides or membrane proteins into the tBLM and study the transport of ions through the membrane.^{7–9} In addition, many physical techniques are available to investigate the structures of assemblies at solid interfaces. These include atomic force microscopy (AFM), surface plasmon resonance

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(SPR) experiments, quartz crystal microbalance (QCM) studies, and electrochemical impedance spectroscopy (EIS).⁴

Apart from AFM, all of the aforementioned experimental techniques determine properties of the overall tBLMs, like the effective thickness (SPR), the mass (QCM) or the capacitance (EIS). Naturally, this leaves questions open relating to the local structure of the tBLMs. One important parameter is the membrane fluidity, which may change due to the binding at an interface. Another important question, especially if the tBLM is formed via a self-assembly route, is whether there are lipid vesicles adsorbed at the interface.¹⁰ From EIS or SPR experiments alone, it is hard to determine whether unfused and unruptured vesicles are adsorbed to a surface, or patches of a lipid bilayer. Although AFM has provided detailed information on the adsorption of vesicles and formation of supported BLMs (sBLMs), the interactions of the sample surface with the cantilever tip can induce vesicle deformation or other imaging artifacts.^{11–14}

Infrared spectroscopy can be used in order to address some of these problems. Different variants of attenuated total reflectance Fourier transform infrared (ATR–FTIR) spectroscopy have been used to study assemblies at metal surfaces.^{15–19} An important application arises when rough Au films are prepared, as these are reported to benefit from a surface enhancement effect, which increases the sensitivity to detect binding of proteins and lipids.^{18,20} Due to the roughness, however, surface enhanced infrared absorption spectroscopy (SEIRAS) is not suited to monitor the orientation of adsorbed molecules. Furthermore, the quality of a tBLM is known to depend on the roughness of the surface.²¹ Here, a different approach was employed. Au films that have been shown to be continuous on ATR crystals were used.^{15,17} Although this approach obtains unenhanced signals, it permits continuous bilayer membranes to be investigated and it permits the study of molecular orientation. A continuous metal film with a mixed self-assembled monolayer (SAM) and a lipid bilayer can in a good approximation be treated as a stratified system. This simplifies the analysis of the results, because it is possible to use the established matrix methods for stratified systems.^{22–25} Bandshape simulations have been performed for isotropic adsorbates on metal layers.²⁴ Here, these simulations are extended to anisotropic films at a metal interface to ensure the validity of the chosen data analysis procedure.

A further advantage of a continuous Au film is the possibility of simultaneously performing electrochemical experiments in a controlled way. So far, applications to sBLMs of such simultaneous experiments have only been reported for external reflection spectroscopy experiments.^{26,27} The use of an ATR–FTIR experiment considerably simplifies the optical design of a cell and the analysis of the results compared to external reflection experiments. Consequently, the use of continuous Au films makes it possible to combine ATR–FTIR spectroscopy and EIS on the same surface. Results on the structure and mechanism of formation of tBLMs obtained from this combination of methods are reported in this work. EIS was used to probe the overall quality of the tBLM. The results of this are put in relation to the conformation of the lipid chains and their average orientation. Typically, the in-situ combination of complementary analytical methods yields information that is hard to obtain from the independent application of the individual methods.²⁸

Here, tBLMs formed by egg yolk phosphatidylcholine (EggPC) tethered to Au surfaces modified by mixed self-assembled monolayers (SAMs) have been studied. The SAMs consist of a mixture of EO₃-Cholesteryl (EO₃Chol) and 6-mercaptohexan-1-ol (MHex).^{10,29} By coupling ATR–FTIR and EIS, it is possible to distinguish between adsorbed flat vesicles, adsorbed spherical vesicles and continuous planar lipid bilayers. The different behavior of the lipid at different surfaces leads to insights into the role of the cholesterol tether in vesicle

rupture and hints at differences in the mechanisms of formation between sBLMs and tBLMs.

Experimental methods and theoretical models

SAMs on ATR crystals

A 20 nm thick layer of Au (99.99 %; Goodfellow, Huntington, UK) was evaporated on ZnSe crystals (45° parallelogram, 64.2×4.2×12 mm; Crystran, Poole, UK) with an Auto 306 evaporator (BOC Edwards, Crawley, UK) at $< 2 \cdot 10^{-6}$ mbar. A mask was used to make sure that one face of the crystal was completely covered with Au. EO₃Chol (Figure 1) was synthesized as previously described.³⁰ SAMs were formed by overnight incubation of the fresh Au side of the crystal in a solution with the appropriate mixture of 6-mercaptohexan-1-ol (Aldrich, Milwaukee, USA; Figure 1) and EO₃Chol in propan-2-ol. The SAMs were then rinsed with propan-2-ol, methanol, and dichloromethane (all HPLC grade; Fisher Scientific, Loughborough, UK). Finally, the crystal was mounted in the spectroelectrochemical cell.

The composition of the mixed SAM was determined from EIS in a similar fashion to that described elsewhere.^{29,31} Briefly, an electrochemical impedance (EI) spectrum of the mixed SAM in buffer was recorded and the surface capacitance estimated from the diameter of the semicircle in the normalized complex admittance plot. The fraction x_{MHex} of surface area covered by MHex in the SAM was determined from the surface capacitance C_M of the

mixed SAM as $x_{MHex} = \frac{C_M - C_{EO3Chol}}{C_{MHex} - C_{EO3Chol}}$, where C_{MHex} and $C_{EO3Chol}$ are the surface capacitances of pure MHex and EO₃Chol SAMs, respectively.

After the experiments, the Au layer was carefully wiped off the ATR crystal with lens cleaning tissue. When necessary, the crystal surface was polished with Al₂O₃ spheres (300 nm diameter to begin with, 50 nm diameter for finishing; Buehler, Lake Bluff, USA). All polishing was done against a polished Si wafer to ensure a smooth surface. The crystal was then carefully cleaned with propan-2-ol, dichloromethane and de-ionized water.

