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Molecular ordering effects in bulk and at lipid/water nanoscopic interfaces: A story of water, light, and order

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En mémoire de mon grand-père In Erinnerung an meinen Großvater

Jochem Bloedhorn (1941-2020)

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N. D.

Abstract

Water is ubiquitous on Earth, playing a critical role for a plethora of structures, processes and chemical reactions in nature. Its unique physicochemical properties originate mainly from its hydrogen bond network. Despite its recognized importance, many aspects of the structure of water as a liquid remain elusive. It is especially difficult to understand the complex nature of fluctuations and ordering in pure water, or to get a complete picture of aqueous interfaces at the molecular level. In this thesis, our aim is to improve our understanding of the underlying interactions between water and ions, macromolecules, and interfaces, especially the long range interactions. To this aim, we use two nonlinear optical techniques, second harmonic scattering (SHS) and vibrational sum frequency scattering (SFS), on liquid water as well as on model lipid membranes mimicking the cell membranes.

We investigate with SHS how the orientational ordering of water molecules is affected by temperature changes and nuclear quantum effects. Scattering measurements of pure water reveal that the intermolecular correlations undergo a symmetry transformation with increasing temperature. On the other hand, with increasing temperature, aqueous electrolyte solutions exhibit a diminished influence of the combined electrostatic field on the waterwater correlations. This trend is predicted qualitatively by a Debye-Hückel model, but not quantitatively due to its non-inclusion of hydrogen bonding and nuclear quantum effects. These insights facilitate the elaboration of future nuclear quantum mechanical models of water.

The same interactions are further studied by a comparison of pure liquid water and pure liquid carbon tetrachloride. SHS measurements and simulations reveal that transient nanoscale voids exist in water and carbon tetrachloride. The coherent SHS emissions observed in water are generated by charge density fluctuations around the cavities. These measurements strongly hint towards non-uniformity in the structure of water, on femtosecond timescale and nanometric range.

Next, we turn towards the structure of water around model lipid membranes. We explore first the three-dimensional confinement of liquid water inside liposomes of various size. By analyzing SHS patterns of zwitterionic and anionic liposomes, we observe that the effect of confinement is visible on a range of a hundred nanometers, larger compared to the previously reported ranges on the order of a nanometer or about ten nanometers. The water orientational ordering at the interface as well as the surface potential of the liposomes are retrieved with a nonlinear light scattering modeling, showing that the confinement effect is mainly attributable to the hydrogen bond network. The effect is in particular 8 times larger for light water than for

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heavy water.

Finally, we investigate how the water structure is modified around lipid monolayers and bilayers. The hydration of lipid membranes is asymmetric with respect to charge. SHS and vibrational SFS reveal that the charge hydration asymmetry is drastically different depending on the nature of the lipid layer. It depends on a delicate balance between electrostatic and hydrogen bonding interactions. The charge hydration asymmetry is further modified by the presence of charges on the lipid headgroups on both sides of the bilayers.

Keywords: water; hydration; long-range order; molecular orientational ordering; lipid membrane; asymmetry; confinement; nonlinear optics; second harmonic generation; light scattering

Résumé

L'eau est omniprésente sur Terre, et joue un rôle critique pour une pléthore de processus, de réactions chimiques, et de structures dans la nature. Ses propriétés physicochimiques uniques proviennent principalement de son réseau de liaisons hydrogène. Malgré cette importance reconnue, plusieurs aspects de la structure de l'eau en tant que liquide demeurent élusifs. Il est tout particulièrement difficile de comprendre la nature complexe des fluctuations et de l'ordre des molécules dans l'eau pure, ou d'obtenir une vision complète des interfaces aqueuses au niveau moléculaire. Dans cette thèse, notre but est d'améliorer notre compréhension des interactions sous-jacentes entre l'eau et les ions, les macromolécules, et les interfaces, particulièrement concernant les interactions à longue distance. À cette fin, nous utilisons deux techniques d'optique non-linéaire, la diffusion de seconde harmonique (SHS) et de sommation de fréquence (SFS), appliquées à l'eau liquide ainsi que sur des modèles de membranes lipidiques qui imitent les membranes cellulaires.

Nous explorons avec la SHS la manière dont l'ordre des molécules est modifié par les changements de température et les effets nucléaires quantiques. Les mesures de diffusion de l'eau pure révèlent que les corrélations intermoléculaires subissent une transformation de symétrie lorsque la température augmente. Par ailleurs, les solutions aqueuses d'électrolytes se caractérisent par une diminution de l'influence des champs électrostatiques combinés sur les corrélations eau-eau, lorsque la température augmente. Cette tendance est prédite qualitativement par un modèle Debye-Hückel, mais pas de manière quantitative, à cause de l'exclusion des liaisons hydrogène et des effets nucléaires quantiques. Ces nouvelles perspectives facilitent l'élaboration de futurs modèles nucléaires quantiques de l'eau.

Les mêmes interactions sont étudiées plus en avant par une comparaison entre l'eau liquide pure et le tétrachlorure de carbone liquide pur. Les mesures de SHS et des simulations révèlent l'existence de vides nanométriques transients dans les deux liquides. Les émissions cohérentes de SHS observées dans l'eau sont générées par des fluctuations de densité de charge autour des cavités. Ces mesures suggèrent fortement la non-uniformité de la structure de l'eau, à l'échelle de la femtoseconde et du nanomètre.

Nous nous tournons ensuite vers la structure de l'eau autour des membranes lipidiques. Nous explorons tout d'abord le confinement en trois dimensions à l'intérieur de liposomes de tailles variées. Par l'analyse des figures de SHS de liposomes zwittérioniques et anioniques, nous observons l'effet du confinement à une échelle de cent nanomètres, plus grande que les échelles précédemment rapportées de l'ordre du nanomètre ou de la dizaine de nanomètres. L'ordre orientationnel de l'eau à l'interface ainsi que le potentiel de surface des liposomes

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sont extraits grâce à une modélisation de la diffusion optique non-linéaire. L'analyse montre que l'effet du confinement peut être attribué principalement au réseau de liaisons hydrogène. L'effet est en particulier huit fois plus sensible pour l'eau légère que pour l'eau lourde.

Enfin, nous étudions comment la structure de l'eau est modifiée autour des monocouches et bicouches lipidiques. L'hydratation des membranes lipidiques est asymétrique par rapport à leur charge. La SHS et la SFS vibratoire révèlent que cette asymétrie d'hydratation par rapport à la charge diffère drastiquement en fonction de la nature de la couche lipidique. Elle repose sur un équilibre délicat entre les interactions électrostatique et de liaison hydrogène. Cette asymétrie est en outre modifiée par la présence des charges sur les têtes lipidiques des deux côtés de la bicouche.

Mots-clefs : eau; hydratation; ordre à longue distance; ordre orientationnel des molécules; membranes lipidiques; asymétrie; confinement; optique non-linéaire; génération de seconde harmonique; diffusion de la lumière

Zusammenfassung

Wasser ist auf der Erde allgegenwärtig und spielt eine entscheidende Rolle für eine Vielzahl von Strukturen, Prozessen und chemischen Reaktionen in der Natur. Seine einzigartigen physikalischen und chemischen Eigenschaften gehen hauptsächlich auf sein Wasserstoffbindungsnetzwerk zurück. Trotz seiner anerkannten Wichtigkeit bleiben viele Aspekte der Struktur von Wasser als Flüssigkeit schwer fassbar. Es ist besonders schwierig, die komplexe Natur von Fluktuationen und Ordnung in reinem Wasser zu verstehen, oder ein vollständiges Bild von wässrigen Grenzflächen auf molekularer Ebene zu erhalten.

Das Ziel dieser Arbeit ist, ein besseres Verständnis von Wechselwirkungen zwischen Wasser und Ionen, Makromolekülen und Grenzflächen im Allgemeinen zu erlangen, und insbesondere auch langreichweitige Wechselwirkungen besser zu verstehen. Zu diesem Zweck verwenden wir zwei Techniken, die auf nichtlinearen optischen Effekten beruhen: die Streuung der zweiten Harmonischen (SHS) und Summenfrequenzspektroskopie (SFS), sowohl von flüssigem Wasser als auch von Lipidmembranen, die als Modell dienen um Zellmembranen zu imitieren.

Wir untersuchen mit SHS, wie die Orientierung von Wassermolekülen durch Temperaturänderungen und nukleare Quanteneffekte beeinflusst wird. Die Streuung der zweiten Harmonischen von reinem Wasser zeigt, dass die intermolekularen Korrelationen mit zunehmender Temperatur eine Symmetrieumwandlung erfahren. Andererseits weisen wässrige Elektrolytlösungen auf eine Abnahme des Einflusses des kombinierten elektrostatischen Feldes auf die Wasser-Wasser Korrelationen mit zunehmender Temperatur hin. Dieser Trend wird qualitativ von einem Debye-Hückel-Modell vorhergesagt, aber nicht quantitativ, da dieses weder Wasserstoffbrücken- noch Kernquanten-Effekte einschließt. Diese Erkenntnisse erleichtern die Erarbeitung zukünftiger quantenmechanischer Kernmodelle von Wasser.

Die gleichen Wechselwirkungen werden durch einen Vergleich von reinem, flüssigen Wasser und reinem, flüssigen Tetrachlorkohlenstoff weiter untersucht. SHS Messungen und Simulationen zeigen, dass transiente nanoskalige Hohlräume in Wasser und Tetrachlorkohlenstoff existieren. Die beobachteten kohärenten SHS- Emissionen in Wasser werden durch Ladungsdichteschwankungen um diese Hohlräume erzeugt. Diese Messungen weisen stark auf Ungleichmäßigkeiten in der Wasserstruktur auf einer Femtosekunden Zeitskala und im nanometrischen Bereich hin.

Als nächstes wenden wir uns der Struktur des Wassers um die Lipidmembranen als Modell zu. Wir erkunden zunächst den dreidimensionalen Einschluss von flüssigem Wasser in Liposomen unterschiedlicher Größe. Durch die Analyse der SHS-Winkelkarten von zwitterio-

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nischen und anionischen Liposomen stellen wir fest, dass die Wirkung von Einschlüssen in einem langreichweitigeren Bereich sichtbar ist als zuvor in der Literatur beschrieben wurde. Sie liegt nach unseren Messungen in der Größenordnung von einigen hundert Nanometern im Vergleich zu einigen zehn Nanometern in der Literatur. Die Orientierung der Wassermoleküle an der Grenzfläche, sowie das Oberflächenpotential der Liposomen, werden mit Hilfe eines nichtlinearen Lichtstreuungsmodels ermittelt, und zeigen, dass der Effekt der Einschlüsse hauptsächlich auf das Wasserstoffbindungsnetzwerk zurückzuführen ist. Der Effekt ist insbesondere bei leichtem Wasser acht mal grösser als bei schwerem Wasser.

Schließlich untersuchen wir, wie die Wasserstruktur um Lipid-Monoschichten und -Doppelschichten verändert wird. Die Hydration von Lipidmembranen ist asymmetrisch bezogen auf die Ladung. SHS und SFS zeigen, dass sich die Asymmetrie der Ladungshydration je nach Art der Lipidschicht stark unterscheidet. Sie unterliegt einem empfindlichen Gleichgewicht zwischen elektrostatischen Wechselwirkungen und Wasserstoffbrückenbindungs-Wechselwirkungen. Die Asymmetrie der Ladungshydration wird darüber hinaus durch die Anwesenheit von Ladungen auf den Lipidkopfgruppen auf beiden Seiten der Doppelschichten modifiziert.

Stichwörter: Wasser; Hydration; weitreichende Ordnung; molekulare Orientierungsordnung; Lipidmembranen; Asymmetrie; Einschluss; nichtlineare Optik; Frequenzverdopplung; Lichtstreuung

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In the beginning God created the heaven and the earth. And the earth was without form, and void; and darkness was upon the face of the deep. And the Spirit of God moved upon the face of the waters. And God said, Let there be light: and there was light. And God saw the light, that it was good: and God divided the light from the darkness. And God called the light Day, and the darkness he called Night. And the evening and the morning were the first day.

- Genesis 1:1-5, King James Version

The first depiction of our world, in the beginning of the Genesis book in the Bible, is one of a "tohu va-bohu" in the hebraic original. This expression has settled as "tohu-bohu" in French, and "tohubohu" in English, to describe a state of disorder and great confusion. Interestingly, the first matter to exist in this original state was water. The next created thing was light, separated from darkness. Later in the Genesis, God separated the waters, organized the disorder into order, transformed the confusion into meaning, to create the world we know. This picture of the original chaos within primordial waters is repetitive in most cosmogonies, for instance in the Sumerian, Egyptian, Aztec, Greek, and Japanese mythologies [1–4]. Therefore, the tryptic water/light/order holds a deep significance in the human psyche.

Water is mostly present on Earth under the liquid form. Liquids are a state of matter that are neither ordered nor disordered [5]. They drive many physical and chemical processes in nature. Among them, water is very peculiar, to the point that it is often referred as the matrix of life on our planet. Light on the other hand is also prominent in our way to perceive the universe. We see the world and we define it through light. For instance, the speed of light is a fundamental constant of the universe, the upper limit of speed at which any matter can travel through space. We use this speed in particular to define the meter unit. Light has been at the core of many conceptual revolutions in science (e.g. the photoelectric effect that was seminal for the development of the field of quantum mechanics [6]). It was also the object of many significant discoveries like the invention of the laser [7]. Finally, the order is fundamental as our universe is organized according to physics laws, and matter assembles or separate through

interactions. At the biological level, the complexity brought by a local decrease of entropy (sometimes simplified as an increase of order) is necessary for many processes [8]. This is especially true for the cellular membrane. Its crucial role of bringing order and segregation of molecules into two distinct spatial regions makes it a necessity for life.

In this thesis, many questions revolving around this tryptic of water, light, and order will be discussed. Water is a liquid with extraordinary physicochemical properties, that make it stand out of the crowd of liquids. These properties are related to the unique ability of water to form hydrogen bonds. In this work, we investigate water in terms of intermolecular interactions. As the structure of pure liquids is governed by a delicate balance of various intermolecular interactions, we need suitable techniques to probe them. In that context, nonlinear optical spectroscopy based on light scattering allows us to get powerful insight on the liquid water structure and its inhomogeneities. Furthermore, the addition of ions in water impacts the balance of interactions. We probe the modifications in the orientational ordering of water molecules caused by temperature changes, on long-range distances. On the other hand, we investigate how the structure of water and the balance of interactions is impacted by the presence of nanoscopic interfaces with lipid membranes, in particular in terms of confinement of water and in terms of charge hydration asymmetries.

1.1 Water and its peculiar properties

Among liquids, water holds a very peculiar place on Earth. Its designation as the "liquid of life" might look like hackneyed but is nonetheless true. Water is the most important thing that scientists are searching for when trying to find life in space. This is because water is an exceptional solvent with unique anomalous properties [9].

The first anomalous property concerns the boiling and melting points. Its main physicochemical properties deviate from the behavior of the series of other liquids with a similar structure, such as the series of primary alcohols or the series of hydrides molecules. For instance, water melts at 0°C under normal conditions, and boils at 100 °C, while the corresponding series of binary hydrides molecules with a central atom surrounded by H atoms (H_2S, NH_3, CH_4, etc) are all gaseous even at negative Celsius temperatures. A high boiling point usually denotes a high cohesive energy of the material. For instance, salts, or metals have a strong cohesion and boil at thousands of degrees, but they are typically made of heavier and bigger atoms, thus water really stands out. The density of water also deviates from the typical behavior of most liquids. It reaches a maximum at 4°C, while normal liquids density monotonically increases when decreasing temperatures [9]. Because of this, ice floats on top of water, enabling aquatic life in cold environments. Another consequence of this density maximum is that the surface of lakes can start freezing only when all their water has cooled down to 4°C. Furthermore, as water has a very high heat capacity compared to other liquids, it acts as a very efficient heat tank. For these two reasons, the Léman (Geneva Lake in English) cannot freeze due to its huge depth. In addition, water is known to have an anomalously high

viscosity and surface tension. Its relative dielectric tension is also unusually high, around a value of 78 at room temperature, meaning that it screens very efficiently electromagnetic fields.

The water molecule is constituted of two hydrogen atoms linked to a central oxygen atom, as shown in Fig. 1.1. The central O atom possesses two lone electrons. As it is more electronegative than the H atoms, it retains more negative charges whereas the H atoms are more positive. Coupled with the H-O-H angle of 104.5°, this induces a dipolar moment of 1.85D [10]. The presence of the electronegative central O atoms makes the water molecule very prone to the so called hydrogen bonding (H-bonding) interaction (Fig. 1.1). The peculiar properties of water are linked to this interaction. H-bonding interaction is indeed stronger than other non-covalent interactions, around 5 - 10 kT, thus it gives a high cohesive energy to water. The hydrogen bond (H-bond) is highly directional compared to Van der Waals interactions [11]. A water molecule can donate two hydrogen-bonds, and accept two. On average, there are 3.6 H-bonds per water molecule in liquid water [12], and 4 in ice. This number of bonds per molecule gives a preferential directionality along the tetrahedral orientation [9, 13] and explains the high coordination number of 36 in liquid H_2O . Still, even though the H-bonds are highly directional, there are still variations in the angles they form. This mobility is essential to keep the molecules in the water network labile. The dynamics of water molecules is in the order of femtoseconds for the intramolecular O-H bending and stretching motions. The intermolecular H-bond stretching is in the order of hundreds of femtoseconds. On the other hand, the reorientation of the H-bond network occurs typically within 8 ps [14, 15]. Moreover, H-bonding is not an additive but a *cooperative* phenomenon [16, 17]. For example, wires of water molecules connected by hydrogen bonds can extend over tenths of nanometers in the liquid. This is one of the reasons for the very high proton mobility in liquid water. The unique ability of the water molecule to realize H-bonding is crucial in its role as a solvent for biological molecules such as proteins. The 3D H-bond network in water has been found very important to hydrate proteins, and for instance to help structuring them in different conformation [18, 19]. Rather than being an inactive background for biological processes, water plays an essential active role in biology [13].

Interestingly, water is sensitive to nuclear quantum effects. Various isotopes of the H and the O atoms exist, allowing different combinations of water molecules. In this work we focus on the deuterium (D) ²H and hydrogen (H) ¹H. We use D₂O, called "heavy water", along with H₂O called "light water". Indeed, the D atom is heavier, increasing the overall mass of the D₂O. Some of the properties of these two molecules are still similar (same dipolar moment, dielectric constant) but most of them are dramatically changing (dynamic viscosity, vibrational changes, etc)[14, 20–22]. The melting and boiling points of D₂O for instance increase respectively to 3.8 and 101.4°C. These are consequences of the change in mass, that makes the nuclear quantum effects stronger in H₂O than in D₂O. This induces a modification of the H-bond by the D atoms. H-bonds in D₂O are around 4% longer than in H₂O, while the intermolecular D to D distance is shorter than the H to H distance [12]. This means that the H-bond network of D₂O is stronger than in H₂O. These differences between H₂O and D₂O are a good starting point to



Figure 1.1 – Schematic of two hydrogen-bonded water molecules (as shown by the dashed line), with oxygen atoms in red and hydrogen atoms in white. The δ symbols indicate the slight asymmetry in charge distributions within the molecule. The dimensions are taken from Ref. [12].

explore the impact of the structure of the water molecule and nuclear quantum effects on its anomalous properties, and to understand why it stands out from the crowd of liquids.

1.2 Intermolecular interactions shape the structure of pure liquids

Liquid water is essential to life and to biological processes in nature. Between solid and gas state, the liquid state is neither completely ordered nor disordered. Liquid holds in a defined volume but has no fixed shape. The properties of a liquid and its structure are based on the nature of intermolecular interactions holding together seemingly disordered molecules [5]. For instance, H-bond networks produce complex structures (such as tetrahedral configuration of water molecules, or various water hexamers [23]) that completely deviate from a structure dominated purely by repulsive forces. Different models have thus been developed to describe the balance of these interactions in liquids, together with various experimental techniques to probe them. These are described here.

