Molecular orientation at biological interfaces: Water and lipids studied through surface-specific vibrational spectroscopy
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Molecular Reorganization at Lipid Monolayers

Proceeding with the exploration of the possibilities of SFG spectroscopy, we here apply it to a highly controllable, but biologically relevant model system of a lipid monolayer with charged nanoparticles (NPs). The interaction of lipid monolayers with charged polystyrene NPs is relevant for NP cytotoxicity and drug delivery research, and may serve as a general model for electrostatics at biomimetic interfaces. In this study, we employ vibrational sum-frequency generation (SFG) spectroscopy as a tool to probe and quantify binding of NPs to lipid monolayers. Strong spectral signatures of the interaction can be identified in both the interfacial water signal, and in the CH vibrations of the lipid alkyl chains. We examine the role of electrostatics on the interaction by using positively and negatively charged NPs, binding to lipids of positive, negative and zwitterionic charge. Although the interaction is strongest for opposing charges, as is to be expected, every combination displays some effect of nanoparticle presence in the SFG spectra. For zwitterionic lipids, a remarkable asymmetry arises that can be ascribed to the distribution of charges in and the flexibility of the lipid head group. The role of the interfacial water molecules in this system is followed in detail, and a schematic model of the molecular alignment is proposed. Scanning electron microscopy images provide a visual confirmation of the interaction.

6.1  INTRODUCTION

In recent years, the interaction of biological matter and nanomaterials has been a topic of discussion in both the scientific world and in society in general. The reason for this interest is the increasing application of nanomaterials in commercial and medical sectors. This rapid growth of exposure of the public to these relatively unknown substances raises health concerns, since our scientific knowledge may not keep up with these developments. The interaction of nanoparticles (NPs) with the human body is dependent on various factors, such as particle size, shape and charge, which determine the spread of particles through different parts of the body and the potential of causing damage there [114–116].
When examining the effect of NPs on the human body on a molecular scale, we should realize that a key interaction is that between the particles and the cell membrane. Firstly, whether the particles are able to move through this barrier greatly influences their spread through the body as a whole. Secondly, the potential disruption of a membrane by NPs is a cause of cytotoxicity, either trough necrosis or the triggering of apoptosis [60,117].

Advances in synthesis methodology have shown that it is possible to produce NPs that pass through the cell membrane without damaging it [114,118,119]. This development confirms that NPs can be applied in a medical context, e.g. as drug carriers or stabilizers of targeted drug delivery systems [120]. To effectively synthesize NPs towards this end, it is crucial to understand the role that physicochemical properties such as size, charge and surface chemistry play in the interaction. More generally then, NPs are of fundamental interest to our understanding of the interaction between nanoscale objects and biological soft condensed matter. The controllability of their composition enables research focused on specific particle properties.

Many studies have focused on the interface between nanoparticles and phospholipids. Nonspecific binding of charged nanoparticles locally changes the fluidity of the lipid membrane in liposomes [46]. The fluidity of the lipids is reflected by the organization of the hydrophobic chains, which are typically more ordered in the gel phase than in the fluid phase. Recent simulations on phospholipid bilayers with nanoparticles of varying charge have confirmed these observations [121]. However, a gap remains in our understanding of the mechanism by which nanoparticles affect the membrane on a molecular level. A direct observation of lipid reorganization would be able to provide a link between the macroscopic observations on the one hand and theoretical and molecular dynamics simulations on the other. To this end, we here employ vibrational sum-frequency generation (SFG) spectroscopy, a second-order and therefore surface-specific optical technique [25]. We study monolayers composed of a single type of lipid, looking at various different lipids. SFG spectroscopy has been shown to be able to directly identify lipid chain ordering by looking at the relative sum-frequency intensity of the various CH-stretch vibrations [122]. It is therefore a suitable tool in studying the changes in molecular organization upon nanoparticle binding to lipid layers. Furthermore, SFG spectroscopy is very well suited to monitor the vibrational response of membrane bound interfacial water, which is known to be very sensitive to changes in the surface charge [24]. The behavior of water around the interface is of added value because of the important role of water in determining the structure and functioning of biological membranes [123].

