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Original article

INTERFACIAL WATER AT SYNTHETIC AND NATURAL LIPID BILAYERS PROBED BY VIBRATIONAL SUM-FREQUENCY GENERATION SPECTROSCOPY

Background: At lipid interfaces, water plays a crucial role in carrying biological processes, so that there is a huge interest in unravelling the behaviour of water close to membranes. At charged bio-interfaces, water dipoles form an organized layer. Probing such an interfacial thin layer buried between macroscopic bulk environments is a real challenge. Vibrational sum frequency generation (SFG) spectroscopy is intrinsically specific to interfaces, and has already proven to be an ideal tool to investigate model membranes and their surrounding water.

Objectives: The goal of this work is to measure the vibrational SFG response of interfacial water around different model membranes - from easiest synthetic lipids to more complex natural lipids -, in order to use it as diagnostic signal able to distinguish the lipid bilayer interface by its charge properties.

Materials and methods: Lipid bilayers made either of synthetic or natural lipids (Avanti Polar Lipids) will be physisorbed on CaF_2 prisms (Crystran), by using the method of the spontaneous fusion of lipid vesicles, to form so called solid-supported lipid bilayers (SSLBs). The model membranes will be investigated by SFG spectroscopy at the solid/water interface.

Results: The SFG response was measured between 3600 cm^{-1} and 2800 cm^{-1} , where OH stretching vibrations of water molecules show-up. The SFG intensity of the OH peak maximum at 3125 cm^{-1} was recorded during the adsorption of lipid vesicles on the surface, and provided knowledge of the changes of the charge properties of the interface due to the adsorption of the model membranes. The SFG signal indicated that the organization of water was larger at negatively charged than at positively lipid interfaces, and reached the highest value with natural E. Coli Cardiolipin layers. Moreover, when the full composition of natural lipids was unknown, the behaviour of the SFG response enabled establishing the charge characteristics of the corresponding lipid interfaces.

Conclusion: The SFG response of water enabled estimating the average charge behaviour of synthetic and natural lipid bilayers in pure water, thus paving the way to use the SFG signal of water as new diagnostic tool to identify lipid interfaces.

KEY WORDS: water, lipid membranes, nonlinear optics, sum-frequency generation, vibrational spectroscopy

Biological interfaces and water make an indivisible pair, where the physicochemical properties and biological processes of biointerfaces are intimately linked to the structure and the behaviour of water.

Among biological interfaces, the cell membrane is an ultimate interfacial system, because it is that two-dimensional region making the barrier between the inner and the outer cell environments. Many physiological processes occurring within the membrane barrier are carried and triggered by water, such as protons and ions transport, metabolites adsorption and translocation, or proteins folding, for instance.[1,2] This is why the properties and the behaviour of this small and ubiquitous molecule have attracted the interest of researchers from different disciplines, from biology and medicine, to chemistry and physics.

A crucial point is that water close to biological interfaces is far from behaving as bulk disordered water, while it owns specific order and organization.[3,4] Indeed, upon an electrical potential, such as the one generated by charged interfaces, water dipoles orient along preferential directions, thus forming an organized layer of water, as long as the electric field associated to the electrical potential is non-vanishing. The degree of organization and the thickness over which the organization take place will depend on the physicochemical properties of the interface, i.e., net molecular charge, charge density per surface area, molecular orientation, structure, and hydrophilicity, as well as pH and ionic strength of the liquid medium.[5] Whatever the process that would modify the interfacial properties, this latter may affect the organisation of such a water layer.

The structure, the order and the behaviour of interfacial water close to membrane interfaces have been extensively modelled with theoretical approaches, while its direct measure remained

long-time unavailable. This limitation has been definitely encompassed with the advent of vibrational sum frequency generation (SFG) spectroscopy. Indeed, being based on a second order nonlinear optical (NLO) phenomenon, which is possible only in non-centrosymmetric environments - like anisotropic bulk materials or interfacial systems –, [6,7] SFG spectroscopy is ideally able to probe an organized layer of water, because this latter owns a non-centrosymmetric structure, contrary to bulk water. Moreover, SFG spectroscopy probes molecular vibrations, so that it provides the chemical fingerprint of interfacial water. These skills made SFG spectroscopy a favorite technique to probe water close to membrane systems. [8-22] The investigation of the SFG response around membrane models demonstrated that the surrounding structure of water strongly depends on the physicochemical properties of the interface, and that is tuned by the interactions and the processes occurring within these biological systems.