These interactions between molecules in pure liquids can be either repulsive or attractive. Attractive interactions are of various nature, strength and dynamical range. Among them, interactions of short $1/r^6$ range where r is the intermolecular distance (namely Debye, Keesom, and London interactions) are grouped as "Van der Waals interactions" [5, 11]. Their dynamics timescale is typically on the order of 100 fs, and they are typically weak (of the order of 1 kT) [5]. In comparison, the hydrogen-bond interaction is stronger (of the order of 10 kT) [5] and has a relaxation time of the order of 1 to 10 ps [15], as is summarized in Table 1.1. Lastly, repulsive interactions are typically short range, and occur on a faster timescale than Van der Waals interactions. Based on these interactions, the Van der Waals picture of liquids describes the strong repulsive interactions as varying faster than the attractive ones, hence dominating. The

dominant repulsion determines primarily the liquid structure, while the attractive interactions stabilize the density of the liquid [24]. In this understanding, the Weeks–Chandler–Andersen (WCA) theory stipulates essentially that the attractive forces act over a much larger distance but are also much slower than the repulsive ones [25]. For computational purposes, it is possible to neglect the attractive part of the potential, and to conserve only the repulsive one [25–27]. Overall, the Van der Waals picture has been found to describe well liquids without hydrogen bonds or ionic forces implied, e.g. carbon tetrachloride, benzene, and formamide [24]. However, this representation does not work well at low density, where the slowly varying part of the interaction potential dominates. In addition, the Van der Waals picture fails when the attractive interactions are stronger than the repulsive forces and dominate. It is also possible for the attractive forces not to compete with the repulsion but still to alter the structure of the liquid. In the specific case of H-bond interactions, they are stronger than Van der Waals interactions and add fast dynamics to the interaction potential, of picosecond order. Therefore, it dominates the repulsive force and alters the liquid structure [24].

Interaction	Range (<i>r</i> is the radius)	Timescale	Energy
Van der Waals	$\sim 1/r^{6}$	100 fs	1 kT
H-bond	$\sim 1/r^2$	1-10 ps	10 kT
Covalent	short range		100 kT

Table 1.1 – Common types of attractive intermolecular interactions present in a pure liquid, and orders of magnitude of their typical range, timescale and energy. The range refers here to the dependence of the interaction energy to the distance.

Several models have been developed to encompass the physical interactions in liquids and to predict their macroscopic properties. A simple way to describe the liquid structure is to model it as driven by a pair potential comprising attractive and repulsive terms, the latter one being referred as the molecular shape [24, 28]. Models of the liquid structure are often classified based on the interactions included in the pair potential [29]. Some of them, such as the hard or the soft sphere model; blip function theory; or Reference Interaction Site Model (RISM) equation [29], report only on short-range repulsive forces. Note for instance that the hard sphere model can be coupled with the adjunction of a dipole inside the sphere, to include dipolar interactions [28]. Furthermore, Van der Waals liquids such as Lennard-Jones liquids rely on a pair potential including Van der Waals interactions. Many simulation models were developed for specific liquids, especially water [30]. In particular, density functional theory (DFT) is the basis for many aqueous models [31]. Dispersion function choices seem to play an important role in their accuracy. However, the description of liquids based on pair potential has limitations. In particular, intermolecular interactions are often not decomposable into sums of pair interactions [28, 32], and better accuracy can be achieved with models including many-body interactions [31]. More recent developments led to the refinement of interaction description, from pairwise to many-body potential energy functions [23]. In particular, MB-pol model has emerged as a more accurate representation of water [33, 34].

In complement to simulations, many experiments have been run to investigate liquid structure. Among scattering techniques, neutron and X-ray scattering are complementary. Neutron scattering probes the nuclei of atoms, whereas X-ray scattering probes the molecular electronic clouds [35]. Therefore, neutron scattering is sensitive to isotopic differences [25]. Considering the interactions in liquids, the two techniques probe pair functions [24]. However, it is not really feasible to derive higher order correlations (more than two body interactions) with these techniques alone [35]. In addition to scattering techniques, femtosecond pump probe techniques are widely used for vibrational spectroscopy of liquids [36]. For instance, optical Kerr effect (OKE) spectroscopy [32, 37-39] is based on the time-dependence of the birefringence alteration, which reveals vibrational dynamics of the liquid. The OKE response is proportional to collective orientational correlation functions, while other techniques like Raman spectroscopy and NMR probe are sensitive to single molecule orientational correlation function [37]. In addition, 2D IR spectroscopy [36] provides insight into coupling between different vibrational modes. Terahertz (THz) spectroscopy has also been used to study liquid structure [40] and coupling between intra- and inter-molecular motions [41]. Furthermore, ¹H NMR spectroscopy is a popular method to study liquids, especially liquids with hydrogen bonding interaction [42]. In the past years, another technique known as second harmonic scattering (SHS) has been developed to probe liquid structure [43–45]. This technique has been further developed in our laboratory and will be the subject of this thesis. As detailed in the next sections, SHS is very powerful to study anisotropy or centrosymmetry breaking due to intermolecular interactions, and it has recently been improved for measurements that are fully elastic, with femtosecond pulses to probe fluctuations below the orientational relaxation time of liquids, and for much shorter experiment duration. In this thesis, we use it to characterize the temperature dependence of intermolecular correlations in light and heavy water, and we successfully probe transient anisotropies in the structure of water that generate coherent scattering emissions.

1.3 The impact of electrolytes on the water structure in aqueous solutions

In nature, liquids are not perfectly pure. They contain impurities and ions in solution. Ions are critical for many physiological, medical, chemical processes [13]. For instance, ions are involved in the functioning of ion channels, in permeation and osmosis processes, in the occurence of action potential in neurons, in interfacial charging, in aggregation and self-assembly, in the denaturation of proteins, and in aerosol formation [13, 46–48]. A lot of these effects are actually ion specific effects: for example surface tension, viscosity, or hydration free energy [49–53]. The most famous historical example concerns the ion-specific impact of salts on proteins, known as the Hofmeister effect, further extended to other kinds of interfaces. This effect is still discussed nowadays, illustrating the challenge of understanding the ion interactions with water and other molecules [13, 47, 48, 54, 55].

In comparison to pure water where only water-water interactions are present, in ionic

solutions ion-water interactions occur and perturb the liquid structure [56]. First, the ions modify the H-bond network of water: water has to adapt around it to fill the spaces around the ionic solute. At the same time, the ionic charge generates an electric field that aligns the water molecules via charge-dipole interaction. This field extends into the liquid and vanishes progressively with the distance to the ion, forming successive hydration shells [11, 56].

Most studies have focused on electrolyte solutions at high concentration, with short range water-ion and water-water interactions. The structure of water has been shown to be strongly modified by the presence of the ion in the first few hydration shells below 1 nm [56–58]. Recently, our laboratory has shown that these interactions also occur at low concentration and extend on a long-range, up to 20 nm from the ion [59]. This effect is non ion-specific but exhibit differences between water isotopes. This shows that the collective hydrogen bonding, thus water-water correlations, plays an important role in this long-range effect.

Despite all of these progresses in our understanding of the ion hydration, there are still many open questions. For instance, increasing temperature impacts heavily many of the anomalous properties of water, and it has been shown to perturb the H-bond network of water [9, 60, 61]. In this context, it is yet not clear what is the effect of temperature on the long-range interactions, and on the interplay between the ion-water and water-water intermolecular interactions.

1.4 Water at nanoscopic interfaces

Water behavior is also impacted by the insertion of objects made of several molecules, larger than ions. This creates what we call a nanoscopic interface, in the sense that 1) it creates a boundary between water and another insoluble material, and 2) this interface has nanoscopic dimensions. Nanoscopic interfaces take various forms such as emulsions, nanoparticles in suspension, large proteins or macromolecules, clathrates, pores, or membranes of cells and organelles. They play a significant role in biology, physics, and chemistry, due to their two-dimensional nature [13, 62]. Nanoscopic interfaces with water are governed by a subtle balance of interactions. For instance, the hydration is critical to assist biomolecules in folding and adopting specific conformations [13, 18, 19]. The interaction between water and the interface modifies also the structure of water that becomes very different from bulk water [13].

The modification of the structure of water is even more dramatic when it is fully confined by the interface, as can be observed in many different systems in nature. The range at which confinement effects occur remain a particular matter of debate nowadays, and is the focus of Chapter 5. Another topic of interest is the lipid / water interface, a specific form of aqueous interface that is crucial for life. It is stabilized by a complex and intricate balance of intermolecular interactions between lipids and water molecules. The structure of water there is consequently dependent on the nature of the lipids (in terms of charge and presence of hydrogen bonding sites), as investigated in Chapter 5 and 6.

1.4.1 Confinement of water

The structural changes of water caused by nanoscopic interfaces are dramatically increased when the dimensions and topology of said interface cause a confinement, i.e. when most of the degrees of freedom of water molecules are constrained. The confinement, as a restriction of the available space, is ubiquitous and can be found in many systems like carbon nanotubes, nanopores, graphene sheets, biomolecules, or clathrates [63–67]. Such confinement has many effects on the structure and dynamics of water, for example freezing transition, ice-like behavior, reduction of the number of H-bonds per water molecule, and slow-down of the dynamics. The origin of these effects remains elusive. Do these effects come from electrostatic, van der Waals, or H-bonding interaction? Or are there also important collective and entropic effects takes place, most studies showing an extent of a few nanometers, but others pointing at larger scale. This is something worth investigating, even more since long-range ordering effects have been shown to occur in aqueous solutions [59].

One of the most impactful example of confinement is the biological cell. Water in the cell is confined within one or more lipid bilayers, without any direct contact with the exterior of the cell. Therefore, the answers to these questions might have a deep impact on our understanding of the molecular biology of the cell. What does this confinement mean for the structure of water, and for the lipid membrane itself?

1.4.2 Lipid membranes

In biology, the nanoscopic interfaces with water are usually stabilized by amphiphile molecules called lipids, forming lipid membranes. Lipids are made of a hydrophilic headgroup and of one or two fatty acid chains that are hydrophobic [8]. When immersed in water, the hydrophobic part positions itself to be away and protected from the water molecules. On the contrary, the hydrophilic part remains in contact with water. This asymmetry of the lipid molecule makes it highly insoluble in water, hence lipids tend to assemble together or at an interface between water and a hydrophobic material such as water because of the hydrophobic effect [68–71]. Lipids can form several kind of structures [8, 70]. They can self-assemble into micelles, where the polar headgroups point towards the water, and the lipid tails point toward each other. However, as the tails are quite bulky, it is more favorable for the lipids to self-assemble into a lipid bilayer. Finally, lipids can also form a monolayer at the surface of a hydrophobic body immersed in water. Lipid bilayers and monolayers are the basis for cellular membranes.

Cellular membranes can be very sophisticated and play a critical role for the cells [8, 72]. The primary role of the outer cellular membrane is to form a permeability barrier and to stabilize the inner environment of the cell. Moreover, a cell can contain multiple inner membranes, either bilayers or lipid monolayers on organelles (lipid droplets) [73, 74]. These additional membranes constitute specialized compartments and subunits, increasing the biological efficiency of the cell and expanding its capabilities.

1.4. Water at nanoscopic interfaces



Figure 1.2 – Schematic cellular membrane. It is composed mostly of lipids, and proteins embedded within the bilayer. Picture from Ref. [75].

The two-dimensional conformation of the membrane, and its ability to embed proteins makes it a crucial place for phenomena such as molecular transport and chemical reactions [74]. Proteins can attach to the surface of the lipid membrane, or insert themselves into it (see Fig. 1.2), providing new functions to the membrane. For instance, ion-channels can ensure the regulated and selective transport of molecules [8, 72]. The control over the molecular transport is fundamental to the energy storage of the cell. One way for the cell to store energy is the synthesis and storage of specific lipids by lipid droplets [8, 76–78]. These droplets are made of oil and covered by a lipid monolayer [78]. Some proteins are specifically involved in the emergence of lipid droplets from the endoplasmic reticulum membrane [79].

Overall, the interplay of interactions between lipids and other lipids, between lipids and proteins, and the hydration, drives the formation and stability of the membranes. In that sense, lipid membranes are noncovalent assemblies of lipids, fluid structures that "can be regarded as two-dimensional solutions of oriented proteins and lipids" [8]. The nature of lipids in the membrane, in terms of acyl chain length, headgroup charge, and propensity to realize H-bonding, impacts the packing and the mobility of lipids, the stiffness, the thickness, and the local curvature of the membrane. It influences also the transmembrane permeability and transport [80–83], and the functioning of proteins.

The variety of lipids and their distribution

There are plethoric kind of lipids in nature, due to the possibilities of variations of the headgroups and the tails. The three family groups of lipids are the phospholipids, the glycolipids, and the cholesterol, the first group constituting the major class of lipids in eukaryotic cells [8, 72, 77]. Within a single living being, the lipid distribution is very different from one organ to another. Also, one single cell can contain thousands of different kind of lipids [73, 77]. Within the cell, the repartition of these lipids in the different membranes depends on their



Figure 1.3 – Chemical structure of the lipids used for the model lipid membranes. DOTAP is positively charged, DOPC is zwitterionic, DOPA and DOPS are negative. The charged lipids are used as a salt powder, hence the presence of the corresponding counterions, Na^+ or Cl^- .

functions. Different processes are linked to lipid synthesis and trafficking within cells [74], and regulate the intracellular distribution of lipids. This distribution varies during the different stages of the cell life. In particular, the normal state of the outer cell membrane is usually one of transmembrane asymmetry with mostly negative lipids on the inner leaflet and neutral ones on the outer leaflet [70, 84]. The disappearance of this asymmetry is usually linked to the cell death - apoptosis [85], or to signaling [76, 86].

Therefore, it seems worth investigating the very peculiar segregation of charged lipids at specific locations in the membrane in terms of hydration. In this thesis, we study membranes made of lipids with different charges: positive 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); zwitterionic 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC); negative 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS). Their chemical structures are shown in Fig. 1.3. All of them are phospholipids containing a phosphate moeity, except for DOTAP that keeps however a very close chemical structure. They possess two acyl chains with one gauche defect on each of them. The choice of this set of lipids allows us to investigate several questions: at the lipid / water interface, are the intermolecular interactions fully symmetric with respect to the lipid charge? How does the nature of the lipids and their ability to realized H-bonding impact the structure of the interfacial water? This requires a precise understanding of the balance of involved interactions.

The interplay of interactions at the lipid-water interface

The diversity of lipid membranes and their respective roles in the cell life are all governed by a subtle balance of interactions. First, the intramembrane interactions are important for the stability of the lipid membranes. There are attractive Van der Waals forces between the lipid tails, but also electrostatic and H-bonding interactions between the lipid headgroups and the molecules in the aqueous interfacial region (mostly water, and ions). The strength of the lipid-lipid interactions for instance is of the order of 1 kT, and hydrogen-bonding of the order of 5-10 kT [11].

But the interactions that are most important for the lipid membrane come from the hydration environment. The structure of water around a lipid membrane is known to be very

different from that in the bulk [13]. The ions in water impact the membrane via electrostatic interactions [87–89], while the water interacts via H-bonding and charge-dipole interaction [90]. But the water-lipid and ion-lipid interactions are directly impacted by the nature of the lipid headgroup, either via charge-dipole or dipole-dipole interactions. The headgroup has more or less propensity to achieve H-bonding with the water molecules in the presence of potential H-bonding sites. Indeed, the ionic groups there interact with water either via charge-dipole interaction or via H-bonding depending on the sign of the charge [91, 92], and its accessibility to the water molecules. For these reasons, hydration is crucial for the membrane assembly and stability, and for the functional processes taking place around it [69, 93–95]. Therefore, it should not be neglected in lipid membrane studies.

There are still many unknown mechanisms at the lipid membrane [78], and a better molecular picture of the nanoscopic lipid/water interface is needed. Numerous molecular dynamics (MD) simulations were performed on the lipid/water interface [19, 93, 96–101]. It is however challenging to study experimentally at the molecular level [102]. Hydration at planar lipid/water interfaces was investigated using several techniques, for instance atomic force microscopy [103–105], surface force apparatus [106], NMR [89], X-ray scattering [89, 107], vibrational sum frequency generation [92, 108], infrared spectroscopy [109], THz spectroscopy [109, 110], and quasi-elastic neutron scattering [111, 112]. However, these experiments were realized on planar lipid membranes, that are not realistic enough compared to the curved cell membranes. Recent progresses were made to tackle this issue with better model membranes and nonlinear scattering techniques, presented in detail in sections 1.4.2 and 1.5.

Model lipid membranes

In order to investigate the lipid membranes and their interactions at the molecular level, there are two traditional approaches [113]. The top-down approach consists of using real biological membranes. The main way to study them spectroscopically is to use dye molecules as probes. However, these additives perturb the distribution of molecules in the membrane, and their mobility and proper functioning. The role of water is also neglected. On the other hand, the bottom-up approach is to study simplistic planar artificial membrane [114] such as supported lipid bilayers, or lipid monolayer on Langmuir troughs (see Fig. 1.4C and D). This approach allows the researchers to have a very good control on the composition of the membrane and the parameters of control, thus to focus on some specific and disentangled properties of the membrane, such as the role of water. Nevertheless, these membranes are quite different from those in the real in-vivo cells (for instance they have no curvature, or are in contact with a support). This does not allow to grasp the complete picture of the cell at the molecular level.

The solution to the respective problems of these two approaches is to use realistic artificial membranes mimicking the neutral membranes. A first example is the planar freestanding lipid bilayers (see Fig. 1.4E) that are fully immersed in water on both sides of the membrane, contrary to supported bilayers or Langmuir troughs. However, they are quite fragile to maintain, and not realistic enough because natural bilayers tend to close on themselves to avoid



Figure 1.4 – Diverse liquid and model membrane systems that can be studied with nonlinear spectroscopic techniques. The systems studied throughout this thesis are highlighted in red.

any contact between their hydrophobic core of lipid tails at their edges [8]. In that sense, lipid droplets (also know as oil nanodroplets) as well as liposomes are much more interesting. They are very similar to cells and organelles such as adiposomes, which allows to study the effects of curvature and confinement of water. Moreover, their full immersion in water prevents any oxidation of the lipids that would denature their molecular structure. In addition, a solution of liposomes or oil nanodroplets possess a surface to volume ratio that is greater by two to three orders of magnitude than planar interfaces [45, 115]. This makes them especially insensitive to contamination by impurities. In this thesis, we use liposomes and oil nanodroplets in water (see Fig. 1.4F and G). We study them suspended in water with nonlinear optical spectroscopic techniques recently developed. This allows to shed light on the collective behavior of water molecules. Water here acts as a non-invasive and label-free probe around the lipid membranes, to investigate how water is impacted by each one of these systems, in terms of size, topology, and lipid composition.

1.5 Nonlinear optical spectroscopy

In this work, we use use techniques based on nonlinear optics. Typically, this involves shining intense laser light on a material. The light interacts with matter and modifies its properties. As the light intensity is very strong, this interaction will involve in particular nonlinear processes. The fundamentals of these processes are briefly introduced in this section, and are developed in detail in Chapter 2.

The interaction between light (the electromagnetic field) and matter is fundamentally described by the polarization term in the Maxwell's equations. When light is shone on a material, the electronic cloud of the molecules can be perturbed, giving rise to a molecular dipole p_v in the molecule v. This dipole can be expressed as a power series of one linear and several higher-order terms that are functions of the electric fields *E* illuminating the material [43]:

$$\boldsymbol{p}_{\nu} = \boldsymbol{p}_{\nu}^{(1)} + \boldsymbol{p}_{\nu}^{(2)} + \boldsymbol{p}_{\nu}^{(3)} + \dots = \boldsymbol{\beta}^{(1)} \cdot \boldsymbol{E} + \frac{1}{2} \boldsymbol{\beta}^{(2)} : \boldsymbol{E}\boldsymbol{E} + \frac{1}{6} \boldsymbol{\beta}^{(3)} : \boldsymbol{E}\boldsymbol{E}\boldsymbol{E} + \dots$$
(1.1)

where $\boldsymbol{p}_{v}^{(n)}$ is the molecular polarization component of *n*-th order, $\boldsymbol{\beta}^{(1)}$ is the first-order polarizability, $\boldsymbol{\beta}^{(2)}$ the second-order polarizability (or first-order hyperpolarizability), $\boldsymbol{\beta}^{(3)}$ the third-order polarizability (or second-order hyperpolarizability), etc. These are mathematical tensors of increasing ranks, and the corresponding tensor products with the electric fields are denoted by the symbols : and \vdots in the equation.