In this study we focus on the role of NP and membrane charge. We choose single-lipid monolayers as model systems. Our charged model membranes are monolayers of DPPG, which is negatively charged due to a phosphate group, and DPTAP containing a positively charged choline group.

The third lipid that we studied is DPPC, which is electrostatically the most complex of the three since it contains both a negative phosphate and a positive choline group. This zwitterionic head group makes its electrostatic influence on
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Figure 6.1. Chemical structure of the three lipids used in this study.

the surrounding water complex [124]. However, it is also the most studied phospholipid due to its prevalence in biological membranes, notably the mammalian pulmonary system [125, 126]. For DPPC, the binding of both positively and negatively charged NPs has been reported, with the negatively charged NPs raising the phase transition temperature by tens of degrees [127] and decreasing the membrane fluidity [46]. These observations can be intuitively interpreted as being caused by a local gelation of the lipids at the NP binding site. Molecular dynamics simulations have confirmed this hypothesis, and have shown an ordering effect due to the pull of the negative NP on the positive terminus of the DPPC head group [121, 128]. The chemical structure of the three lipids is shown in figure 6.1, highlighting the structural similarities between them. Note that while our target system, the actual cell membrane, does not contain positively charged lipids, it has an abundance of negatively charged and zwitterionic lipid species. We opted to include DPTAP to assess the water’s response to a single choline group, in hope to better understand the partial effect of the two separate charge groups of DPPC.

We employ basic polystyrene latex NPs functionalized with small, simple charge groups: amidine provides a positive charge, carboxyl a negative charge. Evidently, the interaction between NPs and monolayers of opposite charge is expected to be electrostatically favored. The influence of NP binding on the monolayer fluidity and alkyl chain order, which are indicators of the membrane structure, is more complex since it depends on the charge distribution and orientation of the lipids head group [121, 128]. The spectral changes will indicate what the role is of electric charge on interfacial molecular reorganization upon NP binding. Scanning electron microscopy images were recorded as an additional and direct means to estimate the interfacial NP density.

6.2 Materials and Methods

Lipids. The negatively charged phospholipid DPPG (1,2-dihexadecanoyl-sn-glycero-3-phospho-(1’-rac-glycerol) with sodium salt), the positively charged lipid DPTAP (1,2-dipalmitoyl-3-trimethylammonium-propane with chloride salt) and the zwitterionic phospholipid DPPC (dipalmitoylphosphatidylcholine,
figure 6.1) and its deuterated alternative d75-DPPC were obtained from Avanti Polar Lipids. Self-assembled monolayers were produced by dropcasting a solution of this lipid in chloroform (0.5 g/L) drop by drop onto a H\textsubscript{2}O or D\textsubscript{2}O subphase. D\textsubscript{2}O used in this study was obtained from Cambridge Isotope Laboratories (MA), 99.96% pure and was used as received. H\textsubscript{2}O used in this study was distilled and then filtered using a Millipore unit to a final resistivity of 18.2 M\textOmega per cm. Samples were prepared in a 20 mL Teflon-coated aluminum trough. Surface pressure in these troughs was quantified using a commercially available tensiometer (Kibron, Finland).

Nanoparticles. Polystyrene latex NPs have a mean diameter of 27 nm with a standard deviation of 6 nm. Negatively charged NPs were functionalized with carboxyl groups, yielding a charge density of 1.1 nm\textsuperscript{2} per charge group (8.2 x 103 charges per particle). A zeta potential of -61.4 mV was measured by means of electrophoretic mobility equipment (Malvern Instruments Ltd). Positively charged NPs were functionalized with amidine groups, yielding a charge density of 4.3 nm\textsuperscript{2} per charge (2.1 x 103 charges per particle) and a zeta potential of 53.3 mV.

After preparation of the monolayer, NPs were injected underneath the surface without puncturing it through a hole in the side of the trough. In all described experiments concentrations are expressed in µL of 4 volume-% NP suspension. The aqueous suspension was injected into the subphase (i.e. the water reservoir underneath the monolayer) while gently stirring with a magnetic stirrer located on the bottom of the sample holder. The resulting NP concentration was 0.32 nM per 1 µL injected suspension. The added NP cross section surface present in the trough at a concentration of 1 µL is enough to cover the entire monolayer surface.