To avoid spectral contributions from contaminants adsorbing onto the back face (i. e. the face not used for experiments) of the crystal during an experiment, a 70-100 nm thick Ag (99.99 %; Advent, Oxford, UK) layer was evaporated onto one face. The Ag layer was only renewed when it was seriously damaged.

Tethered lipid bilayer formation

EggPC was purchased from Avanti Polar Lipids (Alabaster, USA) and used as received. The buffer used for all experiments was 0.02 mol/L 3-(*N*-morpholino)-propanesulfonic acid (MOPS; Sigma, St. Louis, USA) with 0.1 mol/L NaCl (Fisher Scientific) in de-ionized water (Millipore, Molsheim, France) adjusted to pH 7.4. Unilamellar vesicles of EggPC were prepared by dispersing 5 g/L EggPC in buffer, vortexing and extruding 11 times through a track-etched membrane with a pore diameter of 100 nm. The vesicle dispersion was diluted to give a final lipid concentration of 0.5 g/L and was injected into the spectroelectrochemical cell, where the formation of a bilayer was monitored by EIS and ATR-FTIR spectroscopy. All experiments were performed at 23°C room temperature.

Spectroelectrochemical cell

The cell (53 × 22 mm), which is shown in Figure 2, was cut from a Teflon block (Goodfellow) of 5 mm thickness. A well of approximately 43 × 7 mm houses the electrolyte. The ATR crystal covers one side of the opening. The other side is covered by a piece of

silicone rubber (Goodfellow). The Au layer on the ATR crystal serves as working electrode. From the size of the opening given above, an electrode area of $\sim 3 \text{ cm}^2$ is calculated. The Ag/AgCl reference electrode is fixed in the middle of the cell. A Pt wire loop serves as counterelectrode. Liquid can be injected into the closed cell through an inlet, and leaves the cell through an outlet.

Electrochemical impedance spectroscopy

Electrochemical impedance spectra were recorded on an Autolab PGSTAT30 potentiostat equipped with a FRA2 impedance analyser (Eco-chemie, Utrecht, The Netherlands). All experiments were performed at a potential of -59 mV vs. Ag/AgCl/0.1M NaCl (222 mV vs. normal hydrogen electrode). A $200 \text{ }\Omega$ resistor was used in series with the working electrode to enhance the stability of the potentiostat at high frequencies. The working electrode was connected by putting a Cu wire in contact with the Au covered face of the ATR crystal. The quality of the connection can easily be assessed from the high frequency modulus of the impedance, which was usually found to be only slightly higher than the $200 \text{ }\Omega$ from the external resistor.

FTIR spectroscopy

ATR-FTIR spectra were recorded on a Bruker IFS-66 v/S FTIR spectrometer (Bruker, Ettlingen, Germany). The Specac (Specac, Orpington, UK) variable angle unit was used for the ATR experiments. 1000 Scans at a resolution of 2 cm^{-1} were averaged to measure buffer and sample spectra. To follow the kinetics of the vesicle adsorption, consecutive spectra of 100 scans each were recorded and automatically analyzed. In all cases, the recorded interferograms were zero-filled with 2 levels, apodized with a Blackman-Harris-3-Term function and Fourier transformed. Finally, the respective sample and buffer spectra were ratioed as $-\lg(\text{sample/buffer})$ to give an ATR spectrum. Consecutive measurements were performed with perpendicular (s) and parallel (p) polarization.

For the analysis of spectra, two different strategies were used. The spectra collected at the end of an adsorption experiment were baseline-corrected manually, and both the peak heights at the maximum and the area under the peaks used to determine the intensities and hence the dichroic ratio. The dichroic ratios from peak heights and areas have been found to agree. The results shown in section Structure were obtained using this first approach. To ensure an objective analysis of the spectra, as a second approach, a homebuilt software package was used in an entirely computer-based analysis of the spectra to check the results obtained through manual processing. The latter approach was also used to analyze the time evolution of the spectra as presented in section Kinetics. In the computer-based analysis method, a region of interest in the spectra was selected first. This region contains the peaks of interest and a sufficiently large region of a baseline without peaks, say $2500\text{--}3050 \text{ cm}^{-1}$. The region with peaks is then removed from the dataset. A third or fourth order polynomial is fit to the remaining baseline. That polynomial is finally subtracted from the initial spectrum to yield a baseline-corrected spectrum. Again, two strategies were used to determine the band intensities and positions. The first strategy uses the "Peakpick" function of the Bruker OPUS software to find the maximum position and absorbance within a certain wavenumber interval. In the other strategy, a Lorentzian peak function is fit to a certain range of the baseline-corrected spectrum. The software programs used for this type of analysis are available for download on the world wide web.³²

The band absorbance values were corrected for the polarizer inefficiency as defined by Flach et al.³³ The value of ϵ around 2900 cm^{-1} was determined from ATR spectra of propan-2-ol as an isotropic liquid in direct contact with a Si crystal with 45° angle of incidence. The polarizer angle was varied 1° steps over one 360° turn. Background and

sample spectra were recorded for every polarizer setting. The polarizer settings where the absorbance is maximal gives the polarizer angle for p-polarization, the setting with minimal absorbance corresponds to s-polarization. The theoretical dichroic ratio obtained with a 45° prism for an isotropic two-phase system is always 2.³⁴ The observed value was 1.94, yielding ~ 0.02 .