At the macroscopic level, the overall polarization *P* per unit volume of the material under illumination is made of the sum of all individual molecular dipoles in said volume. Similarly to the molecular dipole, the macroscopic polarization can be expressed as a power series:

$$\boldsymbol{P} = \boldsymbol{P}^{(1)} + \boldsymbol{P}^{(2)} + \boldsymbol{P}^{(3)} + \dots = \epsilon_0 \left(\boldsymbol{\chi}^{(1)} \cdot \boldsymbol{E} + \boldsymbol{\chi}^{(2)} : \boldsymbol{E}\boldsymbol{E} + \boldsymbol{\chi}^{(3)} : \boldsymbol{E}\boldsymbol{E}\boldsymbol{E} + \dots \right)$$
(1.2)

where $\mathbf{P}^{(n)}$ is the polarization component of *n*-th order, ϵ_0 is the vacuum permittivity, and $\boldsymbol{\chi}^{(n)}$ is the *n*-th order susceptibility tensor of the illuminated medium. The latter is a (n+1)-th order tensor describing the intrinsic local response of the material. The presence of this polarization means that new light fields are generated in the material, with frequencies dictated by the polarization processes. In particular, the second-order nonlinearities in the material (non-zero $\boldsymbol{\chi}^{(2)}$) generate sum-frequency (SF) and second harmonic (SH) light [43]. In this work, we focus on these two processes. In the case of sum-frequency generation (SFG), the two incoming electromagnetic fields have frequencies ω_1 and ω_2 and interact within the material to produce a third field of frequency $\omega_0 = \omega_1 + \omega_2$. SHG is a specific case of SFG, where $\omega_1 = \omega_2$.

It is important to note that the higher the order, the weaker the polarization component is. The nonlinear polarization is thus inherently weak compared to the linear polarization. This is why historically the field of nonlinear optics emerged with the discovery of the laser in 1960 [43]. However, the efficiency of the generation process is greatly increased when the frequencies involved are in resonance with an energy transition in the material (vibrational or electronic). The energy level transitions for SFG and SHG are shown in Figure 1.5, in the different combination cases of resonance and non-resonance. In this work, we focus on the cases of fully non-resonant second harmonic scattering (1.5 A) and half-resonant sum frequency second-harmonic scattering (1.5 C). In the first case, two incident photons with same frequency ω will excite the molecule from a ground state to a virtual state that does not correspond to any real electronic excitation level. Then the molecule relaxes by emitting a third photon at double the frequency and returns to its original ground state. In the case



Figure 1.5 – Energy levels of second-order nonlinear processes. (A) Non-resonant second harmonic generation. (B) Half-resonant second harmonic generation. (C) Half-resonant sum frequency generation. (D) Non-resonant sum frequency generation. (E) Fully resonant sum frequency generation.

of half-resonant SFG, the photon at frequency ω_2 resonates with a vibrational mode of the molecule and excites it. Then the photon of frequency ω_1 excites the molecule further to a virtual state of higher energy, before it relaxes by emitting a third photon at the sum frequency ω_0 . As it is half-resonant, this type of SFG is particularly suited to probe the vibrational modes of specified molecules by tuning the frequency ω_1 .

Due to intrinsic symmetry properties, second-order (and other even-order) nonlinear optical interactions occur only in non-centrosymmetric medium, i.e. in a material without inversion symmetry [43, 116, 117]. This translates as the vanishing of the components of the tensor and the absence of light emission. For this reason, the second-order nonlinear processes represent powerful tools to probe selectively the structural anisotropy in a bulk medium (e.g. crystal or liquid), or to study the molecular organisation at the interface between two centrosymmetric media.

SFG and SHG can be experimentally performed on different geometries. Planar interfaces can be probed via reflection geometry (Fig. 1.6, left) where there is a phase-matching of the beams in the normal plane of incidence. The second-order nonlinear spectroscopy can also be combined into a scattering geometry (Fig. 1.6, right), where the light is emitted at various angles. This is particularly suitable to probe bulk media like liquids, or particles and droplets in suspension, where there is no planar interface. The SFG and SHG processes are identical for both geometries at the molecular level. The theoretical treatment of scattering differs from reflection only in the transformation of the local susceptibility $\chi^{(2)}$ into an effective susceptibility $\Gamma^{(2)}$ that takes into account the scattering geometry and depends among other parameters on the $\chi^{(2)}$ values at the interface of the scattering objects. This is presented with more details in Chapter 2.



Figure 1.6 - Geometry of second-order nonlinear spectroscopic techniques.

1.5.1 Vibrational Sum Frequency Scattering

The development of the theory for SFG was initiated in 1962 [118] shortly after the invention of the first working laser in 1960 [7]. The first experiments started in 1987 [119, 120], while further theoretical developments were performed [121]. In the last decades, SFG has been applied on a large variety of planar interfaces [122–125].

Nevertheless, studies on planar interfaces are very sensitive to impurities, thus the samples are challenging to prepare. Also, these interfaces do not represent well the real interfaces in nature, where flatness is scarce. These problems were solved in 2003 by combining vibrational SFG with light scattering into vibrational SFS [126] measurements, as demonstrated by Roke et al. SFS allows in-situ probing of more realistic model membranes. Moreover, the higher surface/volume ratio that it offers enhances the sum frequency signal and decreases the sensitivity to impurities. By using a theoretical framework [127–129], it is possible to retrieve information on the molecular structure of the interfaces from the SFS measurements. This has been successfully applied to probe the interfacial structure of liposomes [130], colloids [131], and oil nanodroplets [87, 115, 132–137].

1.5.2 Second Harmonic Scattering

Historically, the study of second-order nonlinear processes started with the discovery of SHG in a quartz crystal in 1961 by Franken [138]. Bloembergen and Pershan established the theoretical framework in 1962 [118] that was further developed in the next years [139–141].

SHG was used first to study liquids in scattering configuration. The first resonant SHS from bulk liquid was reported by Terhune in 1965 [44]. The corresponding theory in liquids

was developed by Cyvin in the same year [142] and subsequently refined [143–145]. A first aspect of SHS in liquids is that noncentrosymmetric molecules uncorrelated with each other generate a weak incoherent SH light. This is the so-called hyper-Rayleigh Scattering (HRS) [146], and it is proportional to number density of the molecules [147] in the probed volume. The second aspect is that intermolecular correlations increase the SH emission: constructive interferences of the signal of individual molecules build up into an additional coherent contribution to the overall SH light. Therefore, SHS is a very powerful technique to study anisotropy or centrosymmetry breaking due to intermolecular interactions. However, non-resonant processes such as SHS are inherently weak, leading to very long measurement duration [44]. Our group has recently addressed this issue by using a specific combination of laser parameters to get a higher throughput. As the number of emitted photons scales with the repetition rate and the square of the pulse energy, we increase the energy and decrease the repetition rate in comparison to traditional measurements. In that way, we are able to improve the scattering intensity by 2 to 3 orders of magnitude [148], reducing the duration of the measurement from a day down to seconds. More importantly, unlike in the case of traditional SHS measurements performed with nanosecond pulsed lasers, femtosecond SHS consists only into fully elastic scattering [59] without any undesired non-second-order contribution to the signal. Using femtosecond pulses rather than nanosecond pulses allows one as well to probe instantaneous fluctuations at a shorter timescale than the orientational relaxation time of liquids [15]. This is referred as femtosecond elastic SHS (fs-ESHS). It has been used for instance to probe longrange correlations in ionic solutions [59, 149], and even the impact of nuclear quantum effects on structure of liquid water [22, 59, 150-153].

SHG was also performed extensively on interfaces. A first resonant SHG surface experiment was performed by Brown et al. in 1969 [154]. Other planar interfacial studies followed [155–157]. More specifically, the study of liquid/liquid interfaces by SHG started in 1996 [158]. For liquid/air interfaces, it has been performed since the 80's [157, 159, 160].

SHG was also applied on the interfaces of particles in solution, in scattering configuration. It provides advantages similar to SFS, compared to planar interface configuration. The first resonant SHS was realized in 1996, from resonant malachite green particles adsorbed onto the particle surface [161]. In 1998, Yan et al. reported the first elastic non-resonant SHS from polystyrene particles, and describing the relation between the SHG and surface potential of the particles [162]. SHS experiments were then conducted on liposomes [163, 164]. This has been used extensively since then [45, 165–170].

As SHG depends quadratically on the number density of molecules, and as water is the most abundant species at the interface of particles in solutions, the dominant SHS response comes from interfacial water molecules [171, 172]. This was used to develop angle-resolved SHS (AR-SHS) in 2010 [172]. Many studies followed, to investigate nanoparticles in water [87, 115, 130, 133, 134, 173–177]. Later improvements were made to the AR-SHS theory to include the case of charged interfaces and allow a better match between theory and experiments [88, 171, 178]. Furthermore, it allowed to extract the surface potential of particles from AR-SHS

measurements. This was applied for liposomes [88, 178], and nanoparticles [179, 180].

1.6 This thesis

This work is divided into seven chapters. Experimental and theoretical details are provided in Chapter 2, while the results of the thesis are presented in Chapter 3 to 6. The first part (Chapters 3 and 4) was adapted from two manuscripts (respectively published and submitted) and focuses on pure water and aqueous solutions studied by SHS. The second part (Chapters 5 and 6) revolves around the hydration of lipid membranes, using mainly SHS, and SFS in Chapter 6.

Chapter 2 provides the theoretical background of the techniques used in the thesis: SHS from bulk liquids, and SHS and SFS from scatterers in solution. This is followed by the experimental details of the AR-SHS setup used during the experiments. Two complementary measurement techniques known as Dynamic Light Scattering (DLS) and ζ potential measurements are also presented, as well as their data analysis.

Chapter 3 presents the SHS measurements of the temperature dependence of intermolecular correlations in pure light and heavy water, and in NaCl aqueous solutions, as a function of temperature. The changes in orientational order are interpreted in terms of the symmetry of H-bonds and are indicators of nuclear quantum effects in the structure of water.

In Chapter 4, we investigate in more details the intermolecular interactions in water by comparing SHS measurements of pure liquid water and carbon tetrachloride. We observe a deviation in water compared to the incoherent SHS expected for a seemingly isotropic liquid. These coherent emissions of SH light are interpreted with the aid of MD simulations, as arising from charge density fluctuations around transient nanoscale voids in the liquid.

In Chapter 5 we extend our study to the structure of water around lipid membranes. We study the confinement of H_2O and D_2O within liposomes of different sizes, using AR-SHS and surface potential analysis. Three-dimensional confinement manifests itself primarily in the amount of orientational ordering of the hydrogen bond network. For D_2O the effect depends on the change in interfacial area. For H_2O the effect is larger and depends on the inner volume of liposomes.

In Chapter 6, the discussion is further brought to the impact of the charge of phospholipids on the hydration around liposome bilayers and oil droplets monolayers. The measured charge hydration asymmetries result from a delicate balance of electrostatic and hydrogen bonding interactions.

Finally, Chapter 7 contains a summary of the results presented in this work, as well as an outlook of the opportunities for future studies.

2 Experimental details and methods

This chapter presents a theoretical background for the principal techniques used throughout this thesis, followed by experimental details of their implementation. First, the theory of nonlinear scattering is detailed, from pure liquids to uncharged and charged particles. The apparatus for experimental measurements using these scattering techniques are then presented. This is followed by a section on additional supporting techniques (dynamic light scattering and zeta potential measurements). Finally, the sample preparation and characterization are detailed.

2.1 Theory of nonlinear light scattering

This section focuses on the theoretical framework of the nonlinear scattering techniques implemented in this thesis: second harmonic scattering (SHS) and vibrational sum frequency scattering (SFS). First, the theory of SHS in pure liquids is developed, with a distinction between incoherent and coherent scattering. This is followed by the derivation of the scattering theory for spherical particles in solution: first the vibrational SFS, then the more specific case of SHS. Afterwards, this framework is extended to particles in solution bearing a charge on their surface, and the application for surface potential measurements is discussed.

2.1.1 Second harmonic scattering in bulk liquids

Second harmonic generation (SHG) is a nonlinear optical process where two identical photons of frequency ω interact with the molecules of a non-centrosymmetric medium to be converted into one photon of the doubled frequency 2ω . This interaction is described by the polarization vectors of equations (1.1) and (1.2). In the case of scattering in liquids, SHS can be generally described by the sum of an incoherent and a coherent contribution. The SH intensity can therefore be expressed as follows:

$$I(2\omega) \propto N \left\{ \left\langle \left(\beta_{ijk}^{(2)} \right)^2 \right\rangle_{\text{incoh}} + \left\langle \left(\beta_{ijk}^{(2)} \right)^2 \right\rangle_{\text{coh}} \right\}$$
(2.1)

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where N is the number density of molecules in the volume illuminated by the exciting light, and $\beta_{ijk}^{(2)}$ is the tensor element of the second-order polarizability $\boldsymbol{\beta}^{(2)}$ (also referred as first-order hyperpolarizability) that describes the response of the molecule to the excitation. The brackets $\langle ... \rangle$ represent the ensemble orientational average over all involved molecules. The subscripts _{incoh} and _{coh} denote respectively the incoherent and coherent contributions to the intensity.

The first term in equation (2.1) is also known as Hyper Rayleigh Scattering (HRS). It originates from the electronic non-centrosymmetry, i.e. the anisotropic distribution of electrons, within individual molecules. The summation of these individual contributions is incoherent as the SH electric fields have no fixed phase difference between each other. The second term in the equation originates from the non-centrosymmetry of the distribution of molecules. The SH electric fields generated individually by each molecule are summed up coherently with a phase difference induced by any correlation between the molecules, such as hydrogen bonding or dipole-dipole interaction. As a consequence, the second term of the equation sheds light on the intermolecular correlations, thus on the liquid structure.

When molecules are distributed in a random or centrosymmetric manner, the individual electric fields emitted by the molecules cancel each other out via destructive interference and the overall coherent SH contribution vanishes, leaving only the incoherent contribution to the SH intensity. On the contrary, when the centrosymmetry is broken due to intermolecular correlations, the coherent contribution is non-zero.

Molecular correlations can be induced by hydrogen bonding, Coulombic, dipole-dipole, or other interactions. For water, the orientational correlation of a pair of water molecules can be changed by breaking an H-bond through a rotational or bending / libration motion, but not by stretching. The orientational correlation modifies the coherent contribution of the correlated molecules to the SH intensity.

Derivation of the SH intensity

The SH intensity originates from electronic anisotropy induced by an electric field within every single non-spherical molecule. This anisotropy is described at the microscopic level by the second-order polarization $\tilde{p}_{v}^{(2)}$ of a molecule *v* induced by an electric field \tilde{E} at fundamental frequency ω [43]:

$$\widetilde{\boldsymbol{\rho}}_{\nu}^{(2)}(2\omega) = \boldsymbol{\beta}^{(2)} : \widetilde{\boldsymbol{E}}(\omega) \widetilde{\boldsymbol{E}}(\omega)$$
(2.2)

where $\boldsymbol{\beta}^{(2)}$ is the tensor of second-order polarizability (or first-order hyperpolarizability) of the molecule. At the macroscopic level, the polarization of the medium (i.e. the average response of the material) is composed by the sum of the induced molecular polarizations per unit of volume [43]:

$$\boldsymbol{P}^{(2)}(2\omega) = \epsilon_0 \boldsymbol{\chi}^{(2)} : \widetilde{\boldsymbol{E}}(\omega) \widetilde{\boldsymbol{E}}(\omega) = N \left\langle \boldsymbol{p}^{(2)} \right\rangle$$
(2.3)
where $\chi^{(2)}$ is the second-order susceptibility tensor, characteristic of the material, and ϵ_0 is the vacuum permittivity.

The SH electric field is obtained from the polarization with the relation $\tilde{E}(2\omega) = \epsilon_0^{-1} P^{(2)}(2\omega)$. From there, the SH intensity is derived as the square of the sum of all SH electric fields $\tilde{E}(2\omega)$ emitted individually by each molecule excited by the electric field $\tilde{E}(\omega)$ in the probed volume. In the case where all molecules are considered as independent scatterers, the SH electric fields can be simply summed up, allowing to separate the total SH intensity into two contributions in terms of momentum transfer:

$$I(2\omega) = \frac{cn\epsilon_0}{2} \left| \sum_{\nu} \widetilde{E}(2\omega) \right|^2$$
(2.4)

$$= \frac{cnc_0}{2} \left\{ \underbrace{\sum_{\nu} |E_{\nu}(2\omega)|^2}_{\text{self-correlations,}} + \underbrace{\sum_{\nu \neq \nu'} E_{\nu}(2\omega) E_{\nu'}^*(2\omega) e^{i\boldsymbol{q}\cdot(\boldsymbol{r}-\boldsymbol{r}')}}_{\text{cross-correlations, coherent}} \right\}$$
(2.5)

where *c* is the speed of light in vacuum, *n* is the refractive index, ω is the frequency of the excitation light, *q* is the momentum transfer in the SH emission process and *r* and *r'* are the positions of molecules *v* and *v'*.

In this equation, the self-correlations are always present and are at the origin of the HRS [143]. On the contrary, the cross-correlations are dependent on intermolecular correlations. Also, the coherent contribution to the SH intensity contains momentum transfer in the form of the scalar product $\mathbf{q} \cdot (\mathbf{r} - \mathbf{r}')$. The computation of this coherent term require extensive path-integral computation. On the contrary, the first term (HRS) can be analytically derived under some assumptions.

Hyper Rayleigh Scattering

The polarization combinations are indicated with a three letters code for the three photons involved in the SH process. The letters correspond to increasing wavelength from left to right, representing the polarization of the scattered SH light and the incident fundamental light with P (S) referring to linearly polarized light parallel (perpendicular) to the scattering plane.

Under the assumptions of an isotropic (with a random orientation of molecules), lossless and dispersionless medium, and with the use of symmetry properties of $\beta^{(2)}$, the calculation of the incoherent SHS intensity is simplified. The HRS intensity in the (*x*,*z*) scattering plane can be expressed for the following polarization combinations [145]:

$$I_{SSS}(2\omega) = \frac{cnk_0^4 N_m V E_y^4}{32\pi^2 \epsilon_0 R^2} \left\langle \left(\beta_{yyy}^{(2)}\right)^2 \right\rangle$$
(2.6)

$$I_{PPP}(2\omega) = \frac{cnk_0^4 N_m V E_x^4}{32\pi^2 \epsilon_0 R^2} \left\{ \left\langle \left(\beta_{yyy}^{(2)}\right)^2 \right\rangle \cos^2 \theta_R + \left\langle \left(\beta_{yxx}^{(2)}\right)^2 \right\rangle \sin^2 \theta_R \right\}$$
(2.7)

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$$I_{SPP}(2\omega) = \frac{cnk_0^4 N_m V E_x^4}{32\pi^2 \epsilon_0 R^2} \left\langle \left(\beta_{yxx}^{(2)}\right)^2 \right\rangle$$
(2.8)

$$I_{PSS}(2\omega) = \frac{cnk_0^4 N_m V E_y^4}{32\pi^2 \epsilon_0 R^2} \left\langle \left(\beta_{yxx}^{(2)}\right)^2 \right\rangle$$
(2.9)

where *c* is the velocity of light in vacuum, *n* is the refractive index of air, k_0 is the second harmonic wave vector, N_m is the molecular density of the liquid, *V* is the volume of the liquid contributing to the SHS intensity, E_x and E_y are the incoming electric fields along the *x* and *y*-direction of the laboratory frame, and *R* is the distance between the far-field observation position and the scattering position. θ_R is the scattering angle in the (*x*,*z*) plane, with respect to the *z* direction.

Coherent SHS

According to the selection rule of SHS, molecular correlations of spherical symmetry only contribute to the SH intensities of PPP and PSS combinations [45, 129]. Consequently, the PPP and PSS combinations mainly report on the collective orientational order and the SH intensity consists of both coherent contributions from correlated molecules and incoherent contributions from uncorrelated molecules. The SSS and SPP combinations report on the electronic structure of individual molecules and the SH intensity is dominated by the incoherent contributions from uncorrelated molecules.

2.1.2 Nonlinear scattering from particles in solution

The addition of a solid/liquid interface by the introduction of particles in the liquid changes the way the second harmonic light is generated. We focus now on the light scattered because of the presence of the particles, and neglect the HRS light. We assume that the particles are spherical, and we use the Rayleigh-Gans-Debye (RGD) approximation [181]:

$$\left| 1 - \frac{n_p}{n_s} \right| \ll 1$$

$$\frac{2\pi}{\lambda} d \left| 1 - \frac{n_p}{n_s} \right| \ll 1$$
(2.10)

where n_p and n_s are the refractive indices of respectively the particle and the solvent, λ is the wavelength of the light, and d is the diameter of the particle. We also focus on particles with a homogeneous, isotropic, and achiral surface. The case of chiral surfaces is out of the scope of this thesis.