Tensiometry measurements. In tensiometry, the two-dimensional pressure along the circumference of a needle tip is measured. This surface pressure reflects the surface free energy, which changes upon the binding or insertion of new molecules to or into an existing monolayer [129, 130]. Tensiometry measurements were performed using the Wilhelmy plate method (Kibron Inc.). In tensiometry, the two-dimensional pressure along the circumference of a needle tip is measured. This surface pressure is indicative of the surface free energy, which changes upon the binding or insertion of new molecules to or into an existing monolayer. Tensiometry measurements were performed using the Wilhelmy plate method (Kibron Inc.).

Sum-frequency generation spectroscopy. SFG spectroscopy is able to probe interfaces specifically and background-free through the second-order non-linear susceptibility $\chi^{(2)}$ of the sample material.

In the SFG experiments, 1 mJ of the output of a regeneratively amplified titanium sapphire system (Spitfire Ace, Spectra Physics, Inc.) producing $\sim$35 fs pulses centered at 800 nm was used to generate tuneable mid-IR pulses using a homebuilt optical parametric amplifier and difference frequency generation unit [68]. 0.5 mJ of the amplifier output was spectrally narrowed to $\sim$15 cm\textsuperscript{-1} using a Fabry-Perot etalon. The IR beam passes through a half wave plate and polarizer before being focussed with an f = 50 mm lens onto the sample together
with this spectrally narrowed visible beam, focussed with an f = 200 mm lens, with angles of incidence of 45° and 40° with respect to the surface normal and a power of 5 mW and 25 mW. The SFG signal was detected in reflection mode and focused into a spectrograph (Acton, Princeton Instruments) in which it was dispersed, via a grating, and focused onto an electron multiplied Charge Coupled Device (emCCD) camera (Newton, Andor). All spectra reported in this study were collected under the ssp-polarization condition (SFG and visible s, IR p polarizations). All measurements were conducted at 23°C.

The increase in CH vibrational intensity can be thought to originate from a direct spectral response of CH modes within the NPs themselves. To check this, control experiments were performed on a monolayer deuterated DPPC (d75-DPPC). In the SFG spectrum of this deuterated lipid, the alkyl chain vibrations are removed from the 2800-2950 cm\(^{-1}\) region. By doing so, spectral changes caused by the introduction of new vibrational modes can be unambiguously separated from those caused by lipid reorganization. Upon injecting NPs underneath a d75-DPPC monolayer, no emerging peaks were observed in the CH region, ruling out the possibility of the presence of molecular vibrations within this region of moieties within or on the polystyrene NPs. At first sight, this observation may be surprising given the abundance of CH modes within polystyrene polymers. However, the small size of the nanoparticles in conjunction with their internal symmetry causes their SFG response to cancel out [131].

Scanning electron microscopy (SEM). Samples for SEM were prepared by means of the exact same protocol as the SFG spectroscopy samples, and then deposited on a silicon template-stripped gold (40-45 nm) on glass substrate by Langmuir-Schfer deposition. The SEM images were all taken with an FEI GEMINI SEM-system for a 10 µL 4% volume suspension of NPs, amounting to a concentration of 3.2 nM, and stirred for 20 minutes.

Reference measurements without lipids were performed observe the possible direct interaction between gold substrate and NPs. These images showed no deposition of NPs, indicating that all NPs observed in the SEM images in this chapter are lipid-mediated.

6.3 Results and discussion

DPTAP

Pure monolayers of the positively charged lipid DPTAP were prepared at a surface pressure of 28 mN/m. Charged NPs were injected underneath the monolayer while stirring with a magnetic stirrer. To visualize the binding of NPs to the monolayer and estimate the interfacial concentration, the monolayer was deposited on a gold substrate and recorded by means of SEM. Figures 6.2 and 6.3 show the resulting images for monolayers with 3.2 nM of carboxyl (-) and amidine (+) coated polystyrene latex NPs, respectively. Although with this technique no lipid structures are discernible, the 30 nm NPs are readily iden-
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Clearly, electrostatics dominate the interaction: adding the negatively charged NPs to the positively charged surface causes large clusters of NPs to congregate at the surface (figure 6.2). Combining equal charges, on the other hand, shows no trace of NP binding, with only a few particles visible in the image (figure 6.3, right image).