Optical models used for spectrum analysis

Detailed descriptions of the basic theory of reflection in general and of ATR spectroscopy in particular, can be found elsewhere in the literature.^{22,34–37} The coordinate system used in this work is the usual coordinate system for the optics of reflection from stratified media, where the x–y plane is the reflecting interface, the surface normal the z–direction and the projection of the incident beam onto the interface defines the x–direction.²²

The absorbance A_s of s-polarized light in such a coordinate system is related to the absorption coefficient α_y of the thin film in y-direction and the time-averaged mean-square amplitude of the electric field (which will be denoted as “intensity” throughout this work), I_y , at the interface in y-direction,

$$A_s = \alpha_y I_y. \quad (1)$$

The absorbance A_p of p-polarized light depends on the respective quantities of the film in x- and z-direction,

$$A_p = \alpha_x I_x + \alpha_z I_z. \quad (2)$$

The dichroic ratio R_{ATR} is defined as^{35,36}

$$R_{ATR} = \frac{A_p}{A_s} = \frac{\alpha_x I_x + \alpha_z I_z}{\alpha_y I_y}. \quad (3)$$

The absorption coefficients α_i are related to the average tilt angle $\langle G \rangle$ of the transition dipole moments in a uniaxial distribution of transition dipole moments with respect to the z-direction (i. e. the surface normal) by^{36,38}

$$\alpha_z = F \left(S_2^G + \frac{1}{2} (1 - S_2^G) \right) \text{ and } \quad (4)$$

$$\alpha_y = \alpha_x = F \left(\frac{1}{2} (1 - S_2^G) \right), \quad (5)$$

where F is a factor related to the magnitude of the transition dipole moment, the surface coverage and the number of reflections in the internal reflection unit, and

$$S_2^G = \frac{3 \langle \cos^2 \theta_G \rangle - 1}{2}, \quad (6)$$

where angular brackets denote an ensemble-average. The factor F cancels when the ratio of A_p and A_s is calculated. Subsequently, S_2 and hence the tilt angle follow. For the tilt angles, the notation $\langle \cos^2 \theta \rangle^{1/2}$ will be used in this work, as in the literature in general, as a shortcut for $\arccos(\langle \cos^2 \theta \rangle^{1/2})$.³⁵ Both terms become equivalent only if there is no distribution of tilt angles. Using model distributions, the different averages of $\langle \cos^2 \theta \rangle$ can be evaluated.³⁸

In this work, the focus will be on CH₂ groups. If θ_G is calculated as described above, the result is the average tilt angle of the direction of the bisection of the H–C–H plane with respect to the surface normal, because the transition dipole moment of both the symmetric and the antisymmetric stretching mode are located within the H–C–H plane. In the case of all-*trans* chains, the tilt angle θ_C of the chain axis is very often discussed in the literature. In an all-*trans* chain, the axis of the molecule, i. e. the director of the hydrocarbon chain, is perpendicular to all H-C-H planes of the CH₂ groups. The value of θ_C can be calculated from S_2^G with the knowledge of the angle θ between hydrocarbon chain and transition dipole moment from³⁶

$$S_2^C = S_2^G \cdot S_2^\gamma. \quad (7)$$

Here, S_2^C and S_2^γ are defined in analogy to S_2^G in eq. 6 with θ_C and θ respectively as arguments of the cosine. To allow direct comparisons with different literature values, both tilt angles will be given in the results section. The directions with respect to an individual CH₂ group which each of the two refers to is shown in Figure 3. The tilt angle θ_G of the CH₂ group, will be denoted as θ_{CH_2} . The tilt angle of the chain is abbreviated as θ_C . If the chains are not in an all-*trans* conformation, θ_C becomes the average tilt angle of a chain segment.¹⁵ It should be noted that in an isotropic situation $S_2^G = S_2^\gamma = 0$, and

consequently $\langle \theta_C \rangle = \langle \theta_G \rangle = \arccos\left(\frac{1}{3^{1/2}}\right) \simeq 54.7^\circ$.

In order to determine any R_{ATR} from R_{ATR} , the knowledge of I_x , I_y and I_z is essential. For stratified media in general, I_x , I_y and I_z can be calculated from matrix methods.^{25,35} It should be pointed out that these values are not constant over an absorption band due to the dispersion of the optical constants. In the analysis used in this work, the effect of the thin adsorbed film of organic material on the intensities in all directions is neglected, i. e. a three phase system consisting of ZnSe, Au and water was used to calculate I_x , I_y and I_z . This type of analysis has previously been found to lead to more reliable results than taking the thin organic film into account.³⁹

A more sophisticated analysis method is the simulation of whole absorption bands, which has been done by several authors.^{24,40} In this work, simulations were carried out for the system ZnSe – Au – (anisotropic organic film –) water. The matrix method of Schubert was used to calculate the reflectivity $|r_{ref}|^2$ for a system ZnSe – Au – water for each wavenumber $\tilde{\nu}$.²³ Subsequently, the reflectivity $|r_{smp}|^2$ for the system ZnSe – Au – anisotropic organic film – water was calculated for each $\tilde{\nu}$. The two reflectivities were then ratioed as $-\lg(|r_{smp}|^2 / |r_{ref}|^2)$ as in an experiment to give the reflectance absorbance. The frequency dependence of the optical constants of ZnSe and Au has been taken from literature values and interpolated by polynomials.^{41,42} The frequency-dependent optical constants of water from Bertie et al. were used and interpolated piecewise.⁴³ The computer program used for the calculations together with the complete sourcecode is available online.³²

The dielectric tensor of the anisotropic layer has got three components in its own Cartesian coordinate system with the three axes a, b, and c. The three Eulerian angles α , φ and β are used to describe the orientation of the coordinate system of the layer with respect to the overall coordinate system defined above.⁴⁴ The complex refractive index m_j (j is one of a, b or c) in each of the three spatial directions in the a–b–c coordinate system of the layer is described with the dielectric function of a damped harmonic oscillator,^{24,45}

$$m_j^2(\tilde{\nu}) = n_j^2 + \sum_{k=1}^N \frac{B_{jk}}{\tilde{\nu}_{0k}^2 - \tilde{\nu}^2 - i\gamma_k \tilde{\nu}} \quad (8)$$

The summation is over all N absorption bands. Each band is defined by a wavenumber of the absorption band maximum $\tilde{\nu}_{0k}$, a factor B_{jk} that determines the intensity and a damping constant γ_k determining the width. The refractive index n is the real part of the refractive index far away from the absorption band maximum. It is worth noting that $\tilde{\nu}_{0k}$ and γ_k are the same in all three directions for physical reasons in the case of molecular vibrations. The relationship between the B values of an absorption band in the different directions in space is governed by the nature of the film.