Here, we will probe the vibrational NLO response of interfacial water at different models of lipid membranes, made of synthetic or natural lipids, supported on CaF_2 . Indeed, the average charge properties of the interfaces trigger the signal of interfacial water accordingly. The goal is testing the potentiality of using the SFG response of interfacial water to identify the underlying lipid bilayer, and then explore new solutions to make SFG spectroscopy a bio-diagnostic tool.

MATERIALS AND METHODS

Lipid bilayers. We prepared lipid bilayers adsorbed on CaF_2 prisms from different lipids Figure 1), namely 1,2-dihexanoyl-sn-glycero-3-phosphoethanolamine (6:0 PE or DHPE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (16:0 PE or DPPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (18:1 Δ^9 -Cis PE or DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (18:1 PS or DOPS), 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(3-lysyl(1-glycerol))] (18:1 Lysyl PG), E. Coli Cardiolipin (E. Coli CA), E. Coli Total Lipid Extract (ETLE) and Yeast Total Lipid Extract (YTLE). All lipid molecules were purchased from Avanti® Polar Lipids, Inc. (Alabaster, Alabama, USA). SSLBs were obtained through the method of the spontaneous fusion of lipid vesicles on CaF_2 (Crystran, UK). CaF_2 prisms were cleaned by immersion in a diluted *piranha* solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2$, 2:1 diluted 10 times) for 1 minute and then rinsed thoroughly with Milli-Q water (18.2 M Ω .cm, pH = 5.5). Lipid vesicles were prepared by i) solubilizing 2 mg of lipid powder in 300 μL of chloroform (CHCl_3), ii) evaporating CHCl_3 under N_2 flow, and iii) adding 1 mL of Milli-Q water. The obtained solutions were sonicated for 10 min and then centrifuged for 10 min at 6000 rpm. The vesicle solutions were injected into a Teflon cell containing 1 mL of milliQ water (18.2 M Ω cm $^{-1}$) and holding the CaF_2 substrate, so that the vesicles concentration during the fusion process at the surface were half of the native concentration. After 3 hours of contact with the prism, SSLBs were expected to be formed.[21,23,24] The remaining vesicles in solution were fully removed by exchanging the solution with Milli-Q water. This procedure was carried out by adding 0.5 mL of Milli-Q water and then removing 0.5 mL of the diluted solution. These steps were repeated at least 20 times in order to leave the SSLBs in contact with an almost pure Milli-Q water solution.