Figure 6.2. SEM image of DPTAP (+) monolayer with 3 nM carboxyl (-) NPs deposited on a gold substrate.

Figure 6.3. SEM image of DPTAP (+) monolayer with 3 nM amidine (+) NPs deposited on a gold substrate. Hardly any particles are present due to the electrostatic repulsion.

SFG spectra of the aqueous sample were recorded for a range of NP concentrations. A few of these spectra are shown in figure 6.4. First, the response of the DPTAP monolayer was measured without any NPs present; this spectrum is shown in red. The most prominent features are the double water (OD) feature between 2200 and 2600 cm\(^{-1}\) and the various CH vibrations around 2900 cm\(^{-1}\). The two apparent water peaks do not, in fact, arise from separate water structures, but are due to a dip caused by intramolecular coupling between bend and stretch vibrations of the water molecule [132]. The CH vibrational modes of saturated lipid alkyl chains give rise to six SFG peaks in the 2800-2950 cm\(^{-1}\) region [133]. For most lipids at high concentration, the three most intense of these are the CH\(_2\) symmetric stretch (CH\(_2\)SS, 2854 cm\(^{-1}\)), the CH\(_3\) symmetric
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6.3

Figure 6.4. Vibrational SFG spectra of a DPTAP (+) monolayer with varying concentrations of carboxyl (-, blue) and amidine (+, green) functionalized latex nanoparticles.

Figure 6.5. OD stretch resonance intensity (water intensity) and order parameter R extracted from the fits in figure 6.4 plotted as a function of concentration for carboxyl (-, left, blue) and amidine (+, right, green) nanoparticles. Note the negative sign of the water intensity, a large negative value implying a strong negative signal.

Adding NPs beneath the monolayer induced significant and distinct changes in the spectra within minutes after injection. Both for the carboxyl (-) and
amidine (+) NPs, the water signal intensity and the order parameter R change dependent on NP concentration, which proves that the NPs interact with the lipid monolayer. To quantify the observed spectral changes, the VSF spectra were fitted by assigning Lorentzian line shapes to the CH modes and Voigt profiles to the water bands [25,63].

Apart from the double water feature and the three aforementioned CH vibrations, two Lorentzians were needed to obtain an accurate fit of the spectra. The first is a weak, broad feature around 2650–2700 cm$^{-1}$ that can be attributed to weakly hydrogen bonded water bordering the lipid and/or air-water interface [35]. The second is a very weak and narrow (33 cm$^{-1}$) feature at 2750 cm$^{-1}$ that corresponds to an overtone of the CH$_3$ bend mode [134]. Figure 6.4 shows the fitted spectra for a selection of NP concentrations. The increase in noise level around 2350 cm$^{-1}$ is due to IR absorption by the CO$_2$ asymmetric stretch vibration in air.

The decrease of the water intensity is the most striking effect that the injection of NPs underneath the DPTAP monolayer has on the SFG spectrum. This decrease implies that water alignment at the air-lipid-water interface is disturbed, which is likely to be caused by the changes in the electrostatic potential at the surface upon introducing the highly multivalent charged NPs. While SFG spectroscopy is highly surface specific, both the second-order susceptibility (2) and the third-order susceptibility (3) contribute to the signal at a charged interface [135, 136]. The former arises from water molecules aligned at the interface, the latter from the fact that a static potential is present at a charged interface so that a signal from the isotropic bulk appears. While spectral changes may originate from both (2) and (3) and their relative contribution is hard to estimate, any such change must be caused by a modulation of the electrostatic potential. An additional effect may be that the presence of NPs at the surface pushes a part of the water contributing to the SFG signal out of the interfacial region.

Figure 6.5 displays the concentration dependence of the water intensity defined as the sum of the intensities of the two water peaks between 2200 and 2600 cm$^{-1}$ for carboxyl (-) NPs (left) and amidine (+) NPs (right). The fitted intensity does not only have a magnitude, but also a sign, indicating the phase of the vibration, i.e. whether the molecular bond is pointing up or down with respect to the surface. For water aligned by a positive monolayer, which is to say with the negative oxygen pointing up and the positive hydrogens pointing down, commonly a negative sign is assigned, as is indicated on the left axes in figure 6.5.