From molecular hyperpolarizability, to effective particle susceptibility

For the computation of the scattered electromagnetic field, we need to transition from a molecular view to a broader perspective encompassing the geometry of individual particles and the scattering geometry. In other terms, we need to link the microscopic description of

the material response $\boldsymbol{\beta}^{(2)}$ to the macroscopic description in the form of $\boldsymbol{\chi}^{(2)}$, and then to encompass the scattering geometry of the experiment by defining an effective susceptibility $\Gamma^{(2)}$ [129, 182].

The general way of proceeding is the following. From equations (2.2) and (2.3), the macroscopic $\chi^{(2)}$ tensor is first computed from the molecular hyperpolarizabilities of the material. A simplification is done here by assuming that the particles are made of a centrosymmetric material, and that the the solvent around is centrosymmetric as well. Then the centrosymmetry is broken only at the interface. This means that the $\chi^{(2)}$ tensor is non-zero only at the particle/solvent interface [43]. Therefore, we can express it at the position r as a function of the delta distribution δ , as $\chi_s^{(2)}(r) = \chi^{(2)}(r)\delta(|r| - R)$, where R is the radius of the particle.

Next, we include the geometry of the experiment by defining an effective particle surface susceptibility $\Gamma^{(2)}$, realizing a change of coordinates to encompass the experiment's geometry. The element i j k of the $\Gamma^{(2)}$ tensor is defined by an integration over the whole particle surface Ω of a particle. It is related to the element *abc* of the $\chi^{(2)}$ tensor by:

$$\Gamma_{ijk}^{(2)} = \sum_{abc} \int^{\Omega} T_{ia} T_{jb} T_{kc} \chi_{s,abc}^{(2)} e^{i\boldsymbol{q}\cdot\boldsymbol{r}} \mathrm{d}\Omega$$
(2.11)

where the matrix T is the transformation matrix of coordinates between the laboratory frame and the scattering frame, and **r** is the position of the point of integration at the surface of the particle (different from the **r** vector of equation (2.4)). **q** is the scattering vector. These $\Gamma_{ijk}^{(2)}$ elements are finally used to compute the amplitude of the outgoing electric field scattered by the particle.

Vibrational sum frequency scattering

In the general case of sum frequency scattering, two beams with electric field amplitudes E_1 and E_2 are shone on a solution containing particles, with two different incidence angles. Figure 2.1 displays the geometry of this type of experiment. Here θ is the scattering angle between the scattered light wavevector k_0 , and the sum of the incoming wavevectors $k_1 + k_2$. The scattering wavevector is defined as $q = k_0 - (k_1 + k_2)$. Similarly to the subsection 2.1.1 on the SHS from liquids, we define the polarization as S when perpendicular and P when parallel to the scattering plane (x,z), and the polarization combinations are indicated with a three letters code for the three photons involved in the nonlinear scattering process.

In this configuration, we compute the susceptibility tensor $\chi_s^{(2)}(\mathbf{r})$ at the surface. This surface tensor contains $3^3 = 27$ elements. Due to symmetries at the surface [43], the number of non-zero independent tensors is reduced to seven, and in the case of particles with achiral surfaces it reduces further to four: $\chi_{\perp\perp\perp}, \chi_{\parallel\parallel\perp}, \chi_{\parallel\parallel\parallel}, \chi_{\perp\parallel\parallel}$. Here, the index \perp denotes the radial direction in spherical frame centered on the particle, while \parallel denotes the two directions perpendicular to that (thus tangent to the surface).



Figure 2.1 – Sketch of the scattering configuration for SFS/SHS experiments, top view, adapted from Ref. [183]. The scattering plane is in the (x,z) plane. The parallel and perpendicular notations for $\chi^{(2)}$ and $\Gamma^{(2)}$ are shown at the surface of the particle and next to the scattering vector.

With this, we compute the effective susceptibility. The element $a_0 a_1 a_2$ of $\Gamma^{(2)}$ is related to $\chi_s^{(2)}$ elements by the following relation [129]:

$$\Gamma_{a_0a_1a_2}^{(2)} = \sum_{c_0c_1c_2} \int d\mathbf{r}^{\prime 3} \left\{ \chi_{c_0c_1c_2}^{(2)} \delta\left(|\mathbf{r}^{\prime}| - R \right) e^{-i\mathbf{q}\cdot\mathbf{r}^{\prime}} \prod_{i=0}^{2} \left(\hat{\mathbf{e}}_{c_i} \cdot \widehat{\mathbf{q}}_{a_i} \right) \right\}$$
(2.12)

where *R* is the radius of the particle, $\hat{\boldsymbol{e}}_{c_i}$ are the unit vectors of the spherical (particle) coordinate system, and $\hat{\boldsymbol{q}}_{a_i}$ are the unit vectors of the coordinate system defined with respect to the scattering vector \boldsymbol{q} . The spatial coordinates a_1 , a_2 , and a_3 can take the values x, y, z.

The computation of equation (2.12) yields the following $\Gamma^{(2)}$ components:

$$\begin{pmatrix} \Gamma_1^{(2)} \\ \Gamma_2^{(2)} \\ \Gamma_3^{(2)} \\ \Gamma_4^{(2)} \end{pmatrix} = \begin{pmatrix} 2F_1 - 5F_2 & 0 & 0 & 0 \\ F_2 & 2F_1 & 0 & 0 \\ F_2 & 0 & 2F_1 & 0 \\ F_2 & 0 & 0 & 2F_1 \end{pmatrix} \begin{pmatrix} \chi_{s,1}^{(2)} \\ \chi_{s,2}^{(2)} \\ \chi_{s,3}^{(2)} \\ \chi_{s,4}^{(2)} \end{pmatrix}$$
(2.13)

where the effective particle surface susceptibilities are rearranged as $\Gamma_1^{(2)} = \Gamma_{\perp\perp\perp}^{(2)} - \Gamma_{\parallel\parallel\perp}^{(2)} - \Gamma_{\perp\parallel\parallel}^{(2)} - \Gamma_{\perp\parallel\parallel}^{(2)} - \Gamma_{\perp\parallel\parallel}^{(2)}$, $\Gamma_3^{(2)} = \Gamma_{\parallel\perp\parallel}^{(2)}$, $\Gamma_4^{(2)} = \Gamma_{\perp\parallel\parallel}^{(2)}$. Here, the index \perp denotes the direction in the scattering frame that is perpendicular to the plane defined by \boldsymbol{q} (thus parallel to $\hat{\boldsymbol{q}}_z$), while \parallel denotes the two directions perpendicular to that. The surface susceptibilities are also redefined as $\chi_{s,1}^{(2)} = \chi_{\perp\perp\perp}^{(2)} - \chi_{\parallel\parallel\perp}^{(2)} - \chi_{\perp\parallel\parallel}^{(2)} - \chi_{\perp\parallel\parallel}^{(2)}$, $\chi_{s,2}^{(2)} = \chi_{\parallel\parallel\perp}^{(2)}$, $\chi_{s,3}^{(2)} = \chi_{\parallel\perp\parallel}^{(2)}$, $\chi_{s,4}^{(2)} = \chi_{\perp\parallel\parallel\parallel}^{(2)}$. It is important to remember that the \perp and \parallel indices for surface susceptibilities and effective surface susceptibilities have a different meaning in each case. The rearrangements of the susceptibilities allow to write the relation matrix in an elegant way, with the form factors F_1

and F_2 . These factors are, in the case of spherical particles:

$$F_1(qR) = 2\pi R^2 i \left(\frac{\sin(qR)}{(qR)^2} - \frac{\cos(qR)}{qR} \right)$$

$$F_2(qR) = 4\pi R^2 i \left(3 \frac{\sin(qR)}{(qR)^4} - 3 \frac{\cos(qR)}{(qR)^3} - \frac{\sin(qR)}{(qR)^2} \right)$$
(2.14)

The amplitude of the electric field E_0 scattered by the particle when illuminated by two incoming fields E_1 and E_2 is expressed as [182], [129]:

$$E_0 = C E_1 E_2 \sum_{a_0 a_1 a_2} \Gamma^{(2)}_{a_0 a_1 a_2} \prod_{i=0}^2 \left(\hat{\boldsymbol{q}}_{a_i} \cdot \hat{\boldsymbol{u}}_{i,l} \right)$$
(2.15)

where $C = \frac{ick_0^3}{2\pi |\hat{r}||\hat{I}|} \frac{e^{ik_0 r_0}}{r_0}$ with \hat{r} and \hat{I} as unit vectors of distance and current used to preserve the coherence of units in the formula. $\hat{u}_{i,l}$ is the unit vector of polarization of the beam with amplitude E_i . Therefore, the l index can refer to either S or P polarization. The unit polarization vectors corresponding to S or P are defined as:

$$\begin{pmatrix} \widehat{\boldsymbol{u}}_{0,P} \\ \widehat{\boldsymbol{u}}_{1,P} \\ \widehat{\boldsymbol{u}}_{2,P} \end{pmatrix} = \begin{pmatrix} \cos\theta & 0 & -\sin\theta \\ \cos(\beta - \alpha) & 0 & \sin(\beta - \alpha) \\ \cos\alpha & 0 & -\sin\alpha \end{pmatrix} \begin{pmatrix} \widehat{\boldsymbol{x}} \\ \widehat{\boldsymbol{y}} \\ \widehat{\boldsymbol{z}} \end{pmatrix}$$
(2.16)
$$\begin{pmatrix} \widehat{\boldsymbol{u}}_{0,S} \\ \widehat{\boldsymbol{u}}_{1,S} \\ \widehat{\boldsymbol{u}}_{2,S} \end{pmatrix} = \begin{pmatrix} 0 & 1 & 0 \\ 0 & 1 & 0 \\ 0 & 1 & 0 \end{pmatrix} \begin{pmatrix} \widehat{\boldsymbol{x}} \\ \widehat{\boldsymbol{y}} \\ \widehat{\boldsymbol{z}} \end{pmatrix} = \begin{pmatrix} \widehat{\boldsymbol{y}} \\ \widehat{\boldsymbol{y}} \\ \widehat{\boldsymbol{y}} \end{pmatrix}$$
(2.17)

In the case of achiral particle surfaces between two isotropic media, only four polarization combinations scatter a non-zero electromagnetic field: PPP, SSP, SPS, and PSS. Equation (2.15) then yields the following amplitudes of scattered electromagnetic fields in these different polarization combinations [129, 171]:

$$E_{PPP} = C \left[\cos \left(\frac{\theta}{2} \right) \cos \left(\frac{\theta}{2} - \alpha \right) \cos \left(\frac{\theta}{2} - \alpha + \beta \right) \right] \Gamma_1^{(2)} + \cos(\theta - \alpha + \beta) E_{SSP} + \cos(\theta - \alpha) E_{SPS} + \cos(\beta) E_{PSS} E_{SSP} = C \cos \left(\frac{\theta}{2} - \alpha \right) \Gamma_2^{(2)} E_{SPS} = C \cos \left(\frac{\theta}{2} - \alpha + \beta \right) \Gamma_3^{(2)} E_{PSS} = C \cos \left(\frac{\theta}{2} \right) \Gamma_4^{(2)}$$

$$(2.18)$$

Second harmonic scattering

In the specific case of second harmonic scattering generated from particles with an achiral surface, a single beam with amplitude $E(\omega)$ is shone on the sample, thus the two incoming beams in the scattering configuration in Fig. 2.1 are now collinear along \hat{z} ($\alpha = \beta = 0$) and with the same frequency. Due to the degeneracy in frequencies, the set of non-zero elements of

 $\chi_s^{(2)}$ reduces from seven to four and $\chi_{s,2}^{(2)} = \chi_{s,3}^{(2)} = \chi_{s,4}^{(2)}$. Because of the single beam geometry of the SHS experiment, the last two polarization indices are identical for each polarization combination. This leaves PPP, PSS, SPP and SSS as unique combinations.

For these reasons, we get $\Gamma_2^{(2)} = \Gamma_3^{(2)} = \Gamma_4^{(2)}$, and equation (2.18) thus simplifies in the following way:

$$E_{PPP}(2\omega) = CE(\omega)^2 \left[\cos\left(\frac{\theta}{2}\right)^3 \Gamma_1^{(2)} + 2\cos(\theta)\cos\left(\frac{\theta}{2}\right) \Gamma_2^{(2)} \right] + E_{PSS}(2\omega)$$

$$E_{PSS}(2\omega) = CE(\omega)^2 \cos\left(\frac{\theta}{2}\right) \Gamma_4^{(2)} = CE(\omega)^2 \cos\left(\frac{\theta}{2}\right) \Gamma_2^{(2)}$$
(2.19)

while the other polarization combinations are null.

The contributions from the bulk liquid solvent (incoherent HRS, as well as any coherent contribution from intermolecular correlation in the solvent) are still generated and add up to the SHS from equation (2.19). This means that experimentally, for instance, the SSS polarization combination will not be null. In order to study the particles without the bulk, one has to measure SHS from one sample with particles in suspension in the solvent, as well as the SHS from the solvent alone. Then, the contribution from the particles only is retrieved by a normalization procedure described in equation (2.27).

Second harmonic scattering from charged particles

We consider now the case of charged spherical particles. The surface charge brings an additional effect on the overall nonlinear scattering process, by generating an electrostatic field $\widetilde{E}_{DC}(0)$ extending into the solvent. $\widetilde{E}_{DC}(0)$ is perpendicular to the surface, and its magnitude is linked to the so-called surface potential Φ_0 [5, 184] of the particle. Indeed, $E_{DC}(r) = -\frac{d\Phi(r)}{dr}$, and Φ at the surface of the particle, $\Phi(R)$, is equal to Φ_0 . This field will perturb the orientational order of the solvent molecules around the particles. The extent to which the molecules are perturbed is related to Debye length [45, 162, 183, 185]. Indeed, the electrostatic DC field induces a third-order response of the material by two pathways [183]: 1) the two incoming varying fields plus the DC field interact by generating a purely third-order response from solvent molecules via the $\boldsymbol{\beta}^{(3)}$ tensor; 2) the DC field induces a symmetry-breaking reorientation of solvent molecules both at the interface and further into the solvent in the so-called diffuse double layer, this reorientation then resulting in a second-order process via the $\beta^{(2)}$. Since the second-order responses in both pathways depend linearly on the incident electric fields and on the electrostatic field, we group them under the effective third-order susceptibility $\chi^{(3)'}$ [183]. We include this in our description by implementing an additional polarization term $P^{(3)}(2\omega) = \epsilon_0 \left(\chi^{(3)'} : \tilde{E}(\omega) \tilde{E}(\omega) \tilde{E}_{DC}(0) \right)$ into the polarization vector for the second harmonic generation. Consequently, the total polarization vector describing the second harmonic response is given by:

$$\boldsymbol{P}(2\omega) = \epsilon_0 \left(\boldsymbol{\chi}^{(2)} : \widetilde{\boldsymbol{E}}(\omega) \widetilde{\boldsymbol{E}}(\omega) + \boldsymbol{\chi}^{(3)\prime} : \widetilde{\boldsymbol{E}}(\omega) \widetilde{\boldsymbol{E}}(\omega) \widetilde{\boldsymbol{E}}_{DC}(0) \right)$$
(2.20)

As a consequence, the equation (2.19) for SHS has now to be modified to include some additional terms due to $\chi^{(3)'}$. This can be computed in a similar fashion as that of the second-order susceptibility. A first point to note is that the direction of $\tilde{E}_{DC}(0)$ is radial, along the normal to the particle surface, meaning that the last index of $\chi^{(3)'}$ elements can be excluded : $\chi^{(3)'}_{ijkl} = \chi^{(3)'}_{ijk,r}$. For this reason, we can simplify $\chi^{(3)'}$ from a fourth-rank tensor to a third-rank tensor (similar to $\chi^{(2)}$).

Next, the calculation of $\Gamma^{(3)}$ is done in the same way as for $\Gamma^{(2)}$ in equation (2.12), but the integration is also performed in the radial direction. The radial variation of the electrostatic field is also included in the integral:

$$\Gamma_{a_0a_1a_2}^{(3)\prime} = \sum_{c_0c_1c_2} \int d\mathbf{r}^{\prime 3} \left\{ \chi_{c_0c_1c_2,r'}^{(3)\prime} E_{DC}(r') e^{-i\mathbf{q}\cdot\mathbf{r}'} \prod_{i=0}^2 \left(\mathbf{e}_{c_i} \cdot \mathbf{q}_{a_i} \right) \right\}$$
(2.21)

Due to symmetries, we get $\chi_{s,1}^{(3)\prime} = 0$ and $\Gamma_1^{(3)} = 0$. Because of this, the other elements in equation (2.13) simplify as $\Gamma_n^{(3)} = 2F_1\chi_n^{(3)\prime}$ for all n = 2, 3, 4. By using $E_{DC}(r) = -\frac{d\Phi(r)}{dr}$ and $\Phi(R) = \Phi_0$ at the surface of the particle, we can compute the effective third order susceptibility $\Gamma^{(3)\prime}$ elements as:

$$\Gamma_n^{(3)\prime} = -\int_R^{+\infty} \frac{\mathrm{d}\Phi(r')}{\mathrm{d}r'} \Gamma_n^{(3)} \mathrm{d}r'$$
(2.22)

$$=2\chi_{n}^{(3)'}\left[F_{1}\Phi_{0}+\int_{R}^{+\infty}\frac{\mathrm{d}F_{1}}{\mathrm{d}r'}\Gamma_{n}^{(3)}\Phi(r')\,\mathrm{d}r'\right] \quad \text{for all } n=2,3,4 \tag{2.23}$$

The second term of this equation requires an analytical expression of the potential $\Phi(r)$. We can use the diffuse double layer equation $\Phi(r) = \Phi_0 \frac{R}{r} e^{-\kappa(r-R)}$, with κ being the inverse Debye length [171, 186]. However, this expression is derived under the Debye-Hückel approximation and is valid only when the values of potential difference are low. Due to interferences, the second term of equation (2.22) only contributes to the expression several nanometers away from the interface [171]. Therefore we can use the diffuse double layer equation without loss of generality [171], even though we do not use a Debye-Hückel model.

Knowing all of this, we can compute the effective third order susceptibility $\Gamma^{(3)\prime}$ elements as:

$$\Gamma_n^{(3)\prime} = 2\chi_n^{(3)\prime} \Phi_0 (F_1 + F_3) \text{ for all } n = 2, 3, 4$$
 (2.24)

with the form factor $F_3 = 2\pi i R^2 \frac{qR\cos(qR) + \kappa R\sin(qR)}{(qR)^2 + (\kappa R)^2}$

In the end, we can correct the equation (2.19) by replacing $\Gamma^{(2)}$ by $\Gamma^{(2)} + \Gamma^{(3)\prime}$ [171]. The final result for SHS light scattered by charged spherical particles with an achiral surface is:

$$E_{PPP}(2\omega) = CE(\omega)^2 \left[\cos\left(\frac{\theta}{2}\right)^3 \Gamma_1^{(2)} + \cos\left(\frac{\theta}{2}\right) \left(\Gamma_2^{(2)} + \Gamma_2^{(3)'}\right) (2\cos(\theta) + 1) \right]$$

$$E_{PSS}(2\omega) = CE(\omega)^2 \cos\left(\frac{\theta}{2}\right) \left(\Gamma_2^{(2)} + \Gamma_2^{(3)'}\right)$$
(2.25)

Surface potential fitting

It is worthy to remember here that $\Gamma_n^{(3)\prime}$ is directly related to the surface potential Φ_0 by the equation (2.24). The measurement of the surface potential from particles in solution has usually been a difficult task [187]. However, it has been shown recently [178] that the angle-resolved second harmonic scattering (AR-SHS) measurements can be used to extract the Φ_0 value in a simple and elegant way. By measuring two scattering patterns in the two polarization combinations of equation (2.25), namely PPP and PSS, it is indeed possible to solve the sets of equations above via the fitting of angle-resolved SHS (AR-SHS) patterns, in order to extract two unknowns: the values for Φ_0 and the effective surface second-order susceptibility $\chi_{s,2}^{(2)}$. To this aim we use the procedure described in Refs. [171, 178].