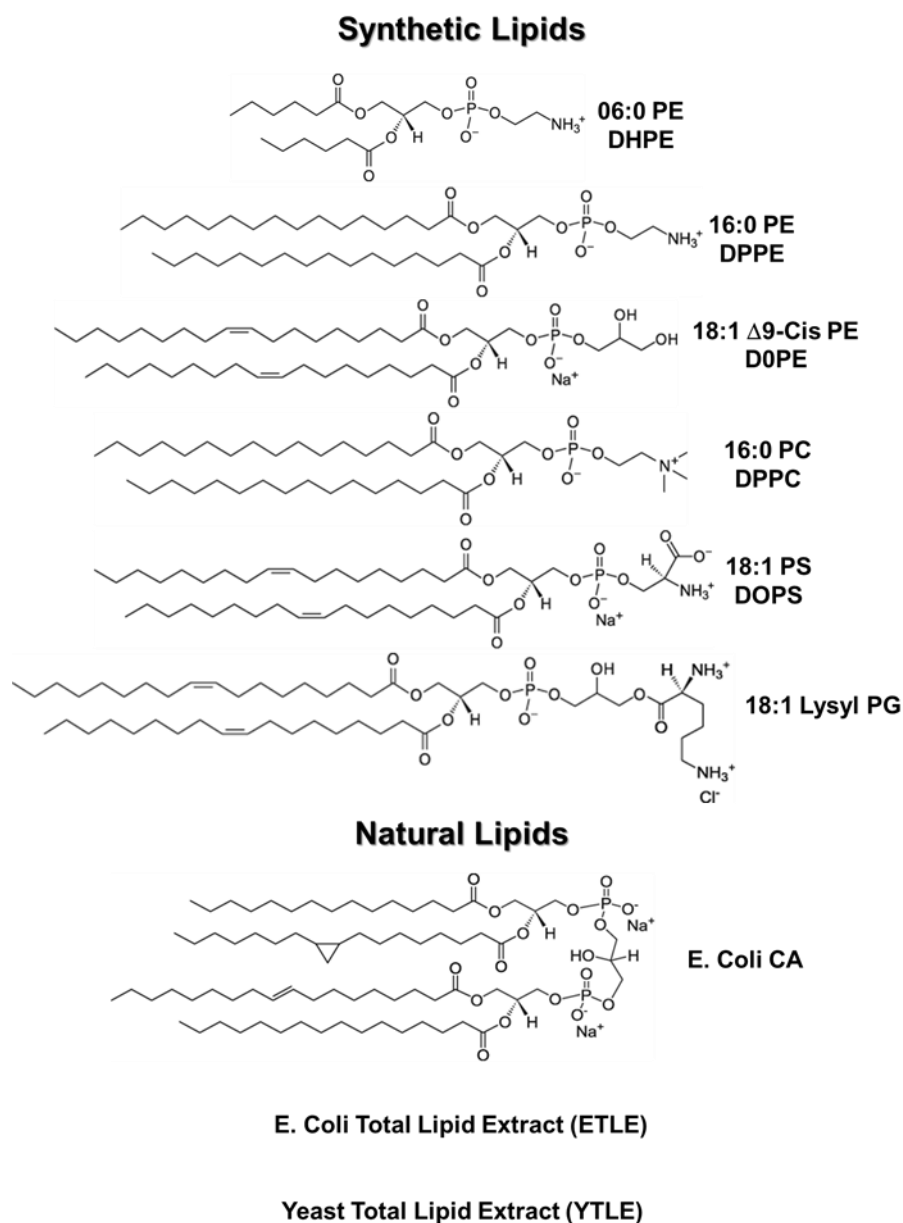


Figure 1. Chemical structures of the lipid compounds used to prepare SSLBs as obtained from Avanti® Polar Lipids supplier (for E. Coli Total Lipid Extract and Yeast Total Lipid Extract full compositions refers to Avanti® Polar Lipids website).

SFG setup. The SFG response of water at the interface was recorded with a homemade SFG spectrometer. Two pulsed incident beams (15 ps, 25 Hz), namely, an IR beam tuned between 3600-2800 cm^{-1} (average power = 25 mW) and a visible (Vis) beam fixed at 532 nm (or 18796.99 cm^{-1} , average power = 10 mW), were superimposed spatially and temporally at the CaF_2 /water interface in a total internal reflection geometry through the prism (Figure 2). An SFG beam was generated with a frequency equal to the sum of the two incident beam frequencies. All measurements were performed at room temperature (22.5°C). To perform comparisons between samples, the SFG responses were normalized by the IR and Vis beams, and by the SFG response of the neat CaF_2 /water interface, which was measured before each lipid bilayer adsorption. All experiments were repeated at least twice.

SFG configuration in a Total Internal Reflection geometry

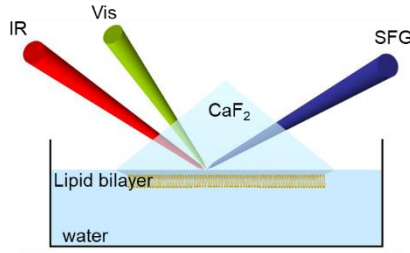


Figure 2. Schematic representation of a SFG measurement at the CaF_2 /lipid/water interface in a Total Internal Reflection (TIR) geometry of the incident IR and Vis beams and of the generated SFG beam.

Theoretical background. Vibrational SFG spectroscopy is based on a 2nd order NLO process, in which the three photon mixing process enables probing the vibrational response of chemical groups in a nonlinear regime. In a SFG process (Figure 2), one tunable infrared (IR) and one fixed visible (Vis) beams are focussed at the interface, where their interaction with the second order susceptibility ($\chi^{(2)}$) of matter generates a new beam at their sum frequency (SFG, $\omega_{\text{SFG}} = \omega_{\text{IR}} + \omega_{\text{Vis}}$). The key selection rule of 2nd order NLO processes is that the $\chi^{(2)}$ susceptibility tensor, in the dipole approximation, is zero for centrosymmetric media, while it is non-zero where the inversion symmetry is broken, like at interfaces. It follows that SFG spectroscopy provides the vibrational signature of interfacial regions only, while it is insensitive to their surrounding bulk environments, either solid, liquid or gaseous.