Looking at figure 6.5, left panel, we can conclude that the carboxyl (-) NPs interact strongly with the oppositely charged choline in the lipid head group. With increasing concentration of NPs, more and more negative charges are added to the positively charged surface, and we see the water signal go to zero. This tells us that the carboxyl (-) NPs are closely bound to the DPTAP head group; if a layer of water would be present between the two, a strongly aligned negative signal would remain. At higher NP concentrations (> 2 nM) the water intensity becomes positive; this effect can also be seen in figure 6.4
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as a remarkable change in spectral shape due to interference of the overlapping vibrations. This is a strong indication that charge inversion takes place at the interface: the overcompensation of the potential at a charged surface by opposing charged ions or particles, which various studies have reported to occur with multivalent charge groups [137–142]. In these studies, the multivalence of the charged particles was crucial to changing the surface charge. This explains the seemingly low influence of counterions in the current experiments. In terms of molecular structure, the charge inversion in this case may be caused by the negative carboxyl group being in direct contact with the water molecules, while the positive charge on the lipids choline group is shielded by the head groups methyl groups and by the carboxyl NPs themselves.

When adding amidine (+) NPs, the water signal decreases (the solid green line in figure 6.5, right panel), but much less strongly than for the carboxyl NPs. This result may appear surprising at first glance, since a) the SEM images revealed no interaction of the DPTAP monolayer and the amidine (+) NPs at all (figure 6.3), and b) the charge on these particles, when located at the surface, should only enhance the water alignment. However, combining these two observations, we may conclude that the amidine (+) NPs do not bind to the DPTAP monolayer, but remain in a suspension in the subphase. The electrostatic potential of this suspension of positively charged NPs below the interfacial water opposes the alignment effect of the charged lipids. As a consequence the water SFG response decreases.

In addition to variations in the water intensity, a second large spectral change can be identified in the CH stretch vibrational region, around 2900 cm$^{-1}$ (figure 6.4). The order parameter R calculated from these changes is plotted in figure 6.5 as a function of NP concentration (dashed lines). It is immediately clear that R closely follows the water intensity. Given the negative sign of the water peak, this means that the water intensity is actually rather inversely proportional to R. So what does this relation imply about the interaction on the molecular level? Considering carboxyl (-) NPs first, the order of the lipids increases with NP concentration. This effect has been reported before for DPPC membranes in both theory and experiment, and was ascribed to the electrostatic pull of the NPs negative charge on the flexible lipid head group [46, 121, 128]. This pull straightens the lipid, decreasing its footprint size (the space the head group takes at the interfacial plane), increasing the local lipid density and the phase transition temperature. We are able to detect these changes with SFG spectroscopy due to the accompanied increase in alkyl chain ordering. It is remarkable that DPTAP shows the same effect, since its smaller head group size does not allow equally large changes in footprint size as those occurring in DPPC. Possibly, the change in charge distribution over the atoms of the lipid head group is enough to induce the molecular order.

For amidine (+) NPs, the change in lipid order parameter R is not significant. Note that although in figure 6.5 (right panel) the change appears to be similar to that of the water signal, the error on R is larger than on the water signal because it originates from much smaller spectral features. The observation that R remains unchanged (or decreases by a very small amount) is consistent with
the conclusion that amidine (+) NPs do not come into close contact with the monolayer but rather remain suspended in the subphase.

**DPPG**

![Figure 6.6. SEM image of DPPG (-) monolayer with 3 nM carboxyl (-) NPs deposited on a gold substrate. Hardly any particles are present due to electrostatic repulsion.](image1)

![Figure 6.7. SEM image of DPPG (-) monolayer with 3 nM amidine (+) NPs deposited on a gold substrate.](image2)

The SEM and SFG measurements were repeated in the exact same way with monolayers prepared of the negatively charged lipid DPPG. Monolayers were prepared at a surface pressure of 30 mN/m. Figure 6.6 and 6.7 show SEM images of deposited monolayers of DPPG with 3.2 nM of carboxyl (-) and amidine (+) coated polystyrene latex NPs, respectively. Clearly, the result is the exact opposite of DPTAP: now the carboxyl (-) particles do not bind, while the amidine (+) NPs cover a significant area of the surface. Basic electrostatics is responsible for this behavior: the repulsion and attraction of carboxyl (-) and amidine (+), respectively, of NPs by the negatively charged phosphate group of DPPG. Two other, more subtle differences of DPPG with the DPTAP case can be identified: firstly, on a larger scale some inhomogeneous features can
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Figure 6.8. Vibrational SFG spectra of a DPPG (-) monolayer with varying concentrations of carboxyl (-, blue) and amidine (+, green) functionalized latex nanoparticles.