Briefly, in order to perform the fitting procedure, we have to use the known SHS response from the solvent as a reference. The measured AR-SHS intensities are thus normalized by the intensity $I_{SSS}(\theta)$ of the pure solvent in the SSS polarization. Also, the AR-SHS patterns are normalized following equation 2.27 to remove the response of the pure solvent and to keep only the pure SH intensity corresponding to the particles surface. And finally, the $\Gamma_1^{(2)}$, $\Gamma_2^{(2)}$ and $\Gamma_2^{(3)\prime}$ are corrected to account for changes in the refractive index between the solvent and the particle. In the end, the fitting procedure is performed on the following normalized patterns:

$$\frac{I_{PPP}(\theta)}{I_{SSS}(\theta)} = \frac{\epsilon_0^2 \left[E(\omega)^2 \left[\cos(\frac{\theta}{2})^3 \Gamma_1^{(2)} + \cos(\frac{\theta}{2}) (\Gamma_2^{(2)} + \Gamma_2^{(3)\prime}) (2\cos(\theta) + 1) \right] \right]^2}{\overline{\mu}^2 N_b / N_p} = S_{PPP}(\theta)$$

$$\frac{I_{PSS}(\theta)}{I_{SSS}(\theta)} = \frac{\epsilon_0^2 \left[E(\omega)^2 \left[\cos(\frac{\theta}{2}) (\Gamma_2^{(2)} + \Gamma_2^{(3)\prime}) \right] \right]^2}{\overline{\mu}^2 N_b / N_p} = S_{PSS}(\theta)$$
(2.26)

where $\overline{\mu} = \overline{\beta}^{(2)} E(\omega)^2$ is the averaged induced dipole moment of the solvent molecule; N_p is the number of particles, and N_b is the density of bulk solvent (3.34 · 10²⁸ molecules/m³ in the case of pure light water). N_b/N_p is the number of bulk solvent molecules per particle.

This technique of fitting has been applied successfully on various systems such as liposomes, and silica and titania nanoparticles [88, 180, 188].

2.1.3 AR-SHS setup

In this subsection, we cover the implementation of angle-resolved second harmonic scattering (AR-SHS) into an experimental setup. The scheme of the apparatus is shown in figure 2.2. It has already been described in detail in Ref [148]. Briefly, laser pulses at 1032 nm wavelength and 190fs pulse duration are generated with a repetition rate of 200 kHz by a Yb:KGW laser (Light Conversion Pharos). The linear polarization of the laser beam is controlled by a Glan-Taylor polarizer (GT10-B, Thorlabs) followed by a zero-order half wave plate (WPH05M-1030, Thorlabs). Afterwards, a long-pass filter (FEL0750, Thorlabs) removes any second harmonic light generated by the preceding optical elements of the setup. The laser beam is then focused by a plano-convex lens (f=75mm) into a cylindrical glass sample cell (4.2 mm inner diameter, high precision cylindrical glass cuvettes, LS instruments) placed into a holder, with a beam



Figure 2.2 – Experimental setup for AR-SHS measurements, top-view. The z-axis is defined along the optical axis of the incident beam, and the scattering plane is the (x,z) plane. The linear polarization of the laser pulses is defined as "S" when along the y-axis, and as "P" when it is contained in the scattering plane. The scattering angle is defined with respect to the forward direction along the z-axis. The optics parts are abbreviated as follows: GT = Glan-Taylor beam splitter; HW = half-wave plate; HP = high pass filter; L = lens; BP = band pass filter; PMT = photomultiplier tube. The cylindrical cell containing the sample is typically inserted along the y-axis in the hole of the sample holder.

waist around $27\mu m$ and a Rayleigh length of 2.20 mm. The temperature of the sample is tuned precisely by a customized controller (Quantum Northwest), in the range of 253.15 K to 423.15 K with a precision of 0.1 °C. The SH light scattered by the sample is collected by a series of optics mounted onto a rotating arm controlled by computer. First, a plano-convex lens (f=50mm) collimates the light, that passes afterward through an iris set typically at an acceptance angle of 3.4° for patterns or 11.4° for single-angle measurements. The SH light is then analyzed by a Glan-Taylor polarizer (GT10-A, Thorlabs), passes through a band-pass filter (Chroma ET516/10x) and is finally focused (f=30mm) into a gated photomultiplier tube (PMT) (H7421-40, Hamamatsu). The detected signal is amplified by a GHz wide-band amplifier (HFAC-26dB, Becker & Hickl) and finally measured by a two-channel photon counter (SR400, Stanford Research systems).

2.1.4 Characterization

Characterization of incident near infrared laser pulses

We verify the proper functioning of the AR-SHS setup by recording the spectra of the laser beam with a spectrometer (USB4000, Ocean Optics). Fig. 2.3 shows the spectrum of the fundamental beam, and the spectrum of the SH generated by the fundamental beam when passing through a β -barium borate (BBO) crystal. The spectrum of the fundamental is limited because of the cutoff of the spectrometer at 1037 nm. The wavelength of the fundamental beam is thus centered around 1032 nm.

In addition, the profile of the beam is measured at the focal point at the position of samples. The profile, shown in Fig 2.4, is inevitably slightly distorted compared to the Gaussian fit as it has been focused. The beam waist w_0 is ~ 26.9 μ m, and the corresponding Rayleigh



Figure 2.3 – Spectra of the incident laser beam before (red circles) and after (green circles) transmission through a BBO crystal. The solid lines are fits to the data with Gaussian functions. The peak wavelengths are respectively 1032.4 nm and 515.9 nm. Original data measured by A. Marchioro, graph by N. Dupertuis.

length is 2.20mm.

Finally, it was proven previously that there is no higher order harmonic generation in the samples at the conditions we use for our experiments (200kHz, 60mW, 38 μ m diameter) [59, 148]. This means that the SHS response is fully elastic and that there is no transfer of energy from the laser pulse to the sample, as well as no sample heating. For this reason, unless specified, we use a power of 60mW in our experiments.

Alignment of the setup

Liquids are usually assumed to be isotropic [43]. As discussed above, only incoherent HRS can be generated in isotropic liquids. If the optics in our setup are properly aligned and configured, one expects scattering patterns to be symmetrical around the forward scattering direction, and matching the HRS theory. In addition, the SSS intensity should be constant at every scattering angle [143, 145]. We probed a model liquid, carbon tetrachloride, with our AR-SHS setup to verify these aspects. Results are shown in figure 2.5. We exclude the data points in forward direction (at 0°), as it contains additional contributions to the SH light originating from optics in the detection arm.

The patterns in figure 2.5 are indeed fully symmetrical around 0°. The measured patterns correspond to HRS theoretical predictions and validate the setup configuration. In particular, The PPP and SSS intensities are equal at 0° angle (within the error margin). Furthermore, the SSS intensity is equal at all angle. For this reason, we record the SSS intensity pattern at the beginning of every series of measurements and verify its flatness in order to check the alignment of the setup.



Figure 2.4 – Beam profile (cross-section) of the incident laser beam at the focal point, at the place of the sample. The repetition rate is 200kHz and the power 60mW. The solid line is the gaussian fit on the data. The full width at half maximum is ~ $63\mu m$, and the beam waist w_0 is ~ $26.9\mu m$.

Normalization of SHS data of particles

For scattering patterns, we want to extract the meaningful contribution to SH intensity from the surface structures within the sample. For this reason, we substract the corresponding background signal coming from the bulk of the liquid solvent (e.g. an ionic solution) in the same polarization combination. Then, we correct for differences coming from daily variations in the focal volume of the laser beam or in measurements conditions by dividing it by the SH intensity of pure solvent in the SSS polarization combination:

$$S(\theta) = \frac{I_{\text{SHS,scatterers,...}}(\theta) - I_{\text{SHS,solvent,...}}(\theta)}{I_{\text{SHS,pure solvent,SSS}}(\theta)}$$
(2.27)

where the index \cdots indicates the same polarization combination for the sample with scatterers and the sample with only solvent. Using this normalization procedure, we can consistently compare $S(\theta)$ scattering patterns from different measurements, with an absolute scale.

Multiple scattering in liposomes

In general, the intensity generated in scattering experiment depends linearly on the number density of scattering particles. However, when this density goes over a specific threshold, multiple scattering occurs and reduces the detected intensity, altering the linear dependence [189].

In order to verify the onset of multiple scattering and other effects (such as the overlap of the hydration shells of scattering particles), we recorded AR-SHS patterns of negatively charged liposomes made of DOPS (dioleoylphosphatidylserine) phospholipids. Figure 2.6 shows the normalized intensity $S(\theta)$ integrated from -90 to +90° angle, as a function of the



Figure 2.5 – A) Hyper-Rayleigh Scattering predicted by equation (2.6) as a function of the scattering angle θ . B) Hyper Rayleigh Scattering from pure CCl₄ in PPP and SSS polarization combinations. 0° corresponds to the forward propagation direction of the fundamental beam. The patterns were recorded in steps of 5° with an angle of acceptance of 3.4°. Error bars represent the standard deviation from 20 measurements per angle. The solid lines are fits according to equation (2.6). Data measured by Y. Chen, graphs made by T. Schönfeldová.

number density of liposomes in the sample. The data for both liposome sizes have the same trend within the investigated concentration range: first, a strong linear increase, then it slows down. The linear fits of these two distinct regimes show that the onset of multiple scattering may be around a critical number density of $3.0e^{12}mL^{-1}$. In order to avoid possible biases, we correct the measured patterns when the number density is above this value, by scaling the intensities with the intensity ratio of the two linear fits of figure 2.6.

2.2 Complementary methods

2.2.1 Dynamic Light Scattering

In order to determine the hydrodynamic size distribution of particles in our samples, we use an autocorrelation spectroscopy technique called Dynamic Light Scattering (DLS). This technique is based on measurements of the temporal autocorrelation of light scattered by particles dispersed in a solution [190]. In this work, we used a Malvern Nanosizer ZS (Malvern Ltd., UK). The samples are illuminated by a laser at 632.8 nm wavelength, and a photodiode placed at an angle of 175° records the intensity I(t) of nearly backscattered light. The intensity autocorrelation *G* as a function of time *t* and time delay τ is computed from the intensity as:

$$G(\tau) = \int_{-\infty}^{\infty} I(t)I(t+\tau)dt$$
(2.28)

The scattered light is analysed with respect to the random Brownian motion of particles [191]. In that case, *G* can be expressed as a function of the correlation function *g* as:

$$G(\tau) = A[1 + Bg(\tau)^2]$$
(2.29)

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Figure 2.6 – Integrated SH intensity of DOPS liposomes extruded through pores of 30 and 200 nm diameter, and diluted to different number densities. The recorded AR-SHS patterns were normalized as detailed in equation (2.27), and integrated from -90 to +90°. The data for 30 nm pore were multiplied by a scale factor of 4. The two lines are linear fit of the data points at number densities lower than $2.66e^{12}$ mL⁻¹ and higher $2.99e^{12}$ mL⁻¹, respectively.

where A represents the baseline of *G* and B the intercept of *g*. For monodisperse solutions, *g* decays exponentially as $g(\tau) = e^{-Dq^2\tau}$, with *D* as the translational diffusion coefficient and $q = (4\pi n/\lambda)\sin\theta/2$ as the scattering wavevector. *q* depends on the refractive index *n* of the solution, on the wavelength λ , and on the scattering angle θ .

For perfectly spherical particles, the relation between the hydrodynamic radius R_H and the translational diffusion coefficient *D* is given by the Stokes-Einstein equation [5, 187]:

$$R_H = \frac{k_B T}{6\pi\eta D} \tag{2.30}$$

where k_B is the Boltzmann constant, *T* the temperature, η the viscosity of the solution. The intensity autocorrelation decay is fit with the cumulant-fit method, using a single exponential function. This allows to get a mean particle radius distribution, and to extract the poydispersity index (PDI) which is a metric of the variance of the distribution. The lower the PDI value, the narrower is the size distribution. As scattering is very sensitive to the size of particles, acceptable PDI values for our experiments are below 0.3 [192]. A PDI value around 0.1 or lower is characteristic of a very narrow size distribution, ideal for scattering experiments.

2.2.2 Size normalization and correction for the difference in size distribution

Our samples are produced in different batches and thus have different particle size distributions. As scattering is very sensitive to the size of particles, we need to correct this in order to get comparable values of intensities scattered by the particles (either nanodroplets or liposomes). For a monodisperse (with a low enough PDI) solution N_p particles that are smaller than 250 nm in radius and that each scatter an intensity $I(\theta, R)$, the total scattered signal $S(\theta, R)$ scales as $S(\theta, R) = N_p I(\theta, R) \propto \alpha(\theta) N_p R^6$ [127]. The scattering efficiency $\alpha(\theta)$ contains all the information that we seek about the SHS response per particle, independent of its size. Thus, in order to compare the water response per particle, we need to compute the following:

$$\alpha(\theta) = \frac{S(\theta, R)}{N_p R^6} = I_{\text{norm}}(\theta)$$
(2.31)

For our SHS experiments, we further take into account the polydispersity of our samples by computing an effective radius, as described in the next section.

2.2.3 Computation of an effective radius

Since the intensity scales as R^6 , it varies dramatically with the different sizes of particles within a sample. The overall intensity is the sum of the individual contributions $S(\theta, R_i) = \sum_i I_i(\theta, R_i)$ where the subscript *i* refers to individual particles. For this reason, we cannot simply normalize the SHS intensity by $\langle R_i \rangle^6$ in order to get only the size-independent surface scattering contribution. To still achieve our normalization, we want to replace the total intensity $S(\theta, R_i)$ of the polydisperse solution by an an ideal intensity $I_{norm}(\theta, R_{eff})$ scattered by a theoretically monodisperse solution of unique radius R_{eff} . This procedure that we describe now is detailed in the supplementary material of Ref. [130]. Because all particles contribute in the same way to the overall intensity of any light scattering experiment, we can use the size distribution obtained from the DLS measurements to compute an effective radius R_{eff} to replace $\langle R_i \rangle$, as follows:

$$\alpha(\theta) = \frac{\sum_{i} I_i(\theta, R_i)}{N_p R^6} = \frac{S(\theta, R)}{N_p R_{\text{eff}}^6} = I_{\text{norm}}(\theta)$$
(2.32)

The size distribution histogram obtained from DLS measurement is a normalized distribution D(R). In the Rayleigh Gans Debye limit (applicable here [193]), the intensity recorded in the DLS experiment also scales with R^6 . D(R) is thus normalized as:

$$D(R) = \frac{P(R)R^6}{\int P(R)R^6 dR}$$
(2.33)

where the particle size distribution P(R) is a probability distribution normalized such that $\int P(R) dR = 1$. It can be obtained simply from D(R) by calculating:

$$P(R) = \frac{\frac{D(R)}{R^{6}}}{\int \frac{D(R)}{R^{6}} dR}$$
(2.34)

Knowing P(R), we can now compute the effective radius for the particles in our samples. For liposomes, we have to take into account the two lipid leaflets separated by a bilayer thickness

d, typically around 4 nm in the litterature [194]. The effective radius is then:

$$R_{\rm eff,lip} = \left[\frac{\int P(R)R^6 dR}{\int \frac{1}{2}P(R)(R^2 + (R-d)^2)dR}\right]^{1/4}$$
(2.35)

The denominator in equation (2.35) takes into account the fact that when the radius of the liposomes change, the number of lipids per liposomes changes also.

For droplets, the effective radius is:

$$R_{\rm eff,drop} = \left[\frac{\int P(R)R^6 dR}{\int P(R)R^3 dR}\right]^{1/3}$$
(2.36)

In this case, the denominator corresponds to a full sphere rather than a hollow shell, as there is only a monolayer at the surface of the oil.

2.2.4 Measurement of the Zeta potential

The zeta potential is a physical property of particles in suspension in a solvent. Its value is in particular indicative of the stability of the suspensions [187, 195]. The liquid medium around a charged spherical particle in suspension is usually described in two parts (see figure 2.7): a first inner layer of ions and water molecules (the Stern layer) is tightly bound to the charged surface of the particle. Further from the particle is a second region in the solution made of ions and water molecules more weakly impacted by the electrostatic potential originating from the particle. This region is the so called electric double layer (EDL) [196], where the potential fades as going away from the particle surface. A theoretical boundary is defined there, within which ions and water molecules are sufficiently bound to remain together with the particle when it moves. This boundary is called the slip plane, and the potential measured there is called the ζ potential. Of course, this kind of definition is heavily dependent on the dispersant medium.

According to the DLVO theory [11, 187], the stability of suspensions depends on the balance between attractive van der Waals forces, and repulsive forces from the electrical double layer around the particles in suspension. Particles with strong enough repulsion will not aggregate and flocculate. In particular, if the particles are charged enough, the suspension will be stable. This charge can originate from the ionization of the surface groups, the adsorption of charged species, or from the intrinsic properties of the particle material. The magnitude of the ζ potential gives an indication of the stability of the emulsions and on the electrostatic properties of the particle. A ζ potential greater than 25-30mV means that the particles will tend to repel each other and that the suspension will be stable.

In our case, we use the Malvern Nanosizer ZS (Malvern Ltd., UK) instrument to measure the ζ potential via electrophoretic mobility measurements. An electric field **E** is applied across the sample, and the particles in suspension move towards the electrode with a charge opposite to their surface charge. The equilibrium velocity **v** of these particles is proportional to the electric field as follows: $\mathbf{v} = \mu_e \mathbf{E}$, where μ_e is the electrophoretic mobility. Here, the velocity of the particle in the electric field is measured through a combination of Doppler velocimetry and light scattering analysis, giving the electrophoretic mobility. Then, the mobility value is converted into potential value via the Henry equation:

$$\mu_e = \frac{\varepsilon_0 \varepsilon}{\eta} \zeta f(\kappa a) \tag{2.37}$$

where η is the dynamic viscosity of the solvent, ε_0 and ε the vacuum permittivity and the relative permittivity of the solvent, f is the Henry's function with arguments κ and a. a is the radius of the droplet, κ is the inverse Debye length ($\kappa^{-1} = \sqrt{\varepsilon_0 \varepsilon k_B T / 2 \cdot 10^3 N_A e^2 I}$, where k_B is the Boltzmann constant, T is the temperature, N_A is Avogadro's number, e is the elementary charge, and I is ionic strength). In the instrument's software, this equation is by default coupled with the well-known Smoluchowski approximation: $f(\kappa a) \rightarrow 1$, being accurate when $\kappa a \rightarrow \infty$. However this is not always valid for our samples. We use instead the approximation proposed by Ohshima [198] that works for values of κa below 1:

$$f(\kappa a) = \frac{2}{3} \left[1 + \frac{1}{2 \left\{ 1 + \frac{2.5}{\kappa a (1+2e^{-\kappa a})} \right\}^3} \right]$$
(2.38)

2.3 Sample preparation and characterization

2.3.1 Glassware cleaning

Glassware for liposomes and nanodroplets preparation is cleaned with a Deconex® (Borer Chemie AG) solution prepared by 1:20 dilution with ultrapure water (Milli Q, Millipore, Inc., electrical resistance of 18.2 M Ω cm), then rinsed thoroughly with ultrapure water at least 15 times. Glassware for the phosphate assay requires a two-step cleaning procedure: first a cleaning with a 3:1 H₂SO₄:H₂O₂ ("piranha") solution is done, which is followed by a cleaning with a 3:1 NH₄OH:H₂O₂ solution at 100 °C, each for 10 minutes. After and in between the cleaning steps the glassware is thoroughly rinsed with ultrapure water.

2.3.2 Liposomes

Small unilamellar vesicles (liposomes) are prepared by extrusion according to Ref. [199–201]. To create multilamellar vesicles, typically 10 mg of lipid powder is dissolved in chloroform in a glass vial. Subsequently, the chloroform is evaporated with a nitrogen gas stream while rotating the vial. The resulting lipid film is further dried in vacuum (<100 mbar, created by an oil-free diaphragm pump) at room temperature for at least 2 hours. Finally, the lipid film is re-suspended in D₂O and vortexed. To create unilamellar vesicles, the resulting multilamellar vesicle solutions are extruded with a Miniextruder (AvantiPolarLipids, Al) using a polycarbonate membrane (AvantiPolarLipids, Al) with a specific pore diameter (200 nm if not specified)

otherwise) at room temperature. Unilamellar vesicles are stored in closed containers up to 4 weeks at 4 °C. The size and ζ -potential distribution of the liposomes are also measured with DLS and electrophoretic measurements at 23 °C (Malvern ZS nanosizer). To determine the size distribution of the vesicles, three subsequent measurements, each 11 runs, are averaged. To determine the ζ -potential of the vesicles, three subsequent measurements, each 30 runs at automated voltage, are averaged. The liposomes are found to have a mean diameter in the range of 58-145 nm with a polydispersity index (PDI) of less than 0.2. The final lipid concentration is determined using a calorimetric phosphorus assay as described in subsection 2.3.4. The concentration of the lipids in the sample is 0.5 mg lipids/mL weight ratio for DLS, ζ -potential measurements, and SHS experiments.