Consider a single all-*trans* hydrocarbon chain. The direction of the chain shall be along the c -direction. The transition dipole moment of the symmetric and antisymmetric stretching modes are oriented in the $H-C-H$ plane.⁴⁶ This means that for the CH_2 stretching modes in an all-*trans* chain, there is no absorption of light along the chain, i. e. $B_c = 0$. When a large number of hydrocarbon chains form a uniaxial film B_c may have a value which is slightly larger than 0 if there is a distribution of the molecular axes around the direction of the c -axis of the film. For simplicity, this is neglected here. Also, in a uniaxial film, $B_a = B_b$ and $n_a = n_b$.^{37,45} The two remaining refractive indices n_a and n_c correspond to the ordinary refractive index and the extraordinary refractive index of the layer. For a uniaxial phase of molecules with cylindrical symmetry, the average values of $\varphi = 45^\circ$, while θ can be identified as θ_c , the average tilt angle of the chain.³⁸

Results and Discussion

Spectrum simulations

ATR spectra of adsorbates on metal surfaces have been simulated by Bürgi.²⁴ He has shown that ATR spectra of samples on metal layers are more complicated than for layers of materials with a low absorption coefficient. Dispersive bandshapes are possible in such a situation.²⁴ With a strongly dispersive bandshape, estimating the peak height or the peak area from experimental data is much more difficult or might be impossible. As a consequence a dichroic ratio as specified above is a quantity that cannot easily be defined.

Bürgi's findings pose the question whether the simple analysis using the dichroic ratio is valid at all in the presence of a metal film. Here, the results of simulations of spectra of an anisotropic layer on a metal layer are presented. As an example, a spectrum modeling the antisymmetric and symmetric CH_2 stretching modes is shown in Figure 4. To get close to the experimental situation of this work, a 20 nm thin Au film on a ZnSe crystal with 45° angle of incidence is used as a substrate. The thickness of the anisotropic organic layer on top of the Au is set to 2 nm. The further parameters used are $n_a = 1.50$ and $n_c = 1.40$, a situation typically encountered in anisotropic organic materials, $B_{a1} = 1900 \text{ cm}^{-2}$, $\gamma_1 = 15 \text{ cm}^{-1}$, $\tilde{\nu}_{01} = 2850 \text{ cm}^{-1}$, $B_{a2} = 2300 \text{ cm}^{-2}$, $\gamma_2 = 10 \text{ cm}^{-1}$, and $\tilde{\nu}_{02} = 2920 \text{ cm}^{-1}$.⁴⁷ The results for two different tilt angles, $\theta_c = 10^\circ$ and $\theta_c = 80^\circ$ are shown in Figure 4 for both s- and p-polarized incident light.

For low tilt angles ($\theta_c = 10^\circ$), only the spectrum with s-polarization shows slightly distorted bandshapes. If the simulated spectra are processed in the way used to process experimental spectra here, $R_{ATR} \sim 3.1$ is found, yielding $\theta_c \sim 8.5^\circ$. This value is in reasonable agreement with the initial value of $\theta_c = 10^\circ$, proving this type of analysis to be self-consistent. However, there are distinct differences in the spectra simulated for high tilt angles ($\theta_c = 80^\circ$). As expected, the p-polarized absorbance for $\theta_c = 80^\circ$ is much higher

than calculated for $\theta_C = 10^\circ$. In the spectra with s-polarized light, however, negative bands are present. The change of sign of an absorption band is also known from external reflection experiments at the air/water interface for certain angles of incidence and tilt angles of the molecule.^{40,48} Dispersive intermediates have been observed under certain conditions.^{48,49}

These simulated spectra confirm Bürgi's conclusion that dispersive bandshapes are possible and predicted from electromagnetic theory.²⁴ Their appearance does however depend on the tilt angle of the dielectric tensor of the layer and hence the molecules within that layer with respect to the surface normal. Above a certain θ_C the dispersive bandshape of the s-polarized absorption determines the behavior. The dichroic ratio is not a well-defined quantity in these cases.

Two further conclusions can be drawn from the simulations shown above. Firstly, the peak maximum in the spectrum is shifted compared to the peak maximum of the imaginary part of the anisotropic layer's complex refractive index m . In the spectra for $\theta_C = 10^\circ$, the peak maxima are shifted to lower wavenumbers by $1\text{--}2\text{ cm}^{-1}$ compared to $\tilde{\nu}_{01}$. The shift is more pronounced with s-polarization, opening up the possibility for the existence of dispersive bands. This shift also implies that great care must be taken when interpreting the maxima of the band positions, e. g. in terms of the fraction of *gauche* conformers of a chain. Secondly, the baseline in the CH stretching mode region of the spectrum is expected to be a line curved downwards. The reason for this is the displacement of water from the surface when material is adsorbed. It means that some kind of baseline treatment will always be necessary for the analysis in terms of the simple dichroic ratio model. This is, however, not a problem, because the CH_2 modes are narrow peaks on a broad background.³⁶

Structure

Electrochemical Impedance Spectroscopy (EIS)—Examples for the normalized admittance plots of impedance spectra of EggPC adsorbed to surfaces with SAMs of different composition are shown in Figure 5. A detailed analysis of these spectra will be presented elsewhere.²⁹ The radius of the semicircle at high frequencies is used to determine the surface capacitance C_S of the sample. For surfaces with $x_{\text{MHex}} = 0$, that is pure EO_3Chol surfaces (curve 1 in Figure 5), $C_S \sim 0.8\text{ }\mu\text{F}/\text{cm}^2$ is found. As shown in Figure 5, already small amounts ($x_{\text{MHex}} = 0.16 \pm 0.03$, curve 2) of MHex lead to a slight increase of C_S . For EggPC, the value of C_S does almost continuously change with x_{MHex} . Overall, these results agree very well with previous findings.^{8,29} For EggPC on similar tethering systems, up to $x_{\text{MHex}} \sim 0.4$, a continuous lipid bilayer is formed on the surface, though at this concentration the EIS data differs significantly from the data at pure EO_3Chol ($x_{\text{MHex}} = 0$). We attribute this behavior to dispersion in the surface capacitance originating from inhomogeneities in the lipid and the SAM.²⁹ The nature of the assemblies for $x_{\text{MHex}} > 0.4$ on the other hand is not as obvious. Possibilities are (a) patches of lipid bilayers, (b) adsorbed spherical vesicles and (c) adsorbed flat vesicles. Importantly, spherical and flat structures have different values of C_S and can be distinguished by ATR-FTIR spectroscopy.