Figure 6.9. OD stretch resonance intensity (water intensity) and order parameter R extracted from the fits in figure 6.8 plotted as a function of concentration for carboxyl (-, left, blue) and amidine (+, right, green) nanoparticles.

be identified, where the concentration of NPs is locally larger (figure 6.6, left panel) or smaller (figure 6.7, left panel). Given the fluidity of the monolayer, inhomogeneities of this scale are unlikely to occur in single-lipid monolayers on an aqueous subphase, and should therefore be ascribed to impurities on the gold substrate. Secondly, the NP concentration and agglomeration rate is lower for DPPG with amidine (+) NPs than it was for DPTAP with carboxyl (-) NPs, even though the charge density on the amidine (+) NPs is lower. At this point, we may hypothesize that the electrostatic field of DPTAP is larger than that of DPPG, but SFG spectroscopy will shine more light on this issue.
Several SFG spectra of DPPG with NPs are shown in figure 6.8. Similar to the SEM images, many of the effects of NPs on the DPPG spectrum mirror the changes observed for DPTAP. Again, both amidine (+) and carboxyl (-) NPs lower the water intensity, but the effect is now much stronger for amidine (+) NPs (figure 6.9). Given the difference in head group charge between DPTAP and DPPG this makes perfect sense, and all conclusions about the mechanism of the interaction drawn for DPTAP equally hold for DPPG.

A difference between the DPTAP and DPPG interaction can be identified in the order parameter R for NPs of charge opposite to that of the lipid head group. While R increased in the case of DPTAP with carboxyl (-) nanoparticles, no such change can be seen for DPPG with amidine (+) NPs. Apparently the chemical structure of the DPPG head group, with the glycerol dangling beneath the negatively charged phosphate group, does not allow for the same ordering effect when feeling the pull of charged NPs underneath. A steric effect of the glycerol group, which may be forced under a non-zero angle with the rest of the lipid in order to allow the minimal distance between the charge on the lipids and on the NP, is a likely explanation for this asymmetry.

A final difference between the spectra of DPTAP and DPPG is the magnitude of the water intensity. Note here that it is customary to give the SFG intensity in arbitrary units, and that care should be given when quantitatively comparing SFG spectra of separate measurements [20, 66]. In this study however, experimental conditions were kept constant enough to obtain fully reproducible results for the different systems, justifying comparison. Looking at the relative size of the water and CH response gives a further sense of scale. The significantly smaller absolute value of the water signal of DPPG (+37) when compared to DPTAP (-52) indicates that the electrostatic potential felt by the interfacial water is lower for this lipid, thus reducing the overall alignment of water molecules. This spectral feature then supports the hypothesis drawn from the SEM images that the amidine (+) concentration at DPPG is lower than the carboxyl (-) concentration at DPTAP due to the size of the interfacial electrostatic potential.

**DPPC**

Monolayers of DPPC were prepared at a surface pressure of 27 mN/m. Figure 6.10 and 6.11 show SEM images of deposited monolayers of DPPC with 3.2 nM of carboxyl (-) and amidine (+) coated polystyrene latex NPs, respectively. The results are now completely different from those obtained with the charged lipids: both carboxyl (-) and amidine (+) NPs show up in the microscopy images, indicating that both NPs of opposite charge interact with and bind to the dipolar head group of DPPC.