2.3.3 Oil nanodroplets

Oil nanodroplets with a lipid monolayer are prepared according to the procedure described in Ref. [115], with a 2 % d₃₄-hexadecane/D₂O volume ratio. We mix the solutions with lipid powders to reach a lipid concentration of 1 mM at a temperature of at least 5 °C above the transition temperature of the phospholipid by using a hand-held homogenizer (TH, OMNI International) for 4 min and an ultrasonic bath (35 kHz, 400 W, Bandelin) for the same duration. The resultant droplet system is used for SFS measurements and is diluted to 0.1 % hexadecane/D2O volume ratio for SHS measurements. The size distribution of the nanodroplets is measured with dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern). The nanodroplets have a mean hydrodynamic radius in the range of 170-200 nm with a polydispersity index (PDI) of less than 0.25. The hydrodynamic radii are calculated from the intensity autocorrelation function using the optical properties of the liquids (d₃₄-hexadecane and D₂O). The samples are stored and measured in sealed cuvettes. All measurements are performed at 24 °C. The whole procedure from sample preparation to actual measurement takes place in less than two days.

2.3.4 Phosphate assay - determination of the total phosphorus content

The lipid concentration in our liposome samples may be different from the intended one due to the following errors: 1) systematic weighting error; 2) loss of lipids when handling with spatulae and transferring the sample in various glassware; 3) loss of lipids during the extrusion through the polycarbonate filter membrane. Therefore, we realize a phosphate assay after the measurements to quantify the real concentration of lipids in our liposome stock samples [202]. It is a colorimetric assay based on the reaction of the phosphate ions (coming from the phospholipid headgroups) with ammonium molybdate that forms an phospho-molybdate complex, fluorescent in the blue. By comparing the light absorbance of the samples with reference solutions using a UV-VIS spectrometer, we can determine the amounts of phosphate in each sample, hence the real concentration of lipids. The complete protocol can be found in Ref. [203]. This method, however, can only be used for phospholipids having a phosphate moiety. Hence we apply it for DOPC, DOPS, and DOPA liposome samples.



Figure 2.7 – Schematic representation of the different potentials defined around a particle in an aqueous solution. Inspired from Ref. [197].

3 Temperature dependence of waterwater and ion-water correlations in bulk water and electrolyte solutions

The temperature dependence of the femtosecond elastic second harmonic scattering (fs-ESHS) response of bulk light and heavy water and their electrolyte solutions is presented. We observe clear temperature dependent changes in the hydrogen (H)-bond network of water that show a decrease in the orientational order of water with increasing temperature. Although D_2O has a more structured H-bond network (giving rise to more fs-ESHS intensity), the relative temperature dependence is larger in H_2O . The changes are interpreted in terms of the symmetry of H-bonds and are indicators of nuclear quantum effects. Increasing the temperature in electrolyte solutions decreases the influence of the total electrostatic field from ions on the water-water correlations, as expected from Debye-Hückel theory, since the Debye length becomes longer. The effects are, however, 1.9 times (6.3 times) larger than those predicted for H_2O (D_2O). Since fs-ESHS responses can be computed from known molecular coordinates, our observations provide a unique opportunity to refine quantum mechanical models of water.

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3.1 Introduction

Water in its liquid form is the medium of life. In organisms, the structural building blocks of cells (lipid membranes, proteins, DNA, etc.) are embedded in electrolyte solutions. The structure of water and the hydration of ions have continuously been of high interest as they are involved in numerous physiological, medical, biological, and chemical processes [13]. Examples are protein folding/precipitation, enzyme, ion channel, and ion pump activity, action and membrane potential generation, transport across membranes, self-assembly, interfacial charging, and aerosol formation [13, 46-48, 50, 204]. Most studies of ion-water interactions have focused on concentrations above 0.1 M, and from these studies it has emerged that ion specific water-ion interactions occur over up to 3 hydration shells [91, 149, 205-213]. Recently, femtosecond elastic second harmonic scattering (fs-ESHS) experiments, in combination with reflection second harmonic generation and surface tension measurements [59], have shown that there is also an interaction that ranges over tens of hydrations shells and displays significant nuclear quantum effects [22, 214-220]. This effect involves the interaction of the combined electrostatic field of all the ions with the hydrogen (H)-bond network in solution [22, 59, 152]. The orientational correlations or orientational order between the water molecules increases in response to an ionic electrostatic field, leading to an increase in the fs-ESHS response (as in Fig. 3.1), a decrease in the surface tension and an increase in the dielectric energy of the bulk medium [153]. Although the qualitative ingredients of this behavior are understood, there are still many open questions. In the present chapter, we provide a further experimental investigation into the temperature dependence of the fs-ESHS response of bulk light and heavy water and their electrolyte solutions.

With fs-ESHS, we probe the second harmonic (SH) response of liquid molecules. The measured SH intensity originates from electronic anisotropy within every single non-spherical molecule and is modified by orientational correlations of the non-spherical molecules. Randomly distributed liquid molecules as in an ideal gas are uncorrelated with each other; SHS from these uncorrelated molecules is an incoherent sum of the SH response of individual molecules, which is also referred to as hyper-Rayleigh scattering (HRS). Molecular correlations can be induced by H-bonding, Coulombic interaction, dipole-dipole interaction, or other interactions. For water, the orientational correlation of a pair of water molecules can be changed by breaking an H-bond through a rotational or bending / libration motion, but not by stretching (see the inset in Fig. 3.1). The orientational correlation modifies the coherent contribution of the correlated molecules to the SH intensity. Note that femtosecond laser pulses that are shorter than the orientational relaxation time of water are necessary to measure snapshots of the static collective orientational correlations in water. As shown in Ref. [59], information on the two origins of SH responses can be obtained from the SH intensity measured in different polarization combinations of the incident and scattered light. The polarization combinations are indicated with a three letters code, from left to right, representing the polarization of the scattered SH light and the incident fundamental light with P (S) referring to linearly polarized light parallel (perpendicular) to the scattering plane. The PPP and PSS combinations mainly report on the collective orientational order and the SH intensity consists of both coherent contributions from correlated molecules and incoherent contributions from uncorrelated molecules. The SSS and SPP combinations report on the electronic structure of individual molecules and the SH intensity is dominated by the incoherent contributions from uncorrelated molecules. According to the selection rule of SHS, molecular correlations of spherical symmetry only contribute to the PPP and PSS SH intensities [221].

Figure 3.1A shows the relative change of the PPP fs-ESHS intensity (at scattering angle $\theta = 90^{\circ}$) for 21 different electrolyte solutions in the ionic strength range of 1 μ M – 0.1 M, as reproduced from Ref. [59]. The SH intensity was recorded at room temperature (296.15 K, 23 °C). The 21 electrolytes include a monovalent Cl⁻ cation series (NH₄Cl, KCl, NaCl, LiCl, and HCl), an Na⁺ anion series (NaH₂PO₄, NaOH, NaCl, NaBr, NaI, NaNO₃, NaClO₄, and NaSCN), divalent cationic-monovalent anionic pairs (MgCl₂, CaCl₂, SrCl₂, and BaCl₂), monovalent cationic-divalent anionic pairs (Na₂S₂O₃ and Na₂SO₄), and divalent cationic-anionic pairs (BeSO₄, MgSO₄, and CaSO₄). For all electrolyte solutions, the same trend in the SH intensity is observed: A continuous rise up to a plateau with a saturated increase by $30 \pm 3\%$. The 50% half-saturation concentration (c^*) is at $55 \pm 5\mu$ M. This non-ion-specific, saturated increase in the PPP fs-ESHS intensity indicates that electrolytes induce orientational order in the Hbond network of bulk water that extends over tens of hydration shells. Figure 3.1B shows a comparison of the PPP fs-ESHS intensity of NaCl dissolved in H₂O and NaCl, KCl, DCl and NaOD dissolved in D₂O. A similar trend in the SH intensity is observed for the D₂O solution; but the intensity changes up to the plateau is only $9 \pm 3\%$ with $c^* = 310 \pm 71 \mu$ M. Additional electrolyte solutions in D₂O display the same behavior as NaCl solutions (Fig. 3.1B). Provided that the differences in the linear dielectric properties of H_2O and D_2O are minimal, such as permanent dipole moment and dielectric constant, this significant difference in the SH response is a manifestation of stronger H-bonding in D_2O than in H_2O , which arises from nuclear quantum effects [12, 22, 214-216, 219, 220, 222-227]. It also indicates the importance of collective H-bonding [59]. Since these studies clearly show the importance of H-bonding, and since temperature can modify the probability that H-bonds are formed and broken [61], it is of interest to investigate the above behavior for different temperatures.

Here we investigate the temperature dependence of the fs-ESHS response of bulk H_2O and D_2O as well as NaCl solutions in light and heavy water. We find that bulk light and heavy water both display a reduction in their intermolecular coherent responses as the temperature is increased from 263.35 K (-9.8 °C) to 321.45 K (48.3 °C). However, both liquids have distinct temperature behaviors with a larger intermolecular coherent contribution in D_2O arising from stronger spherically symmetric orientational correlations in the H-bond network. The relative temperature dependent change is bigger for H_2O than for D_2O . For electrolyte solutions the intensity vs temperature curves, such as recorded in Fig. 3.1B, shift to higher ionic strengths due to a decreased screening of the electrostatic field at higher temperatures as explained by Debye-Hückel theory. However, the half saturation concentrations change more than the model prediction, with D_2O deviating a factor of 6.3 and H_2O a factor of 1.9. These measurements display the complexity of liquid water and provide a challenge for future

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Figure 3.1 – Fs-ESHS of electrolyte solutions at room temperature. (A) fs-ESHS intensities, relative to that of pure water, of 21 different electrolyte solutions obtained at a scattering angle of 90° (PPP polarization combination). The relative intensities of all electrolyte solutions can be fit with a Langmuir equation whose functional form is given by: $\Delta I = A \frac{(c/c^*)}{1+(c/c^*)}$, with *c* the ion concentration and *A* a fitting parameter. The dashed line indicates the concentration of half saturation, *c**, which is extracted from the fitting. The inset illustrates two H-bonded water molecules that are orientationally correlated. The black arrows represent different axes along which H-bonds can be broken. fs-ESHS is mostly sensitive to the breaking of this H-bond via rotation (black curved arrow). In D₂O molecule, H-bond bending mode is predicted to be stronger than in H₂O, due to nuclear quantum effects. Oppositely, the H-bond stretching mode is expected to be weaker. (B) fs-ESHS intensities of NaCl, KCl, H(D)Cl, and NaOH(D) in H₂O and D₂O (PPP polarization combination). The H₂O (D₂O) data is normalized to pure H₂O (D₂O). All the fs-ESHS data of the figure are adapted from Ref. [59] and were measured by Y. Chen.

models of water and electrolyte solutions.

3.2 Materials and Methods

3.2.1 Sample preparations

NaCl (99.999%, Sigma-Aldrich), KCl (99.999%, Acros), NaOH (99.99%, Sigma-Aldrich), HCl (Ultrapur, Merck), NaOD solution (40 wt. % in D₂O, 99.5 at. % D, Sigma-Aldrich), and DCl solution (35 wt. % in D₂O, \geq 99 atom % D, Sigma-Aldrich) were used as received without further purification. All samples were made by dissolving the electrolytes in degassed ultrapure water to obtain a stock solution with a high concentration. For purifying H₂O, we used a Milli-Q UF plus instrument, (Millipore, Inc.) with an electrical resistance of 18.2 MΩcm. For the experiments with heavy water, we used degassed D₂O from Armar (99.8% D, > 2 MΩcm). The stock solutions were filtered (0.1 μ m polyvinylidene fluoride membrane filters, Millex-VV, Millipore) and diluted to the desired concentration.

3.2.2 Femtosecond elastic second harmonic scattering

The SHS setup was used as described in subsection 2.1.3. The detection angle was set to 90° with an acceptance angle of 11.4°. For every data point, 3 to 11 measurements were performed and the averaged results are shown. In every measurement, the SH intensity was acquired with 50×1 s acquisition time (i.e. using $50 \times 2 \cdot 10^5$ pulses in total) and a gate width of 10 ns. The SH intensity of pure H₂O or D₂O (Figs. 1 and 3) was measured between every two samples and was used as a reference. The reproducibility of the fs-ESHS measurements is 1–3 %. Samples were stored and measured in sealed glass sample cells. The obtained relative intensities as a function of ionic strength were fit with a Langmuir-type equation derived from Debye-Hückel theory, whose functional form is given by: $\Delta I = A \frac{(c/c^*)}{1+(c/c^*)}$, with *c* the ion concentration and *A* a fitting parameter. The 50% onset concentration c^* was extracted from the fitting.

3.3 Results and Discussions

3.3.1 Temperature dependence of molecular correlations in water

Figure 3.2A shows the fs-ESHS intensities of pure H_2O and D_2O as a function of the temperature, measured in the PPP polarization combination. The plotted value is the intensity normalized by that at the highest temperature (T = 321.45 K [48.3 °C]). Increasing the temperature from 263.35 K (-9.8 °C) to 321.45 K (48.3 °C), the PPP SH intensity drops by ~ 15% for H_2O and by ~ 10% for D_2O . The inset shows the measured intensities on the same scale. This shows that increasing the temperature of D_2O does not generate the same response as H_2O . In order to understand the behavior of Fig. 3.1A and to possibly assign it to changes in the orientational correlations in the H-bond network, we need to consider the sources of intensity change.

The SH intensity has two contributions: an intermolecular coherent and a molecular incoherent one. The temperature dependence of the incoherent response arises from temperature dependence of the molecular hyperpolarizability, and the dielectric constant that is connected to the local field factors. Thus, with increasing temperature we may expect that the dielectric constant, the second-order hyperpolarizability or the square of the second-order hyperpolarizability decreases or that the orientational correlations in the liquid become less probable. The dielectric constants of H_2O and D_2O differ by 0.5% at different temperatures [228], and the hyperpolarizability is a reflection of the electronic structure of individual molecules and has been reported to be almost the same [151]. Therefore, the difference in Fig. 3.1A stems from different degrees of intermolecular correlations between H_2O and D_2O . D_2O has more intermolecular correlations (or correlations that live longer), but changes less with temperature. This is consistent with the notion that D_2O has stronger H-bonds than H_2O .

In order to separate the molecular incoherent and intermolecular coherent contributions, we compare the response for PPP polarized light (that contains coherent and incoherent contributions for spherically symmetric systems) to the response for SPP polarized light (which

В A 2000 1.50 1.20 (arb.u.) 1800 (T) / I(T=321.45 K) 1300 1.15 1.45 1100 1.40 1.10 280 300 32 Temperature (K) 1.35 1.05 H₂O + H₂O D₂O 1.00 + D₂O 1.30 260 270 280 290 300 310 320 260 270 280 290 300 310 320 Temperature (K) Temperature (K)

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Figure 3.2 – Temperature dependent fs-ESHS of bulk light and heavy water. (A) fs-ESHS intensities of H_2O and D_2O as a function of temperature measured at a scattering angle of 90° (PPP polarization combination). The SH intensity is normalized by the intensity at 321.45 K (48.3 °C). The solid lines are guides to the eye. The inset displays the intensities of both liquids on the same intensity scale. (B) The ratio of the PPP intensity to the SPP intensity

contains only incoherent contributions). The incoherent contribution to the SH PPP and SPP intensity can be computed considering random uncorrelated point dipoles [143, 145], as described in equations (2.7) and (2.8). For a molecular distribution with spherical symmetry the SPP polarization combination contains only molecular contributions. PPP polarized light generally contains both molecular as well as intermolecular contributions. Since at the used scattering angle ($\theta = 90^\circ$) Eq. (2.7) and (2.8) are identical for both polarization combinations, dividing these at $\theta = 90^\circ$ will thus remove the molecular incoherent contribution. The caveat here is that this works only for nonlinear optical scattering theory using spherical symmetry. This spherical symmetry refers to the spatial orientational distribution of H-bonded water molecules. A water molecule with 2 accepting and 2 donating H-bonds is spherically symmetric. A water molecule with a single accepting H-bond is not spherically symmetric just like other H-bond-missing configurations. To go beyond this approximation requires extensive path integral molecular dynamics [151].

Figure 3.2B shows the ratio of the measured PPP and SPP SH intensity at different temperatures. The temperature dependence of this intensity ratio is remarkably different compared to the PPP intensity shown in Fig. 3.2A. Instead of continuously decreasing with increasing temperature, the intensity ratio of both liquids shows an increase and reaches saturation values at ~283.15 K (15 °C) for D₂O and ~293.15 K (20 °C) for H₂O, while H₂O shows a larger increase over the studied temperature range than D₂O. The intensity ratio of D₂O is larger than that of H₂O at all temperatures.

This different temperature dependence of the intensity ratio indicates a more complicated picture of the bulk water structure evolving with temperature than a mere change in the H-bond number. Breaking down the SH intensities, the SPP intensity consists primarily of an incoherent contribution from uncorrelated water molecules. We note that it could also contain a coherent contribution from orientationally correlated molecules that are distributed non-spherically as remarked above (as this is an approximation in the nonlinear optical theory [221]). In contrast, the PPP intensity contains a coherent contribution from spherically correlated molecules (and of non-spherically correlated molecules if the spatial distribution is non-spherical). We thus have two possible ratios ($R = I_{PPP}/I_{SPP}$) under the measurement condition:

$$R = \frac{I_{PPP, \text{ incoh}} + I_{PPP, \text{ coh, sph}}}{I_{SPP, \text{ incoh}}} = 1 + \frac{I_{PPP, \text{ coh, sph}}}{I_{SPP, \text{ incoh}}} \text{ for spherical symmetry,}$$
(3.1)

$$R = \frac{I_{PPP, \text{ incoh}} + I_{PPP, \text{ coh, nonsph}} + I_{PPP, \text{ coh, sph}}}{I_{SPP, \text{ incoh}} + I_{SPP, \text{ coh, nonsph}}} \text{ for any type of spatial arrangement} (3.2)$$

where _{incoh} in the subscripts denotes an incoherent contribution, _{coh}, _{nonsph} and _{coh}, _{sph} denote coherent contributions from spherically correlated molecules and from non-spherically correlated molecules, respectively. The excess amount of PPP SH intensity over SPP SH intensity (Fig. 3.2B) can then be attributed to two possible factors. From Eq. (3.1) we derive that the intermolecular orientational correlations should increase with increasing temperature. This, however, is counter-intuitive because there are less H-bonds and intermolecular correlations with increasing temperature (Fig. 3.2A). Then, using Eq. (3.1) an increase in the ratio *R* with increasing temperature can arise from a reduction in the amount of non-spherical orientational correlations combined with a relative increase in the amount of spherical orientational correlations. This makes sense because the H-bond network of water loses structure when temperature is increased [56, 60, 229, 230]. The larger ratio *R* of D₂O thus indicates relatively more spherical intermolecular correlations than H₂O.

The differences between D₂O and H₂O in Fig. 3.2, with the incoherent contributions removed, are manifestations of nuclear quantum effects. With H having a smaller mass than D the nuclear quantum effect in H₂O is stronger than in D₂O. Nuclear quantum effects weaken the bending / libration mode of the H-bond and lead to less orientational order in H₂O compared to D₂O [12, 22, 216, 219, 231]. At 321.15 K (48.3 °C), the measured PPP SH intensity of D₂O is ~ 61% larger than that of H₂O. At 263.45 K (-9.8 °C), the measured PPP SH intensity of D₂O is ~ 53% larger than that of H₂O. Recent X-ray Raman scattering [231], X-ray absorption spectroscopy [61, 232] and X-ray emission spectroscopy [233, 234] studies of the molecular structures of liquid H₂O and D₂O show that there is enhanced asymmetry in the asymmetric H-bonds for H₂O compared to D₂O due to nuclear quantum effects. These findings agree with this study.

3.3.2 Temperature dependence of ion-water interactions

In a second set of experiments, we measured the temperature dependence of the water-water orientational correlations induced by ions in NaCl solution in H_2O and D_2O . Figure 3.3A and



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Figure 3.3 – Fs-ESHS temperature dependence of NaCl solutions in light and heavy water. (A) Normalized SH Intensity (I_{salt}/I_{water}) of H₂O as a function of ionic strength at (277.15 K, 4 °C) and (321.15 K, 87 °C) measured at a scattering angle of 90° (PPP polarization combination). (B) Normalized SH Intensity (I_{salt}/I_{water}) of D₂O as a function of ionic strength at (284.15 K, 11 °C) and (321.15 K, 87 °C) measured at a scattering angle of 90° (PPP polarization combination). The solid lines are fits to the Debye-Hückel model. All the data of the figure were measured by N. Dupertuis and Y. Chen, and the graphs were made by H. I. Okur.