In addition to the interfacial sensitivity, the SFG response of a vibrational mode of the interface requires this latter is simultaneously IR and Raman active. Definitely, once the tunable IR beam matches an SFG active vibrational mode of the interface, then there is an enhancement of the SFG response, accordingly to the following relations:

$$I_{\text{SFG}} \propto \left| \chi_{\text{NR}}^{(2)} + \chi_{\text{R}}^{(2)} \right|^2 I_{\text{Vis}} I_{\text{IR}} \quad (1)$$

where I_{Vis} and I_{IR} are the intensities of the incident visible and infrared beams, $\chi_{\text{NR}}^{(2)}$ is the non-resonant part of the effective second order susceptibility that can eventually come from the electronic properties of the close bulk environment. The $\chi_{\text{R}}^{(2)}$ resonant contribution is related to the active molecular vibrations as follows:

$$\chi_{\text{R},ijk}^{(2)} \propto \sum_q \frac{A_{q,ijk}}{\omega_q - \omega_{\text{IR}} - i\Gamma_q} \quad (2)$$

$$A_{q,ijk} \propto N \sum_{lmn} \langle T_{lmn}^{ijk} \rangle \beta_{lmn}^{(2)} \quad (3)$$

$$\beta_{lmn}^{(2)} \propto \frac{\partial \alpha_{lm}}{\partial q} \frac{\partial \mu_n}{\partial q} \quad (4)$$

where ω_q and ω_{IR} are the frequencies of the q^{th} molecular mode and of the incident infrared beam, respectively, Γ_q is the damping factor, and $A_{q,ijk}$ is the oscillator strength of the q^{th} molecular mode, which is the macroscopic response N individual oscillators for which the molecular second order hyperpolarisability $\beta_{lmn}^{(2)}$ is linked to the variations of the dipole moment and of the polarizability. T_{lmn}^{ijk} rotates the molecular coordinates lmn into the interface coordinates ijk , and the bracket indicates the average over the molecular orientation distribution and $\frac{\partial \mu_n}{\partial q}$ and $\frac{\partial \alpha_{lm}}{\partial q}$ are the variation of the dipole moment (IR activity) and of the polarizability (Raman activity), respectively, of the q^{th} vibrational mode. [25,26]

In summary, the strict selection rules – that is $\chi_R^{(2)} \neq 0$, $\frac{\partial \mu}{\partial q} \neq 0$ and $\frac{\partial \alpha}{\partial q} \neq 0$ –, combined to the N^2 dependence of the SFG intensity and to the coherent nature of the SFG process, make SFG spectroscopy ideally able to probe a thin interfacial layer of water molecules close to an interface, with a chemical selectivity.

RESULTS AND DISCUSSION

The SFG spectrum of the neat CaF_2 /water interface (Figure 3, top) was systematically recorded before adsorption of lipid vesicles. The broad band centered at 3125 cm^{-1} is due to OH stretching vibrations of interfacial water.[20-23] Indeed, the surface of CaF_2 is known to own a slightly positive charge (its zeta potential in pure water is about +20 mV), which drives the organization of a thin layer of water, possibly with the H atoms pointing out from the surface (as shown in the simplified picture in the bottom of Figure 3, left part).[27-29] The corresponding maximum SFG intensity was about 0.22 arbitrary units (arb. units) (Table 1). This value will represent the reference signal to which all following CaF_2 /lipid/water interfaces will refer.

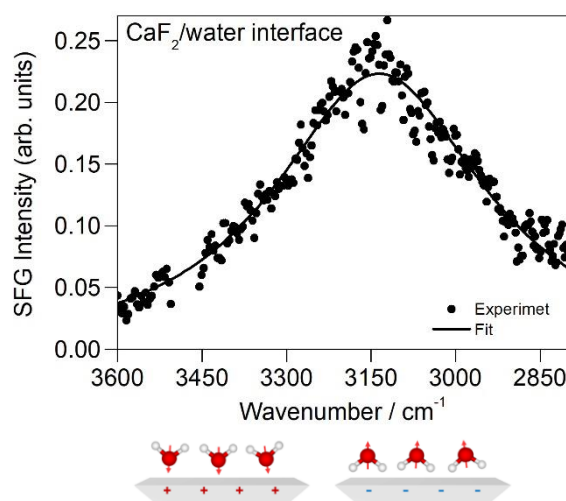


Figure 3. SFG spectrum (top) of the neat CaF_2 /water interface, and schematic and simplified representation (bottom) of the water dipole orientation, with the H atoms pointing out or towards the interface, depending on the sign of the charged interface (positive charge on the left side, and negative charge on the right side).