Attenuated total reflectance infrared spectroscopy—The most prominent features in the IR spectra of the lipid bilayers were the symmetric ($\nu_s(\text{CH}_2)$, $\sim 2850\text{ cm}^{-1}$) and antisymmetric ($\nu_{\text{as}}(\text{CH}_2)$, $\sim 2920\text{ cm}^{-1}$) stretching modes of the CH_2 groups of the lipid acyl chains, as exemplified in Figure 6. Furthermore, the symmetric ($\nu_s(\text{CH}_3)$) and asymmetric ($\nu_{\text{as}}(\text{CH}_3)$) stretching modes of the methyl groups of the lipid acyl chains have been observed. For the analysis of the spectra, the focus of this work was on the symmetric stretching mode of the methylene groups. The analysis of the antisymmetric stretching mode yields essentially the same results. Wavenumber of the maximum, absorbance, and dichroic

ratio of the $\nu_s(\text{CH}_2)$ have been analyzed for lipid bilayers at surfaces of different composition.

Figure 7 shows the wavenumber, $\tilde{\nu}_M$, of the maximum of the $\nu_s(\text{CH}_2)$ mode as a function of the fraction x_{MHex} of surface area covered by MHex. At low x_{MHex} , the maximum of the $\nu_s(\text{CH}_2)$ is found to be $\sim 2850 \text{ cm}^{-1}$. For high x_{MHex} the observed results are somewhat variable.

The maximum $\tilde{\nu}_M$ of the symmetric and antisymmetric stretching modes of the CH_2 groups has been shown to serve as a qualitative indicator of the conformation of the chain and hence the order and fluidity of the bilayer.^{46,50–52} In the case of the $\nu_s(\text{CH}_2)$, $\tilde{\nu}_M$ around or lower than 2850 cm^{-1} is indicative of a chain in an all-*trans* conformation. A shift to higher wavenumbers, up to 2855 cm^{-1} , indicates an increasing number of *gauche* conformers and therefore increasing bilayer fluidity.^{46,50–52}

As already pointed out above, the peaks of resonant modes in the region of the CH stretching modes are predicted to shift under the influence of an Au film. A shift to lower wavenumbers by $1\text{--}2 \text{ cm}^{-1}$ compared to the resonance frequency of the respective damped harmonic oscillator has been obtained as a result of the simulations. Experimentally, a shift to lower wavenumbers is confirmed when the results shown in Figure 7 are compared to transmission spectra of EggPC and ATR spectra of an EggPC bilayer on a Si prism, where $\nu_s(\text{CH}_2) \sim 2854.5 \text{ cm}^{-1}$.

The magnitude of the shift was never larger than 2 cm^{-1} in the simulations. The effect of the shift is predicted to be higher for s-polarized light, where $\sim 2 \text{ cm}^{-1}$ have been observed, while for p-polarization the band maximum was shifted by $\sim 1 \text{ cm}^{-1}$. This results in a predicted difference of $\sim 1 \text{ cm}^{-1}$ between the band maxima for s- and p-polarized light, which is indeed observed.

The maximal effect of the shift predicted from electromagnetic theory is included in Figure 7, showing as black lines the corrected positions expected for the $\nu_s(\text{CH}_2)$ of all-*trans* chains ($\sim 2848 \text{ cm}^{-1}$) and for disordered chains with *gauche-trans-gauche* conformers on an Au film ($\sim 2853 \text{ cm}^{-1}$), respectively. Comparing these two lines with the observed results, it is clear that the predicted shift cannot alone explain the observed low wavenumber for the band maximum.

Consequently, EggPC tethered to surfaces of $x_{\text{MHex}} < 0.3$ appears to show a low fraction of *gauche* conformers and hence decreased fluidity. Increasing the content of MHex increases the fraction of *gauche* conformers in the tBLMs. For $x_{\text{MHex}} > 0.6$ the results do not show a clear trend.

The effect of cholesterol on the chain conformation of phospholipids has been investigated by different groups under a variety of compositions as well as temperature and pressure conditions.^{27,52,53} It is generally found that the presence of cholesterol increases the number of *gauche* conformers in the gel phase, while it decreases the number of *gauche* conformers in the liquid-crystalline phase. It must be stressed that there is a substantial asymmetry in the way cholesterol is distributed between the leaflets of the bilayer in the tBLMs used here. Usually, cholesterol and phospholipids are mixed, the mixture is hydrated and subsequently vesicles with cholesterol in both leaflets are obtained. In the assemblies investigated in this work, the presence of the cholesterol is restricted to one leaflet of the bilayer. EggPC, as a biological lipid mixture, is in the liquid-crystalline phase at room temperature.⁵⁴ Therefore, the behavior found here agrees with the behavior described in the literature: cholesterol does

reduce the overall number of *gauche* conformers in the phospholipid acyl chains, even when it is confined to one leaflet of the membrane.

The low ($\sim 2850\text{ cm}^{-1}$) value of $\tilde{\nu}_M$ might be surprising for a natural lipid mixture. EggPC does however contain a large fraction (~ 0.5) of saturated lipids.⁵⁵

Bin et al. have investigated the potential-dependent adsorption and desorption to unmodified Au surfaces of DMPC vesicles with and without cholesterol. They report for the gel-like cholesterol-rich phase that cholesterol introduces fewer *gauche* conformers in the adsorbed state than in the desorbed state.²⁷ This observation hints to a combined ordering effect of the cholesterol as well as the surface. In the experiments presented here, the effect of the surface might also help to reduce the number of *gauche* conformers.