Remarkably, the NPs now show no signs of aggregation. The fact that they remain perfectly separated here, at the DPPC layer, suggests that they do not aggregate in the subphase suspension, since it is unlikely that the surface potential at the DPPC interface would separate them again. More likely the aggregation observed for the charged lipids takes place at the lipid interface, where
the surface potential overcomes the repulsion of the particles. Despite their overall neutral charge, DPPC monolayers do cause an electrostatic alignment of the interfacial water through dipole-dipole interactions. Molecular dynamics simulations have predicted that the negative charge on the phosphate group dominates the electrostatic potential [143–146]. Phase-specific SFG studies confirmed that the average alignment of the interfacial water is with the hydrogen atoms pointing towards the monolayer, i.e. its dipole moment pointing up, and that the aligned water that contributes most to the SFG signal is located between the phosphate and the choline group [35, 124]. This indicates that the water feels an electrostatic potential similar to that created by a negatively charged lipid like DPPG. The relatively higher concentration of amidine (+) NPs than carboxyl (-) NPs at the DPPC interface observed in the SEM images (figure 6.10 and 6.11) may be explained by this overall negative potential caused by the zwitterionic head group.

In figure 6.12, several spectra of DPPC with carboxyl (-) and amidine (+) NPs are shown. As expected, our fitted SFG spectrum for pure DPPC shows a
Figure 6.12. Vibrational SFG spectra of a DPPC (-/+ monolayer with varying concentrations of carboxyl (-, blue) and amidine (+, green) functionalized latex nanoparticles.

Figure 6.13. OD stretch resonance intensity (water intensity) and order parameter R extracted from the fits in figure 6.12 plotted as a function of concentration for carboxyl (-, left, blue) and amidine (+, right, green) nanoparticles.

large water peak of negative amplitude, implying that the water dipole moment is pointing up. Note that while we measure without phase sensitivity here, the relative sign of the amplitudes can be inferred from the line shapes: the general shape of the DPPC spectrum (figure 6.12) resembles that of DPPG (figure 6.8) much more than DPTAP (figure 6.4). Indeed, it is impossible to obtain a good fit of the DPPC spectrum when assigning a positive amplitude to the hydrogen bonded region.
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The effects of increasing the NP concentration are now highly asymmetrical: carboxyl (-) NPs cause both the absolute water signal and the order parameter R to increase. Amidine (+) NPs cause the water signal and the order parameter to decrease. These changes can be explained by considering the flexibility of the dipolar head group of DPPC. In a typical membrane, the phosphorus-to-nitrogen vector makes only a small angle with the surface plane [147]. The pull of a carboxyl (-) NP on the choline group may increase this angle, while the pull of an amidine (+) NP on the phosphate group will likely decrease it. Since the electrostatic potential of DPPC is highest between the phosphate and choline groups [35, 146], the projection of the DPPC head group dipole vector on the surface normal determines the degree of water alignment, and thus the SFG water signal. The increase of the water signal observed for carboxyl NPs then implies that the lipid is stretched along the surface normal, which is in agreement with the according increase of the order parameter R in figure 6.13 (left panel). The lowering of the water signal to practically zero for the amidine (+) particles implies that the head group vector is parallel to the surface, increasing the lipid footprint and lowering the alkyl chain order, as is reflected by R in figure 6.13 (right panel). The increase in head group order for negative NPs and decrease in order for positive NPs is in good agreement with a recent molecular dynamics simulation study [148]. However, it was reported there that the changes in the head group order always led to a decrease in the order of the alkyl chains, in contrast to what we observe for DPPC with carboxyl (-) NPs. A possible explanation for this apparent discrepancy is the starting lipid surface pressure, which in the simulation was 7 mN/m, corresponding to a mixed lipid expanded and condensed phase, versus 27 mN/m in our measurements, corresponding to an entirely condensed phase. Indeed, preliminary measurements (results not shown) revealed that at lower surface pressure the interaction may display very different characteristics.

6.4 Conclusion

The main results of this chapter are summarized in a schematic graphic. DP-TAP monolayers interact with carboxyl (-) NPs, but not with amidine (+) ones because of the electrostatic repulsion (figure 6.14). Carboxyl (-) NPs induce a local ordering of the lipid by pulling on the positive charge and stretching the lipid, decreasing its footprint. In the case of DPTAP, carboxyl (-) nanoparticles are able to bind directly to the choline charge group. As a result, no water is left between the lipid and the NP in fact, simulations with negatively charged NPs have shown that the binding can be so tight that the lipid membrane may follow the NPs curvature, in a process that may be the first stage of NP endocytosis [121]. The highly multivalent NPs even induce a charge inversion at the surface, resulting in an orientation inversion of the water molecules that can be observed in the SFG spectrum as a change of amplitude sign (figure 6.4, 6.5).