The adsorption and fusion of lipid vesicles at the CaF_2 /water interface was monitored in real time, by recording the SFG intensity at 3125 cm^{-1} for 3 hours of adsorption. Then, the remaining lipid vesicles in solution were removed, and the SFG maximum intensity of the CaF_2 /lipid/water interfaces was measured.

Interface	SFG intensity (arb.units)
CaF_2 /water	0.22
CaF_2 /DHPE/water	0.20
CaF_2 /DPPE/water	0.18
CaF_2 /DOPS/water	1.50
CaF_2 /DOPG/water	0.60
CaF_2 /E. Coli CA/water	3.75
CaF_2 /ETLE/water	0.25
CaF_2 /YTLE/water	0.31

Table 1. SFG maximum signal at the CaF_2 /water and lipids/water interfaces recorded at 3125 cm^{-1} . The error bars are estimated to $\pm 15\%$.

Synthetic Lipid Layers. Neutral zwitterion PE lipids. DHPE, DPPE, DOPE are all neutral zwitterion lipids. Their difference is in the composition of the hydrophobic chains. DHPE holds two full saturated aliphatic chains (i.e. 6 aliphatic carbon atoms/chain). The adsorption of DHPE vesicles on CaF₂ did not modify substantially the interfacial charge properties, as the signal of water remained almost constant along the adsorption process (0.2 arb. units). This is in agreement with both the neutral charge carried by the lipid molecule, and with the weak charge screening effect induced by short aliphatic chains. Compared to DHPE, DPPE has longer aliphatic chains (16 C atoms/chain), made also of all saturated bonds. The adsorption of DPPE decreased the water signal from 0.22 at the neat CaF₂ interface to 0.10 at the DPPE interface, indicating a weak screening of the interfacial charges by the lipid layer, whilst the lipid interface remained positively charged.

DOPE is composed of two aliphatic chains, almost as long as DPPE (18 carbon atoms/chain), while it carries one unsaturated bond in each aliphatic chain. The adsorption of DOPE occurred with only slightly decreasing the SFG signal of water to 0.18 arb. units. The different behavior of DPPE and DOPE in the screening effect can be attributed to the saturated/unsaturated bonds in the aliphatic chains, which trigger the conformational organization of the lipid layers. Indeed, the presence of unsaturated bonds affects the phase transition temperature (T_m) of lipids,[30] so that DOPE ($T_m = -16^\circ\text{C}$) is in a liquid-like phase at ambient temperature, while the full saturated bonds of the DPPE ($T_m = +63^\circ\text{C}$) tails make the corresponding lipid layer in a gel-like phase. The resulting bilayer structures show a more densely packed organization for DPPE than for DOPE, with the consequence that DPPE drives a larger charge screening than DOPE.

Negatively PS and positively Lysyl PG charged lipids. DOPS and Lysyl PG are negative and positive charged lipids, respectively, with one net charge per molecule. DOPS holds two negative and one positive charges, while DOPG holds one negative and two positive charges, which are localized on the polar heads, respectively. Both lipids have the same aliphatic chains, with one unsaturated bond in each aliphatic chain, giving rise to a liquid-like conformation of the corresponding bilayers at room temperature. Starting from the intensity of the CaF₂/water signal (0.22 arb.units), once DOPS vesicles were injected in the cell to adsorb and fuse on the CaF₂ surface and form a lipid bilayer, the SFG intensity decreased to zero in a few seconds, then increased to 1.50 arb. units in the next minute, and remained stable at this value for the next 3h. The passing from zero of the SFG intensity correspond to the flip-flop of water molecules from the up to the down conformation (Figure 3, bottom).[12,31] Indeed, DOPS vesicles carried their negative charge close to the CaF₂ surface, and screened their positive charge. The further saturation of the surface by DOPS vesicles led to a strongly negatively charged interface, so that the SFG signal increased back, which correspond to an organisation of the water dipoles close to the lipid interface. A different behaviour was observed during the adsorption of Lysyl PG vesicles at the CaF₂/water interface. Here, the SFG signal showed a fast increase of the SFG signal to 0.60 arb.units directly, without passing from zero, in agreement with the positive net charge carried by the lipids polar heads, which is of the same sign of the neat CaF₂ surface. Definitely, at the DOPS/water interface, water dipoles are expected to be oriented with a down conformation (Figure 3 bottom right), while for Lysyl PG/water interface they are expected to be predominantly oriented with an up conformation (Figure 3, bottom left).