The dichroic ratios of the $\nu_s(\text{CH}_2)$ mode are shown in Figure 8. At $x_{\text{MHex}} < 0.5$ as well as $x_{\text{MHex}} > 0.95$, dichroic ratios of ~ 2.5 are found. At intermediate concentrations, higher values are obtained. There is one extremely high dichroic ratio $R_{\text{ATR}} \sim 13.1$ at $x_{\text{MHex}} = 0.93 \pm 0.06$.

From the dichroic ratio R_{ATR} , χ_{CH_2} follows as outlined above. For surface concentrations $x_{\text{MHex}} < 0.5$ and $x_{\text{MHex}} > 0.95$, the observed dichroic ratio roughly corresponds to the expected value for CH_2 groups in the plane of the SAM/buffer interface (i. e. the average tilt angle of the CH_2 group $\chi_{\text{CH}_2} \sim 90^\circ$, corresponding to a tilt angle of the chain segment $\chi_C \sim 0^\circ$). The dichroic ratio expected for this orientation under an Au layer (~ 2.7) is shown in Figure 8 as the line label “in plane”. Higher values than ~ 2.7 show that the average CH_2 group is tilted at a smaller angle with respect to the surface normal. For an isotropic systems ($\chi_C = \chi_{\text{CH}_2} \sim 54.7^\circ$), $R_{\text{ATR}} \sim 10.5$ is expected (line “isotropic”), while for an ideal spherical vesicle ($\chi_C = 60^\circ$, $\chi_{\text{CH}_2} = 45^\circ$), $R_{\text{ATR}} \sim 24.6$.

Two causes might contribute to the increase of the dichroic ratio with an increasing content of MHex. The first is the adsorption of spherical vesicles, and the second an increase in the number of *gauche* conformers in the lipid bilayer. For $0.5 < x_{\text{MHex}} < 0.95$, the observed dichroic ratios vary over a large range, while $\tilde{\nu}_M$ changes only in a relatively small range, suggesting that the changes in the dichroic ratio are not primarily based on more disordered chains. Especially the case of $R_{\text{ATR}} > 10$ can best be explained with a substantial amount of spherical vesicles adsorbed to the surface. This interpretation is consistent with EIS data which show that no continuous tBLM is formed. For $x_{\text{MHex}} > 0.95$, a similar line of reasoning applies. While there is some variability in $\tilde{\nu}_M$ of the $\nu_s(\text{CH}_2)$ mode, the dichroic ratios are in the same order of magnitude as for $x_{\text{MHex}} < 0.75$. As the EIS data shows that no continuous bilayer is formed, these dichroic ratios are indicative of the adsorption of flattened vesicles.

It should be pointed out that high dichroic ratios could be obtained from a lipid bilayer with chains tilted with respect to the surface at a high angle, or by aggregates in which the chains are on average tilted at such an angle, as in vesicles. The low dichroic ratios obtained in most experiments on the other hand can hardly be obtained if the material assembled at the interface is arranged in a curved structure. For multibilayer stacks of gel-phase DPPC and DMPC, different experimental techniques find values of $\chi_C \sim 30^\circ$ (corresponding to $\chi_{\text{CH}_2} \sim 70^\circ$), though slightly lower χ_C have also been reported.^{56,57} DMPC adsorbed to Au electrodes shows significantly higher χ_C (lower χ_{CH_2}).²⁷ R_{ATR} expected for an average chain tilt angle of 30° is shown as a line in Figure 8.

Figure 8 shows the measured dichroic ratios to be slightly lower than those expected for the upright chain (or CH_2 groups parallel to the x–y–plane). We have reported such behavior

before.¹⁵ Changes in the background could account for this effect. As the background signal includes the SAM, perturbations of the SAM upon adsorption of the lipids would alter the background. However, no large contributions of the SAM to the background spectra have been observed and it is therefore unlikely that alterations in the background have a significant effect. Also, a thickness dependence of the optical constants of Au is improbable for a 20 nm thick Au film. In addition, the different datasets published for the optical constants of Au yield only slightly different ratios I_x/I_y and I_z/I_y .^{41,58} Thus we propose that there is some evidence for the presence of effects that are not predicted by classic electromagnetic theory. Coupling of the vibrational modes to the electronic transitions of the gold (Fano resonance) is one of these possible causes that may lead to the observed low R_{ATR} .^{24,59} If Fano resonance plays a role, its effect is expected to be highest when the lipids are in closest contact with the Au film, where the observed R_{ATR} is indeed lowest.

After S_2^G has been determined from the dichroic ratio, the prefactor F in eq. 4 and 5 can be calculated. With the knowledge of F , the absorbances A_s^{iso} and A_p^{iso} of an isotropic film ($S_2^G=0$) with s- and p-polarization, respectively, follow. Both quantities are proportional to the amount of material adsorbed to the surface. No systematic dependence of A_p^{iso} and A_s^{iso} on the surface composition was observed, showing that similar amounts of material are adsorbed on all surfaces. This finding confirms that lipid is adsorbing to surfaces where x_{MHex} is close to 1, even though the changes in the EIS are small upon adsorption.

Kinetics

The kinetics of the adsorption process of the lipid vesicles and the formation of tBLMs can be studied by following the time evolution of the intensities of the CH₂ stretching modes and the EIS spectra simultaneously. Typical kinetic traces of these experiments are shown in Figure 9 and Figure 10. From the evolution of the dichroic ratio, qualitative information about the curvature of the lipid membranes in the intermediate states of the adsorption can be obtained.

Figure 9 shows the results for adsorption to a MHex surface ($x_{MHex} = 1$). Two phases in the adsorption can clearly be distinguished. After the injection of the lipid, the absorbance of p-polarized light increases rapidly. This is followed by a slower second phase. The rapid first phase is almost absent in the trace for s-polarization. This absence has a profound effect on the resulting kinetic trace of the dichroic ratio. After an initial phase in which the absorbance with s-polarization is too low for the reliable calculation of the dichroic ratio, the dichroic ratio decreases from between 8 and 10 to a value around 4 at the end of the second phase. The wavenumber $\tilde{\nu}_M$ of the absorption band maximum does not change with time. The same two phases as in the IR spectra are observed in the impedance spectra. In Figure 9b the imaginary parts of the admittance at two frequencies are shown.