3.3B show the normalized fs-ESHS intensity as a function of ionic strength in H_2O (3.3A) and D_2O (3.3B), comparing the effects of Na⁺ and Cl⁻ ions at the temperatures of maximum density for H₂O (277.15 K, 4 °C) and D₂O (284.15 K, 11 °C) to a fixed high temperature (360.15 K, 87 °C). As can be seen there is a significant temperature effect in both solvents: Increasing the solution temperature shifts the increase in the SH intensity to higher ionic strength. The half-saturation points (c^*) in these curves change from $c^*(4^\circ\text{C}) = 44 \pm 5\mu\text{M} \rightarrow c^*(87^\circ\text{C}) = 69 \pm 5\mu\text{M} (\Delta c^* = 25 \pm 5\mu\text{M})$ 7 μ M) for H₂O, and $c^*(4^\circ$ C) = 300 ± 51 μ M $\rightarrow c^*(87^\circ$ C) = 805 ± 130 μ M ($\Delta c^* = 505 \pm 140 \mu$ M) for D₂O. Note that the c^* values at room temperature are at $55 \pm 5\mu$ M and $310 \pm 71\mu$ M. The shift in the SH intensity curves indicate a weakened influence of the electrostatic fields of the ions on the water structure induced by increasing the temperature. The temperature dependent change in concentration can be qualitatively explained by the Debye-Hückel model [59]. With increasing temperature, the Debye length $(\kappa_D^{-1} = \left(\frac{\varepsilon_0 \varepsilon kT}{2N_A I 10^3 e^2}\right)^{1/2}$, where κ is the Boltzmann constant, T the temperature in the unit K, N_A the Avogadro number, I the ionic strength) increases, leading to a reduction of the influence of ions on the orientational correlations in the water. Additionally the ion induced dipole-dipole correlations have a temperature dependence of the form [152]: $C(r) \sim (1/T^2) e^{(-\kappa_D r)/r}$. Here, an increase of temperature results in a reduction of the dipole-dipole correlations. Together, these effects result in [59]: $c^* = \frac{q^2 \varepsilon_0 \varepsilon k_B T}{(2(Ze)^2)}$ where q is the magnitude of the scattering vector and Z the valence of the considered ion.

As such, a shifted intensity curve is expected. However, the model predicts $\Delta c^* = -13 \mu M$ and $\Delta c^* = -80 \mu M$ shifts in the ionic strength for H₂O and D₂O, respectively using the measured values for c^* at the maximum density temperatures as input values. This qualitative

agreement shows that the Debye-Hückel model can partially capture the temperature dependence of the ions' influence on the water structure. Although the direction of the shift in c^* is in the correct direction, for both H₂O and D₂O, the degree of change is significantly different. The screening capacity of the liquid is larger than predicted for both liquids and relatively more for D₂O than for H₂O. This is surprising and somewhat counter intuitive as one might expect that with higher temperatures H-bonds become weaker and so the influence of the electrostatic field on the orientational correlations would be reduced. That we observe the opposite suggests that there are more complicated interactions than the ones considered here. Indeed, the Debye-Hückel theory is not sophisticated enough to achieve a quantitative agreement. The underlying reason for that is that the model neglects any form of H-bonds. Also, nuclear quantum effects are not captured by the Debye-Hückel theory [59]. As recent computational studies show the importance of instantaneous fluctuations in the local H-bond structure and since minute millidegree changes in the orientational order of water can already significantly impact the SH intensity [59], these measurements provide a future opportunity for water models that should include both quantum effects, as well as detailed non-local structures [53].

3.4 Conclusions

In summary, using fs-ESHS we observed clear temperature dependent changes in the molecular structure of the water H-bond network. The ensemble orientational order of water molecules decreases as the temperature increases. H₂O and D₂O show differences in their temperature dependence of the molecular structure of the H-bond network. With stronger H-bonding, D₂O has a more structured H-bond network in which molecular correlations exhibit relatively more spherical symmetry compared to H₂O. An increase in the temperature leads to a transformation of molecular correlations in pure water from correlations with non-spherical symmetry (e.g. single donor species) towards correlations with spherical symmetry (e.g. double donor species). For electrolyte solutions, we measured temperature dependent SH intensity versus concentration curves that show a decrease in the influence of the combined electrostatic field in the solution on the water-water correlations. The trend qualitatively follows the prediction from Debye-Hückel theory, but the effects are larger than predicted: $\Delta c^* = 25 \mu M$ for H₂O and $\Delta c^* = 505 \mu M$ for D₂O was measured while Debye-Hückel theory predicts $\Delta c^* = 13 \mu M$ for H₂O and $\Delta c^* = 80 \mu M$ for D₂O. Since the SH response can be computed from molecular dynamics simulations, the present observations provide a unique opportunity to refine quantum mechanical models of water.

4 Dendritic voids and charge gradients around them cause nanoscale inhomogeneities in liquid water

Liquid water is generally considered as a uniform liquid [235, 236]. However, recent experiments have reported evidence that water is instead a two-state liquid [9, 237]. Subsequently, these findings have been contested by other studies [238–240]. Thus, the structure of water and whether we should think of it as uniform or not remains an open question. Here, we report femtosecond elastic second harmonic scattering (fs-ESHS) measurements [59] of liquid water in comparison to an isotropic liquid (CCl₄) and show experimentally that water is indeed a non-uniform liquid. We interpret the coherent SH emission of water with the aid of molecular dynamics simulations as arising from charge density fluctuations around transient voids. The charge gradients around the cavities originate from topological defects in the hydrogen bond network of liquid water. Although the voids are also present in CCl₄, they are not characterized by hydrogen bond defects and do not show strong polarizability fluctuations, leading to an fs-ESHS emission of an isotropic liquid. The voids increase in number with higher temperatures in agreement with the fs-ESHS data.

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4.1 Introduction

Water is an enigmatic liquid. The structure of liquid water is most commonly represented as isotropic and uniform. Recent small-angle X-ray photoelectron spectroscopy (XPS) measurements by Nilsson and co-workers have suggested that this may be an over- simplification [9, 232, 234]. Based on fluctuations in the measured structure factor in combination with an analysis based on molecular dynamics (MD) simulations [233, 238, 241–246] water is proposed to be a 2-state liquid, composed of high-density locally disordered and low-density locally tetrahedrally ordered patches. A recent theoretical study by the Goddard group refers to liquid water as a 'dynamic polydisperse polymer' consisting of water molecules with only two intact hydrogen bonds [247]. Molecular dynamics simulation studied by others, however, severely contest these interpretations and insist that water is a homogeneous liquid [238–240]. To solve this discrepancy there is a need for alternative experimental techniques that can probe the water structure over nanometric length scales and on time scales comparable to the restructuring time of the hydrogen bond network of water. However, although there are many spectroscopic techniques to probe water structure [36, 38, 40, 41, 248, 249] nearly all methods are sensitive to local structure (sub-nanometric length scale) and spatiotemporal structural averaging, severely limiting our understanding of the nanometric length and femtosecond timescales on which water structures and transforms [15].

Recently, high-throughput femtosecond elastic second harmonic scattering (fs-ESHS, experimental details given in section 2.1.3) has been invented [45, 148]. Illustrated in Figure 4.1A, the interaction of near-infrared femtosecond laser pulse with a liquid can produce second harmonic (SH) photons that report on specific nanoscale structuring of a liquid. Simple aqueous electrolyte solutions were shown to both incoherently and coherently emit SH photons [59]. The incoherent emission has been known since the 1960s as Hyper Rayleigh scattering (HRS) and arises from an isotropic distribution of anisotropic molecules [44, 143, 250], which has been considered as the average structure of a liquid. The coherent emission was previously not associated with liquid structure [44, 143, 250]. It reports on a non-isotropic distribution of anisotropic molecules, and is sensitive to long range effects thanks to the emitted electromagnetic field's cubic distance dependence between the correlated scatterers [59]. These correlations are understood as dynamic fluctuations over nanometer length scales in the polarizability of the liquid. Coherent and incoherent emission are distinguishable by using different polarization combinations of the in- and out-going light [59].

Here, we report fs-ESHS measurements of liquid water in comparison to an isotropic liquid (CCl₄) and show that water is a non-uniform liquid on femtosecond timescales. The SH emission of water is partially coherent and arises from second-order polarization changes that arise from orientationally correlated water molecules. The SH emission of CCl₄, on the other hand, is completely incoherent, and arises from isotropically distributed molecules. MD simulations of water and a Lennard-Jones (LJ) liquid (similar to CCl₄) demonstrate the abundance of cavities or voids with an average life time of 300 fs. In both liquid water and the LJ liquid, space can be cataloged in terms of the presence of spherical and dendritic shaped



Figure 4.1 – Nanoscale femtosecond heterogeneities in water. (A) Illustration of the fs-ESHS experiment, together with drawings of the emitted (\mathbf{k}) and scattering (\mathbf{q}) wave vectors involved in forward and backward scattering. Experiment scheme adapted from the image by C. Macias-Romero in Ref. [178]. B, C: fs-ESHS patterns of neat CCl₄ (B) and neat H₂O (C) collected at room temperature, using beams with all electromagnetic fields oscillating in the horizontal scattering plane (PPP) and the orthogonal vertical plane (SSS). The lines in panel B represent the fs-ESHS emission of isotropic liquid (see equation (2.6)). Graphs made by T. Schönfeldová.

voids [251, 252]. Investigating charge density distributions around the voids, we find that these are present for water, but not for the LJ liquid. In water charge density fluctuations around the voids arise from the presence of coordination water defects in the hydrogen bond network, which dictates the detailed hydration structure around the voids. We propose that these fluctuations are responsible for the coherent SH emission, and verify this by temperature-dependent fs-ESHS experiments that show an increase in the void density at a higher temperature, in agreement with the MD predictions.

4.2 Materials and Methods

4.2.1 Chemicals

Carbon tetrachloride (CCl₄, 99.9%, Sigma-Aldrich) was used as received without further purification. H_2O was purified by a Milli-Q UF-Plus instrument from Millipore, Inc., and it has an electrical resistivity of 18.2 M Ω ·cm.

4.2.2 Femtosecond elastic second harmonic scattering (fs-SHS)

Figure 4.1A illustrates the fs-ESHS setup, already described in Ref. [148] and in subsection 2.1.3. Forward fs-ESHS measurements were realized with an angle of acceptance for the signal collection of 3.4° . The scattering pattern was measured at a scanning step of 5°. Each data point was acquired with an acquisition time of 20×1 s and a gate width of 10 ns.

In the specific case of backscattering measurements at 180°, an additional dichroic mirror (042-5130, Eksma) was placed with 45° angle in the path of the incoming beam, after the long-pass filter and just before the focusing lens in front of the sample cell. This allowed the incoming beam at 1032 nm to be transmitted and focused to the sample, while backscattered light at 516 nm was collimated again with the same lens and then reflected to the side of the setup by the dichroic mirror. This second harmonic light was then collected with the same combination of bandpass filter, Glan-Taylor polarizer, and PMT as for the forward scattering measurements. All measurements were performed in a temperature- and humidity- controlled room (T = 297 K; relative humidity, 26.0 %).

4.3 Results and Discussions

Figure 4.1 shows scattering patterns of neat CCl_4 (4.1B) and neat H_2O (4.1C) collected at room temperature (295 K), using beams with all electromagnetic fields oscillating in the horizontal scattering plane (PPP) and the orthogonal vertical plane (SSS). The first polarization combination reports on both coherent and incoherent SH scattering while the second polarization combination reports only on the incoherent SH scattering [59]. The patterns of CCl_4 display intensity in both SSS and PPP polarization combinations. Close to the forward scattering direction the intensity for both polarizations is equal. Note that it is impossible to measure at exactly 0° due to the presence of the strong fundamental beam. Such an equal intensity is a hallmark of an isotropic liquid and can well be modeled by HRS theory [143, 145, 250] as explained fully in section 2.6. Fits to the incoherent HRS model are shown in Fig. 4.1B (red lines). The scattered SH light arises from electron density or polarizability fluctuations within single CCl_4 molecules that act as uncorrelated point sources. Figure 4.1C displays the same experiment but then for liquid H₂O. Interestingly, it shows remarkably different behavior: The SH intensity in the forward direction is stronger for PPP than for SSS SH emission. Such behavior can point to one of two things: Non-uniformity in the water structure on a ~ 100

fs timescale, or an experimental artifact as previously thought [253, 254]. The latter can be excluded by the CCl_4 liquid data of Fig. 4.1B, which was recorded with the same experimental setup and the same experimental parameters. Additional evidence for the exclusion of the possibility of an artifact is further provided in section 4.6.1 of the appendix, which shows Hyper Raman measurements for both liquids (Fig. 4.3) as well as the difference between elastic and inelastic scattering (Fig. 4.4). The polarization-independence of the setup was also verified with two-photon fluorescence (2PF) measurements in section **??**.

The non-uniformity in the structure and the presence of longer-range orientational correlations between the water molecules on a fs timescale follows directly from the measured intensities. Namely, the PPP polarization combination has a larger intensity than the SSS polarization combination in the near-forward scattering direction ($q \rightarrow 0^{\circ}$). In this direction, the scattering wave vector q lies in the horizontal plane and is nearly perpendicular to the wave vector of the SH light, k_{SH} (Fig. 4.1A). If the emitted SH electromagnetic field oscillates parallel to q, it is possible to transfer momentum. But if the emitted SH electromagnetic field oscillates the structure of individual water molecules while the PPP intensity may be enhanced by their orientational correlations. This simple explanation can be verified by measuring the scattered SH light in the backward scattering direction (Fig. 4.1A). In this direction, q is perpendicular to both the S and P directions of the electromagnetic SH field. One thus expects no difference in the scattered light intensity for either S or P polarized SH light, as neither can probe structural orientational correlations, even if they were present. Table 4.1 (appendix section 4.6.2) shows that this is indeed the case.

To observe such a pronounced coherent response there must be an abundant structural non-uniformity leading to orientational correlations in liquid water at room temperature with a lifetime > 100 fs. Orientational correlations are most pronounced around closed surfaces, and this effect has been used frequently as a way of interrogating the molecular structure of liquid water around nano-objects [45, 188, 193]. The SH intensity depends strongly on size (having an intensity dependence of (size)⁶ for small objects [127]), and shape (with more asymmetric shapes leading to bigger responses [129]). One possible factor that may give rise to these features is the transient variation of empty space in the liquid as seen through the lens of voids. Figure 4.2A shows examples of structural voids in water extracted from the MD simulations. In the appendix sections 4.6.3 and 4.6.4, we provide the details of how these are computed. There are two types of voids: small spherical voids that cover short length scales (< 5 Å), and dendritic shaped voids that are more delocalized, covering length scales of up to 2 nanometers.

Density fluctuations alone do not necessarily lead to orientational correlations between water molecules. A coherent fs-ESHS emission requires the following features: (1) A significant change in charge density or polarization, (2) A lifetime of the voids on the order of at least 100 fs, the time scale of the probing laser pulses, and (3) A sufficient void density at room temperature to generate enough SH photons. To elaborate these possibilities, we employed





Figure 4.2 – Charge density fluctuations around transient nanoscale voids. (A) Illustration of voids found in water. In the center, a spherical void with the water molecules is shown while the other three voids correspond to the dendritic shaped cavities and the hydration waters around them. The color of the oxygen atom of the water molecule represents the total charge of the water molecule, where red shows positive and blue negative charge. For clarity, the bulk water environment is not shown. (B) The integrated charge density of water molecules as a function of distance from the surface surrounding the void. Interestingly, we observe a positive layer of charge around the void followed by a negative region before observing charge neutrality. The LJ liquid does not show such fluctuations. (C) Distribution of the lifetime of voids seen in the simulations. The lifetime is examined starting from various initial conditions, and quantified by the time it takes for the void to disappear, or in other words, for a water molecule to enter the void. (D) The ratio of the coherent to incoherent intensity in the direction where $q \rightarrow 0^{\circ}$ measured as a function of temperature. We observe an increase in the coherent response of 9%. The inset shows the distribution of the void volumes divided by the molecular volume of water $(\sim 30\text{\AA}^3)$ at 300 K and 350 K, which shows an increase in the percentage of large voids with an increase in temperature. The work on MD simulations was realized by N. Ansari, E. Poli, and A. Hassanali. fs-ESHS data of (D) were measured by N. Dupertuis and T. Schönfeldová. The graphs were combined together by T. Schönfeldová.

linear-scaling density functional theory (LS-DFT) allowing for electronic structure calculations of large periodic systems consisting of 1000s of water molecules. From these simulations, density derived electrostatic charges (DDEC) were extracted and charge profiles around the voids examined (See appendix section 4.6.5 for more details). Figure 4.2B shows the integrated charge density around voids in H₂O (blue trace). In bulk H₂O, there is a significant change in charge density leading to charge gradients around the voids. A Lennard-Jones liquid, representative of CCl_4 , does have voids and cavities, but does not display charge density fluctuations, as can be seen from the red trace in Fig. 4.2B. Figure 4.2C shows the lifetime of the voids. The lifetime is on the order of several 100s of femtoseconds, with the mean being at 300 fs. This is exactly what is needed to observe a coherent fs-ESHS response. Figure 4.2D shows the computed void volume at 300 K (inset, green curve). The total volume of voids is found by integrating this curve and amounts to $\sim 3 \text{ vol}\%$ (equivalent to 1.5 M of water), and 20 % of these consist of voids > 1 nm (equivalent to 300 mM of water). Dilute electrolyte solutions having concentrations of 10 μ M of salt ions have been shown to be sufficient to generate a coherent fs-ESHS response. Therefore, the concentration of dendritic voids is certainly sufficient to create the coherent measured SH response.

We can find further evidence for our hypothesis by measuring the amount of coherent response as a function of temperature. Figure 4.2D shows this coherent contribution to the fs-ESHS response as quantified by the PPP/SSS intensity ratio measured for different temperatures. The coherent contribution is seen to increase with temperature. Note that the ratio is obtained in the direction $q \rightarrow 0^{\circ}$ after measuring full patterns, whereas in Chapter 3 on the contrary, we measured the fs-SHS ratios at fixed angle (90°) for considerations of duration of the experiment since it required many more data points. The inset of Figure 4.2D displays the normalized void-volumes computed from the MD simulations at two different temperatures (300 K and 350 K). Increasing the temperature enhances the fraction of dendritic shaped voids. The charge density fluctuations on the larger void surfaces lead to an increase in the coherent SH response. Since the coherent intensity increases with temperature it agrees with the prediction from MD simulations and provides further evidence for both the existence and importance of voids in neat water. Therefore, the presence of voids as computed by MD simulations and the consequential void surface charge density fluctuations lead to nanoscale heterogeneities in liquid water that is detected with fs-ESHS.

Liquid uniform water at elevated temperatures has a decreased hydrogen bond strength [255], and consequently a lower density, viscosity, and cohesive energy [256, 257]. Therefore uniform water, with a decreased hydrogen bond strength should have weaker orientational correlations, charge density fluctuations, and an expected decrease in the coherent fs-ESHS response. Inhomogeneities in water as revealed by XPS experiments have been interpreted as local sub-nanometric density fluctuations involving two dominant hydrogen bond local environments, one being more tetrahedral than the other. If the coherent response would be due to these types of fluctuations as found by XPS measurements, the temperature dependence should also have been reversed. The low and high-density structures reported by XPS have their most pronounced effect at low temperature (in the supercooled regime [9]), and are

virtually absent at elevated temperatures. This is the opposite of what we observe in Fig. 4.2D, which shows an enhancement in the effect with an increase in temperature.

4.4 Conclusions

Summarizing, fs-ESHS measurements of liquid CCl_4 and water in combination with MD simulation of neat water as well as a Lennard-Jones liquid show that transient ~ 300 fs cavities or voids in water lead to non-uniformity in the structure of water, established as nanoscale anisotropies. The charge density fluctuations around the voids generate detectable coherent fs-ESHS emission, which increases with temperature. As the observable in an fs-ESHS experiment is different from that in XPS measurements, the structural anisotropies reported with both methods are likely not exactly the same and occur on different length scales. Both sets of experiments do strongly point towards the notion that water is not a uniform liquid on femtosecond timescales and that transient structures exist on both sub-nanometric and longer distances. Transient structural inhomogeneities in water as reported here can play important roles in chemistry and physics since they ensure non-uniform potential energy landscapes for any type of transition or chemical reaction that might occur.