Similarly, DPPG monolayers interact with amidine (+) NPs, but not with carboxyl (-) ones (figure 6.15). However, in this case the water amplitude does
Figure 6.14. Schematic representation of the interfacial molecular structures during the DPTAP-nanoparticle interaction. Carboxyl-modified (-) particles bind to the monolayer and increase the lipid alkyl chain order.

Figure 6.15. Schematic representation of the interfacial molecular structures during the DPPG-nanoparticle interaction. Carboxyl-modified (-) particles bind to the monolayer.

not change sign: apparently even after NP binding there is water left between the lipid and the particle. This difference with DPTAP can be ascribed to the presence of the somewhat bulky glycerol group beneath the phosphate group of DPPG. This glycerol prevents the NPs to bind directly to the phosphate charge group, and leaves room for strongly aligned water molecules. The glycerol group
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6.4

also adds to the lipid footprint area and prevents the pull of the charged NPs to fully straighten the lipid. This explains why the order parameter R does not increase for DPPG with amidine (+) NPs like it does for DPTAP with carboxyl (-) ones.

**Figure 6.16.** Schematic representation of the interfacial molecular structures during the DPPG-nanoparticle interaction.

The DPPC interface is fundamentally different from the other two systems because a) the aligned water here is located between the charge groups of the lipid rather than below the lipids head group, and b) the head group dipole is able to move relative to the surface normal. The binding of carboxyl (-) NPs induces a pull on the positively charged choline group, stretching the lipid along the surface normal (figure 6.16, left). This causes the dipole projection on the surface normal to increase, and with it the average alignment of the interfacial water, which is detected as an increase of the water signal in the SFG spectrum. Possibly, aligned water underneath the NPs also contributes to this signal. The stretched lipid now has a lower footprint area and more ordered alkyl chains, as can be inferred from the increased order parameter R (figure 6.13, left panel).

The binding of amidine (+) nanoparticles induces a pull on the lipids negative choline group, aligning the head group dipole more parallel to the surface (figure 6.16, right). Since there is now no potential along the surface normal, no net water alignment is present and the SFG signal disappears almost entirely. The lipid footprint area increases and the alkyl chain order decreases, which is reflected in a significant drop of the order parameter R (figure 6.13, right panel). The water around the NPs, which are presumably not tightly bound to the head group due to the presence of the positive choline charge, is symmetrical and does not generate an SFG signal.

Summarizing, we have assessed vibrational SFG spectroscopy as a viable tool to study the interaction between charged nanoparticles and lipid membranes.
From the SFG spectra we were able to infer the location of the NPs relative to the membrane, the changes in water alignment due to the nanoparticles, and the changes in lipid head group and alkyl chain order upon the binding of NPs. SEM images provided further support for the hypotheses drawn from the SFG spectra. Several phenomena are briefly discussed that could have interesting follow-up studies. For one thing, the charged NPs were shown to aggregate at the inversely charged, but not at the zwitterionic lipid interface. Aggregation is an important factor in cytotoxicity of nanomaterials, because even if single particles are harmless, an aggregate may, due to its shape and size, display very different properties [149]. Note however that it cannot be ruled out that aggregation takes place only after deposition on the gold substrate and does not occur at the air-lipid-water interface.

Further, we have shown that the water intensity at saturated NP concentration is an indicator of the vicinity of the NPs to the lipid charge groups. Carboxyl (-) NPs caused charge inversion by tightly binding to a DPTAP monolayer, while amidine (+) NPs at a DPPG monolayer showed no reversal of water alignment. This use of water alignment as a probe of charge proximity and charge inversion may be refined and applied to a range of electrostatic interfacial interactions. Finally, having proven SFG spectroscopy to be an apt tool to probe the charged NP-lipid interaction, further NP parameters such as size, shape and hydrophobicity may be mapped, adding to the understanding of the influence of these factors on a molecular level and providing a link between molecular dynamics simulations and macroscopic observations.