In the presence of more than 20 % EO₃Chol (i. e. $x_{MHex} < 0.8$) on the surface, the curves show a significantly lower dichroic ratio ($R_{ATR} \sim 5-7$) at the beginning of the second phase, as exemplified in Figure 10. Consequently, the dichroic ratio only slightly decreases in the course of the experiment to eventually reach approximately the same level as with pure MHex. The increase in the intensities is similar to pure MHex. Again, $\tilde{\nu}_M$ does not change with time.

The observed two phase adsorption kinetics has been reported before.¹⁰ The combined application of ATR-FTIR and EIS shows however some new aspects of the adsorption process. The initial increase in absorbance and decrease in the imaginary admittance is clearly related to the deposition of material at the interface. The high dichroic ratio found

here at the beginning of the experiment is interpreted as above in terms of (spherical or slightly deformed) vesicles adsorbing to the surface. In the course of the experiment, the adsorbed lipid rearranges. This is shown by the decrease of the dichroic ratio as well as the opposing changes with time in the admittance at two different frequencies. The average tilt angle $\angle_{CH_2}$ of the CH_2 groups decreases. As above, this is interpreted as a transformation from spherical to flat aggregates on the surface as no tBLM is formed at x_{MHex} close to 1. At higher EO_3Chol content, the reorganization of the vesicles seems to be faster than the time resolution of the experiment.

Previously, we have reported differences in the kinetics of lipid adsorption to surfaces with varying EO_3Chol content studied with SPR.¹⁰ These differences have been interpreted in the terms of different adsorption mechanisms. For hydrophilic substrates, vesicle adsorption and rupture were thought to be the first stage, with fusion and bilayer formation as the second phase. For hydrophobic surfaces, the precise mechanism remained unclear.¹⁰ This work supports the assumption that for hydrophilic surfaces the adsorption of vesicles is the first step. The high dichroic ratio does suggest that these vesicles adsorb as distorted spherical vesicles. However, fusion of vesicles may not take place on MHex-surfaces, since EIS shows that no dense lipid bilayer is formed. The flattening of vesicles in the second stage of the adsorption must coincide with the release of water from the vesicles. For EO_3Chol containing surfaces, there are only small changes in the curvature of the adsorbed structures, indicating that the release of the vesicle content must happen at early stages of the adsorption of the individual vesicle.

It is of interest to compare our results with those for the adsorption of vesicles at pure Au and SiO_2 interfaces for which the subsequent structural transformation to lipid bilayers has been investigated in detail.^{28,61} From the systems studied in this work, the pure MHex SAM is the closest to SiO_2 , since there are no tether molecules present. In a combined study with SPR, AFM and QCM it has been shown that vesicles adsorb to the SiO_2 surface and above a certain coverage they transform into supported lipid bilayers.²⁸ This leads to a two-phase kinetics similar to the kinetics observed in this work. The major difference to the system studied here – apart from the timescale of the process – is that as the final result of the adsorption process, a continuous bilayer is obtained on SiO_2 , while for pure MHex surfaces, no continuous bilayer is formed and vesicles are proposed to be adsorbed at the surface.

The situation is modified when a substantial number of cholesterol tethers are present. In contact with a cholesterol-containing SAM, there is an immediate driving force for a vesicle to spread over the surface with cholesterol tethers, since there is an energy gain when the hydrophobic cholesterol is transferred from water into the equally hydrophobic membrane interior.¹⁵ Spreading over the surface can occur in two ways: the vesicle could rupture and form a bilayer patch (which can subsequently fuse with other patches to form a bilayer), or the vesicle could release its content and remain adsorbed to the surface as a flat vesicle. From this point of view, there is no need for a critical surface coverage with vesicles for a lipid bilayer to form, since bilayer patches (or flat vesicles) can form immediately. This assumption is consistent with the kinetics of dichroic ratios as shown in Figure 10. Even in the initial phase of the adsorption, no high dichroic ratios as expected for spherical aggregates at the surface was observed, as opposed to the kinetics of adsorption to a pure MHex-SAM.

Conclusions

The EI spectra show that high-quality tBLMs are formed only for $x_{MHex} < 0.4$.²⁹ The IR data indicates that these bilayers are flat with a reduced fluidity as typically found for cholesterol-containing phospholipids. Surfaces with $x_{MHex} > 0.4$ yield lipid bilayers of a

low quality or adsorbed vesicles. The dichroic ratios in the region between $0.4 < x_{MHex} < 0.95$ show a decrease in CH_2 with increasing x_{MHex} at about the same intensities of the lipid stretching modes. Consequently, lipid is adsorbing to the surface, though its effect on the surface capacitance is small. The average tilt angle of the CH_2 groups is different than on surfaces with low MHex content. To explain this behavior, vesicles are proposed to adsorb to the surfaces at $x_{MHex} > 0.4$ without forming continuous bilayers. The dichroic ratios are however never as high as expected for an undistorted, spherical vesicle bound to the SAM–buffer interface. In a few cases in the concentration range $0.75 < x_{MHex} < 0.95$ there must be a substantial number of spherical vesicles present at the surface. At $x_{MHex} > 0.95$ the adsorbed structures at the interface are flat, and there is no indication of the formation of a significant fraction of dense bilayers.

Though the results of coupled ATR–FTIR and EIS experiments allow for the differentiation between spherical and flat structures at the surface, there is no obvious straightforward way to directly distinguish flat vesicles from patchy bilayers on the surface. Overall, it is however very hard to conceive how for high x_{MHex} about the same number of lipid molecules can adsorb to the surface as for low x_{MHex} without a significant impact on the surface capacitance other than by forming flat vesicles. This assumption is supported by our earlier SPR results.¹⁰

A summarizing sketch of the proposed structures for the different surface compositions is shown in Figure 11. Please note that this sketch is not drawn to scale.