Natural Lipid Layers. Going from the easiest model membranes made of mono-component layers of synthetic lipids of known chemical composition towards more representative models of real systems requires facing that the full composition of the lipid layers is unknown.

E.coli Cardiolipin. E. Coli CA is a mixture of different CA structures, all holding the CA polar head, carrying two net negative charges, and different hydrophobic chains, which are in the amount of four per molecule. Starting from a SFG intensity of 0.22 V at the neat CaF₂/water interface, the injection of E. coli CA vesicles in the liquid cell decreased the SFG intensity to

zero in less than 10 minutes, and then this latter increased back and reached a value as high as 3.75 arb. units after 3h. This behavior indicates that *E. coli* vesicles screened the initially positive charge of CaF_2 , and then the corresponding lipid bilayer provided a strongly negative character to the interface, which drove a higher organization of interfacial water. This is possibly due to the structure of CA compounds, which, due to the presence of four hydrophobic chains per molecule owing strong intermolecular interactions, give rise to densely packed structures, leading to a higher density of charges per surface area. Moreover, so densely packed films led to highly organized structures, where the polar heads are predominantly oriented in a preferential direction, which may favor a large alignment of water close to the lipid interface.

Total Lipid Extracts: ETLE and YTLE. *E. coli* Total Lipid Extract is composed of different neutral and charged lipids. The known fraction of ETLE (as provided by the supplier Avanti Polar Lipids), which account for the 82.4% of the total composition, exhibits an average charge of the lipid composition that is negatively charged, while the remaining 17.6% fraction was unknown. The injection of ETLE vesicles at CaF_2 /water interface induced a decreased of the SFG intensity to zero, and then an increase up to 0.25 arb. units in only one minute. This behavior is in agreement with a very fast screening of the positive charge of CaF_2 by ETLE vesicles, which inverted the sign the interfacial charges from positive to negative. This statement enabled unravelling that an inversion of the orientation of the water dipoles occurred, which testified for an average negatively charged character of the ETLE lipid interface.

Similarly, the exact charge of YTLE was also unknown (see the YTLE composition as provided by Avanti Polar Lipids). The injection of YTLE vesicles decreased the initial SFG intensity to zero, and then this latter increased back up to 0.31 arb. units in the next 3 hours. This behaviour suggests an average negative charge of the YTLE lipid layer, which triggered an inversion of orientation of the water dipoles from the CaF_2 /water interface to the CaF_2 /YTLE/water interface.

CONCLUSION

The SFG response of water was measured at neutral, negative and positive mono-component lipid bilayers, respectively, and showed that the highest signal, corresponding to a high degree of organisation, was reached at the DOPS negative lipid interface. At natural lipid layers interfaces, the SFG response of *E. Coli* Cardiolipin layers indicated an impressive organization of water close to the interface, since the SFG signal increased by two times larger than at the negative mono-component lipid interface. These behaviours enabled estimating the average charge properties of two natural lipids compounds, namely *E. Coli* total lipid extract and yeast total lipids extracts, whose full composition was unknown. The SFG response during the adsorption of the corresponding vesicles onto the surface demonstrated that these lipid layer interfaces were in average negatively charged, with a slightly higher organisation of water at the YTLE than at the ETLE interface. By using synthetic lipid layers of known composition as reference signals, it has been then possible to unravel the average charge properties of natural lipid interfaces. The possibility to distinguish lipid interfaces by the signal of their organized water environment represents a possible solution to be explored for developing new, innovative and label-free biodevices.

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CONFLICT OF INTEREST

The authors report that there is no conflict of interest.

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