Optical simulations have shown that care must be taken in the interpretation of the band positions of ATR–FTIR experiments in the presence of metals, because there is a shift in the band positions.

Overall, from the data of the surface structures together with the data for the adsorption kinetics, it can be suggested that the cholesterol tethers play a prominent role in the rupture and/or spreading of lipid vesicles. When a large number of tethers penetrate the vesicle once it approaches the surface, a major reorganization of the lipid has to take place. This reorganization can eventually lead to the break-up of the vesicle. The vesicle reorganization is however not as important in the case of a low number of tethers, and completely absent without tethers, leading to the major difference in the structures of the EggPC assemblies at low and at high x_{MHex} .

The flat vesicles suggested for pure MHex are consistent with the shapes predicted to be stable for different contact potentials.⁶⁰ Fracture theory has been used to incorporate the possibility of vesicle rupture after adsorption, and the effect of ligands on the shapes has been theoretically analyzed.^{62,63} The theoretical studies reiterate the fact that rupture of adsorbed vesicles depends on the strength of the surface-vesicles interaction. For alcohol terminated SAMs and EggPC this interaction is not strong enough to induce rupture and we have used tether-lipids to induce the formation of planar bilayers. For applications to tBLMs, both concepts are however still lacking the explicit incorporation of the effects of the tether molecules. We note that other strategies like charged surfaces with oppositely charged lipids might also increase the surface-vesicle interaction sufficiently to induce rupture.

From the differences in the kinetics of the dichroic ratio it can be concluded that the mechanisms for the formation of tethered lipid bilayer membranes is substantially different from the mechanisms of the formation of solid-supported lipid bilayer membranes (sBLMs). In the case of tBLMs, and as opposed to sBLMs, the presence of the tether can induce rupture or spreading of the vesicle. Therefore, there may be no need for the existence of critical surface coverage with vesicles in order to form tethered lipid bilayers.

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Figure 1.
Chemical structures of (1) 6-mercaptohexan-1-ol (MHex) and (2) EO₃-Cholesteryl (EO₃Chol) used to form the SAMs.

Figure 2.
A diagram of the spectroelectrochemical cell.

Figure 3.

Different directions used to specify the average tilt angle of the CH_2 group. The tilt angle of the chain segment, θ_c , refers to the average angle between the surface normal (z -direction) and the direction labeled “ c ”. The tilt angle of the group, θ_{CH_2} , refers to the average angle between the surface normal and the direction labeled “ CH_2 ”.

Figure 4.

Simulated spectra with two absorption bands centered at 2850 and 2920 cm⁻¹ of an anisotropic film for two tilt angles, $\langle \theta_c \rangle = 10^\circ$ and $\langle \theta_c \rangle = 80^\circ$ with s- and p-polarized illumination. See text for the further parameters.

Figure 5.

Electrochemical impedance spectra (normalized admittance plot; imaginary part of admittance Y_{im} versus real part of admittance Y_{re} , both divided by angular frequency and electrode area A) of EggPC adsorbed to surfaces with different MHex concentrations. Lines are drawn to guide the eye. The numbers point to the data points Figure 8. The surface concentrations are (1) $x_{MHex} = 0$, (2) $x_{MHex} = (0.16 \pm 0.03)$, (3) $x_{MHex} = (0.36 \pm 0.04)$, (4) $x_{MHex} = (0.79 \pm 0.06)$, and (5) $x_{MHex} = 1$.

Figure 6.

Example of the CH stretching mode region of ATR-FTIR spectra with s- and p-polarized light of EggPC adsorbed to a surface with $x_{MHex} = (0.61 \pm 0.07)$. The spectra were recorded with SAM spectra as background spectra.

Figure 7.

Wavenumber $\tilde{\nu}_M$ of maximum of the symmetric CH_2 stretching mode ($\nu_s(\text{CH}_2)$) of adsorbed EggPC with p-polarized light as a function of the fraction x_{MHex} of the surface area covered by MHex. Each point represents one sample.

Figure 8.

Dichroic ratios of the final assemblies for different fractions x_{MHex} of the mixed SAM surface covered by MHex. Three lines indicate the values expected for three different situations. The line labeled “in plane” corresponds to all CH_2 groups in the plane of the surface. The line labeled “chain tilt 30° ” is the dichroic ratio expected for $\theta_C = 30^\circ$. The line labeled “isotropic” corresponds to the expected dichroic ratio of an isotropic liquid or film. The labels of the right hand side indicate the respective value of $\langle \theta_{CH_2} \rangle$. The numbers refer to the simultaneously measured impedance data shown in Figure 5

Figure 9.

(a) Kinetic trace of adsorption of EggPC to a MHex SAM ($x_{MHex} = 1$). The graph shows the integrated intensities of the $\nu_s(\text{CH}_2)$ mode with p- (●) and s-polarization (▲) and its dichroic ratio (○). (b) Time evolution of normalized imaginary part of admittance (Y_{im} divided by angular frequency ω and electrode area A) at $\omega = 43.7$ Hz (■) and $\omega = 0.66$ Hz (□). The lines are added as an aid to the eye and are prepared by fitting the data to a double exponential using the same time constants in both (a) and (b).

Figure 10. Dichroic ratio of the $v_s(CH_2)$ and $v_{as}(CH_2)$ modes as a function of time at $x_{MHex} = 0.6 \pm 0.06$.

Figure 11.

Overview over the proposed structures of the assemblies on the mixed SAM for different surface compositions. For low x_{MHex} (high fraction of EO₃Chol tethers, shown as ellipses), tethered lipid bilayers are formed. At $x_{MHex} > 0.95$, experimental evidence points to the adsorption of flat vesicles. At small fractions of EO₃Chol tethers ($0.75 < x_{MHex} < 0.95$), the adsorption of spherical vesicles is possible. The sketch is not drawn to any scale.