4.5 Implications for electrolyte solutions

The previous findings of Chapter 3 on electrolyte solutions shall be put in perspective with the existence of transient voids in pure water. Dendritic voids have been proposed as a possible explanation for the unexpected solubility in water of alkane, polymers, and biomolecules [251]. Their asymmetric shape appears indeed to be key to the solvation of solutes. Does the presence of these transient voids also impact the SHS measurements of aqueous salt solutions? The phenomena observed in Chapter 3 are caused by the static electric filed emitted by the charged ions. In contrast, the voids are temporary with a lifetime of 300 fs. Another point to note is that the electric field induced by the ionic charge extends on long range, independently of the presence or absence of voids. fs-ESHS has indeed been used to probe electric field induced correlations on a much longer range [59] than the size of the voids. All of this means that the effects probed by fs-ESHS in Chapter 3 are caused by the presence of the ions, and are independent on the voids.

4.6 Appendix

4.6.1 Exclusion of the possibility of an artifact

To confirm that our measurements are not influenced by hyper-Raman signal, we measured spectra of CCl_4 and H_2O at a +5° scattering angle in PPP and SSS polarization combinations, see Figure 4.3. It can be seen that all the hyper-Raman peaks are outside of the 10 nm bandpass filter (black rectangle) used for the SHS measurements.


Figure 4.3 – Spectra of H_2O and CCl_4 , respectively in the 5° direction with the fundamental wavelength at 1032 nm in SSS (dashed lines) and PPP (solid lines) polarization combination. The black rectangle indicates the 10 nm bandpass filter used for SH measurements. Figure and measurements by T. Schönfeldová.

Another concern that has to be taken into account is the measurement of elastic SH scattering as opposed to inelastic scattering. To be able to measure elastic scattering, fs laser pulses need to be used. Using nanosecond laser pulses leads to inelastic scattering and thus to laser-pulse induced water orientation, which will distort the experiment. This can be verified by comparing power dependencies of the SHS intensity for fs and ns laser pulses. For an elastic second-order process, the SH intensity / (power)² ratio should be constant. Figure 4.4 shows the measured power dependence for the scattered SH intensity comparing fs-ESHS experiments to ns-ESHS experiments [258]. It can be seen that fs experiments are elastic while the others are not. It means that any possible coherence observed in the ns and ps experiments cannot unambiguously be assigned to water-structural properties, as the laser pulses likely induce water-water correlations.

4.6.2 Forward and backward scattering comparison

Figure 4.1A illustrates the SHS measurements from a top view. In this configuration, the momentum transfer q (also called the scattering vector) lies in the scattering plane and S polarization is always perpendicular to the scattering plane. Combining this knowledge with Equation (2.4), we can see that the SSS polarization combination is only comprised of the incoherent contribution, whereas the PPP polarization combination contains not only the incoherent contribution, but also the coherent one, which shifts the PPP patterns above the SSS ones. To verify that the mismatch between the SSS and PPP patterns close to the forward direction is caused by the additional coherent response in the PPP polarization combination, we performed the SH measurements at the backward scattering direction, where the q vector is perpendicular to the P polarization.

The values of the PPP/SSS ratio when close to the forward direction ($\theta = 5^{\circ}$) and for the



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Figure 4.4 – Plots of the measured intensity (I) divided by the square of the incident laser power as a function of the incident power (P), taken from Ref. [258]. Measurements and figure by Y. Chen, N. Dupertuis and H. I. Okur. (a) The power dependence for an experiment employing 190 fs laser pulses in H_2O and D_2O (200 kHz, 35 μ m beam waist). The solid black lines indicate the quadratic dependence of the SH intensity on the incident power. (b) The power dependence for experiments with 100 ns pulses (empty circles) from Ref. [254] and with 5 ps pulses (filled circles). The dashed lines indicate fitting with a quadratic function $a(1 + bP + cP^2)$ to the data from 100 ns measurements.

backward direction are displayed in Table 4.1. In the case of CCl_4 , the PPP/SSS ratio is almost 1 for both directions, confirming the isotropy of this liquid at fs timescales. On the contrary, the PPP/SSS ratio for H_2O is significantly different for those two directions. This is a confirmation that the intermolecular correlations from hydrogen-bonding induce coherent signal, and the additional coherent signal contributes to PPP but not to SSS patterns.

PPP/SSS	H ₂ O	CCl ₄
$\theta = 5^{\circ}$	1.35	0.95
$\theta = 180^{\circ}$	0.92	0.93

Table 4.1 – The ratios of the signal intensity in the PPP over the SSS polarization combination for H₂O and CCl₄, at near-forward (θ = 5°) and backward (θ = 180°) scattering direction. The backward scattering values were measured by Y. Chen.

4.6.3 Molecular dynamics simulations

Molecular dynamics simulations of TIP4P-Ew [259] water (4096 molecules) were performed using the GROMACS 5.0 package [260] with a cubic box with an average side length of 49.68 Å. The system was equilibrated for 2 ns with a time step of 2 fs, and the final production run was carried out over another 40 ns using the NPT ensemble. The Nose-Hoover thermostat [261, 262] and the Parrinello-Rahman barostat [263] were used to fix the system temperature and pressure at 300 K and 1 bar, with coupling time constants of 2 ps and 1 ps respectively. Ewald [264] summation was used to calculate the long-range electrostatic interactions, and a cutoff of 10 Å was used for the Van der Waals interactions. In previous studies, we have also performed extensive benchmarks across a range of different water models and shown that the presence of these large voids is consistently reproduced [252].

4.6.4 Voronoi voids

The voids discussed in this work are the empty space within the hydrogen bond network of liquid water. To analyze the geometry of the empty space, we used the Voronoi-Delaunay method [265] which is implemented in the VNP code [266]. The interested reader is referred to Refs. [251, 252] for more details. In summary, two radii named the probe (R_P) and bottleneck (R_B) radius are needed to construct the Voronoi voids. The former is used for identification of the empty space between the molecules and the latter for merging the connected regions. Here, we used R_P and R_B equal to 1.2 Å and 1.1 Å, respectively. In previous studies we have shown that the R_P and R_B values have no significant impact on qualitative trends, such as the existence of small spherical and large dendritic voids.

4.6.5 Linear scaling density functional theory (LS-DFT) calculations

In a recent work [267], we have shown that asymmetries in the hydrogen bond network around a water molecule can lead to an imbalance in charge transfer between hydrogen bonds being accepted versus donated. Near the air-water interface where there is a breaking of symmetry in the hydrogen bond network, this leads to a triple layer of charge at the interface covering a length scale of 0.5 nm. In particular, we showed that topological defects in water which arise from fluctuations in the hydrogen bond network, serve as important sites for creating swings in the total charge of water molecules.

In this chapter, we show the coupling between the creation of transient voids (femtosecond) and charge gradients that form between water molecules around the voids. In order to do so, 20 frames were sampled from the TIP4P/EW simulations described earlier, and linear scaling density functional theory (LS-DFT) calculations were performed using the ONETEP code [268]. The LS-DFT calculations allow us to conduct electronic structure calculations of periodic boxes consisting of over 4000 water molecules. From the electron density, density derived electrostatic charges (DDEC) are used to assign net atomic charges to each atom and therefore determine the total charge of each water molecule. All technical details regarding the LS-DFT calculations and DDEC charges are documented in our previous work [252]. Here, we briefly summarize the main ideas.

LS-DFT as implemented in ONETEP, takes advantage of the near-sightedness of electrons in dealing with the density matrix representation of the system [269]. Essentially, the physical principle behind this is the fact that at constant chemical potential, properties such as the

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electron density n(r) are strongly modulated by external potentials arising only from nearby points. By expanding the density matrix in terms of non-orthogonal generalized Wannier functions (NGWF) and applying a real-space cutoff, a sparse density matrix $\rho(r, r')$ is formed (see more details in [252] allowing for the application of efficient diagonalization protocols.

In our LS-DFT simulations, the kinetic energy cutoff was 1000 eV and four NGWFs were used for O atoms and 1 NGWF was used for the H atoms. A localization radius of 10 Bohr was used in all cases. The DFT functional used for the simulations was the generalized gradient approximation (GGA) functional BLYP [270, 271], along with Grimme's D2 correction functional [272]. The Kleinman–Bylander [273] norm-conserving pseudopotentials were constructed using the Opium code [274].

DDEC charges are used to assign atomic charges derived from the underlying electron density. Specifically, the DDEC3 [275] scheme is used as implemented in the ONETEP code. The DDEC3 charges are built on ideas involving a combination of iterative Stockholder and iterative Hirshfield approaches. We have compared the DDEC charges we obtain to other charge schemes including Hirshfield and Natural Bond Order (NBO) charges and find that our results of the charge transfer being linked to the topology of the water molecules is consistently reproduced [252].

In our recent work [252], we performed extensive tests to determine how sensitive our results were to the use of standard GGA functionals (BLYP). Specifically, we compared the charge profiles obtained with BLYP to a hybrid functional B3LYP [270] as well as to a more accurate Van der Waals functional rVV10 [276]. We also compared our results to wave-function based approaches such as MP2 [277]. Finally, we also compared the charge profiles we got using configurations sampled from the most accurate potential for bulk water, MB-pol [23] and also various charge schemes (NBO and Iterative Hirshfield). Our results are consistently reproduced across all these various benchmarks.

5 The structural 3D confinement of water leads to structural changes involving 10⁷ water molecules

Water is critical for life and has a highly adaptive three-dimensional hydrogen bond network. Confinement of this 3D H-bond network may lead to dramatic changes in the macroscopic properties of water. While there is agreement that such effects exist on a < 1 nm length scale, it is not clear what they are if larger length scales are also affected. Here, we investigate 3D confinement effects over lengths scales ranging from 58-140 nm, by measuring the change in water structure between the inside and outside of neutral and charged hollow lipid bilayer shells. The structural changes are measured with polarimetric angle resolved second harmonic scattering and the order of water as well as the surface potentials are retrieved by nonlinear light scattering modeling. We find that 3D confinement manifests itself primarily in the amount of orientational ordering of the hydrogen bond network. For heavy water the measured change in water ordering depends on the change in available interfacial area. For light water, however, the effect is 8 times larger and depends on the inner volume of the shells, demonstrating 3D structural confinement effects in water can involve up to 10⁷ water molecules.

The work for this chapter was done together with O. B. Tarun, B. Chu, C. Lütgebaucks and S. Roke. N.D., B.C., and C.L. performed experiments on the liposomes. O.B.T. performed experiments on freestanding lipid bilayers. N.D., O.B.T., and S.R. interpreted the experimental data. S.R. conceived and supervised the work.

5.1 Introduction

As the primary solvent of life water is a fascinating liquid. Liquid water is structured in a three-dimensional dynamic network that is highly adaptive to its surroundings [13]. Interfaces [278] are able to impose a structure on water, electric fields align water molecules [279, 280], and macromolecules [18, 19] such as DNA [281, 282] and aquaporin [283] can impose chirality on the water molecules adjacent to it. Furthermore, nanopores may induce freezing [284], and water in between graphene sheets displays non-bulk like behavior that is presently not understood [63, 65]. Thus, confinement – the restriction of available space – influences water structure. But the way in which it does is not well understood: Are confinement effects, purely stemming from local interactions, such as ion-dipole, van der Waals and direct hydrogen (H)-bonding interactions, or are collective, entropic effects involving many water molecules also important? Likewise, what is the length scale over which confinement plays a role? If a collective effect plays a role, we may expect the length scale for confinement effects to greatly exceed 1 nm.

Currently available experimental and theoretical studies agree that the length scale over which confinement effects occur is at most a few nanometers: Vibrational dynamics studies of water in reverse micelles that are a few nanometers in size show a non-bulk like vibrational relaxation [285-288]. Carbon nanotubes modify water dynamics and thermodynamics compared to bulk water [66, 284, 289, 290]. Freezing transitions are observed in pores with diameters < 2 nm [291–293]. Besides such small systems, structural changes have been probed on macroscopic extended planar interfaces, using atomic and surface force microscopies, whereby the water is confined between two macroscopic surfaces, or between an AFM tip and an extended planar interface. These studies show structural changes at separations of only a few water layers [294-297], and the structural changes due are explained in terms of water-interface [298] interactions. However, collective entropic interactions could be very important: As one confines water the number of hydrogen bond network configuration changes [288], which is for example very clearly seen in the infrared spectra of the gas to liquid phase transition of small water clusters [299, 300]. That these have not been reported yet, is caused by the fact that probing interactions that exceed several nanometers under 3-dimensional (3D) confinement is a great challenge. Most experimental techniques that probe the structure of water, such as dielectric and THz spectroscopy [301-303], X-ray and neutron scattering [19, 237, 304], vibrational spectroscopies [305, 306], vibrational dynamics studies and NMR spectroscopy [64, 289, 304, 307] are sensitive to local, around or < 1 nm perturbations to the H-bond network of water. Likewise, computational methods on the quantum chemistry level have been performed over length scales smaller than a few nanometers [67, 293, 308, 309]. Additionally, most confinement experiments that would allow for longer length scale effects, have been done using partial 1D and 2D confinement [64] in combination with time scales that are much larger than the reorganization time of water. Therefore, it is possible that equilibration of the aqueous system has obscured longer-range, collective confinement effects.

Here, we induce 3D confinement in light and heavy water by encapsulating water using

neutral and charged liposomes. This produces bilayer shells with diameters in the range from 60 to 140 nm. We compare the resulting changes in water structure to the water structure adjacent to an extended 2D lipid bilayer made of the same lipids. To determine structural changes due to confinement, we quantify the structural difference between the inner and outer water using femtosecond (fs) second harmonic imaging and angle resolved [172] (AR) second harmonic scattering (SHS). Non-confined water adjacent to a planar bilayer produces a vanishing SH signal. All measured liposomes display differences in the water structure and the size normalized response increases with decreasing size. Modeling the data using nonlinear light scattering theory we find that the structural differences are caused by spatial restrictions rather than by electric field effects. Remarkably, the SHS water response of D_2O is significantly (~8 times) smaller than that of H_2O and the size dependence is different. Whereas the structural changes in D_2O can be explained by an increase in the relative amount of interfacial water per liposome, the structural changes in H_2O depend on the available confined volume and are therefore due to 3D confinement of the hydrogen bond network.

5.2 Materials and Methods

5.2.1 Chemicals

Sulfuric acid (95-97%, ISO, Merck), ammonium hydroxide (30%, Sigma-Aldrich), hydrogen peroxide (30%, Reactolab SA), chloroform (Emsure, ACS, ISO, Merck), cholesterol, phosphorus standard solution (0.6 M, Sigma-Aldrich), L-ascorbic acid (ACS, \geq 99%, Sigma-Aldrich), ammonium molybdate (VI, ACS, 81-83%, Sigma-Aldrich) were used as received. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS, sodium salt), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (DPPS, sodium salt) were purchased in powder form (>99%) from Avanti Polar Lipids (Alabama, USA) and stored at -20 °C until further use. Ultrapure H₂O was obtained with a Milli-Q UF plus instrument (Millipore, Inc.), with an electrical resistance of 18.2 M Ω cm. D₂O used was 99.8% D atoms with an electrical resistance of >2 M Ω cm (Armar).

5.2.2 Formation of freestanding horizontal planar lipid bilayers

Freestanding horizontal planar lipid bilayers were formed following the procedure of Montal-Müller [310–313]. Two separated lipid monolayers on an air/water interface were combined in a ~80-120 μ m aperture in 25- μ m thick Teflon film. The presence of a bilayer was confirmed with white light imaging and electrical recordings with specific capacitance, $C_m>0.7 \mu$ F cm⁻², specific resistance, $R_m \sim 108 \Omega \cdot \text{cm}^2$ (Refs. [312, 314]). The composition of the leaflets and the aqueous solution where the bilayer leaflets reside are controllable in-situ. All measurements were performed at pH neutral conditions.

5.2.3 Electrical characterization of freestanding lipid membranes

Ag/AgCl pellet electrodes were placed on each side of the bilayer and electrical measurements were recorded through the HEKA patch clamp amplifiers. Capacitance and resistance measurements were made with HEKA's built-in software-based lock-in amplifier [315]. For more details, see Ref [316].

5.2.4 Second harmonic imaging

The imaging setup has been characterized in detail in Refs [316–318] based on principles of second harmonic scattering [148]. Two counter-propagating beams from a Yb:KGW femtosecond laser (Light Conversion Ltd) delivering 190 fs pulses, 1032 nm with a 200 kHz repetition rate were incident at 45° with respect to the membrane. Each beam was loosely focused using an f = 20 cm doublet lens (B coating, Thorlabs) and polarization controlled using a linear polarizer (Glan-Taylor polarizer, GT10-B, Thorlabs) and a zero-order $\lambda/2$ wave plates (WPH05M-1030, Thorlabs). The average power for each arm was set to ~110 mW. The phase-matched SH photons were collected with a 50x objective lens (Mitutoyo Plan Apo NIR HR Infinity-Corrected Objective, 0.65 NA in combination with a tube lens (Mitutoyo MT-L), a 900 nm short pass filter (FES0900, Thorlabs), a 515 nm band-pass filter (FL514.5-10) and an intensified electronically amplified CCD camera (IE-CCD, PiMax4, Princeton Instruments). A 400 mm meniscus lens was placed behind the objective lens to remove spherical aberrations induced by the coverslip. The transverse resolution, and thus the pixel width was 430 nm. All images were recorded with the beams polarized parallel to the plane of incidence (P). The acquisition time of the images was 560 ms.

5.2.5 Liposome Preparation and Characterization

Small unilamellar vesicles were prepared by extrusion according to Ref. [199–201] and section 2.3.2. The extrusion of multilamellar vesicle solutions was done by using a polycarbonate membrane (AvantiPolarLipids, Al) with pore diameters of 30, 50, 100, and 200 nm at room temperature. Unilamellar vesicles were stored in closed containers up to 4 weeks at 4 °C. The size and ζ -potential distribution of the liposomes were also measured with DLS and electrophoretic measurements at 23 °C (Malvern ZS nanosizer). To determine the size distribution of the vesicles, three subsequent measurements, each 11 runs, were averaged. To determine the ζ -potential of the vesicles, three subsequent measurements, each 30 runs at automated voltage, were averaged.

The liposomes were found to have a mean diameter in the range of 58-140 nm with a polydispersity index (PDI) of less than 0.166. The final lipid concentration was determined using a calorimetric phosphorus assay; for further details see Ref. [202] and the protocol at Ref. [?]. The weighted concentration of the lipids in the sample was 0.5 mg lipids/ ml weight ratio for DLS, ζ -potential measurements, and SHS experiments. The ionic strength

of the solutions was estimated to be 4μ M in the case of DOPC liposomes to account for H⁺ and HCO₃⁻ ions in solution. In the case of DOPS liposomes, it was computed from the counterions concentration coming from salt powder form of the lipids, divided by 2 as half of the counterions are entrapped to ensure electroneutrality inside the vesicles. All parameters are given in Table 5.1 in the appendix section 5.5.1.

5.2.6 Angle-resolved second harmonic scattering

Angle-resolved fs-ESHS measurements were performed by moving the detector arm in 5° steps between -85° and +85° with an opening angle of 3.4°. Each data point was acquired with an acquisition time of 20 × 1 s and a gate width of 10 ns. The normalized intensity at the angle θ , S(θ), was normalized to the intensity of the neat water (H₂O or D₂O) using the formula 2.27.

5.3 Results and Discussion

5.3.1 Quantifying confinement effects in water

To determine if structural changes occur under 3D confinement, we compare the structure of water in contact with a free-standing lipid bilayer with water in contact with different sized liposomes composed of the same bilayers. We quantify the effect of confinement by measuring the structural differences between water in contact with the top/bottom and outer/inner leaflets of both systems, using coherent second harmonic (SH) imaging (bilayer) and scattering (liposomes). SH generation is a process in which two near infrared photons are combined into a visible photon with the double frequency (Fig. 5.1A, energy level scheme). SH photons are generated by anisotropic distributions of molecules [116, 157, 319–324]. Isotropic bulk liquids do not generate coherent SH photons, and neither do fully symmetric systems. A single interface will change the isotropic distribution of water and induce SH contrast, while a bilayer with two leaflets facing opposite sides will not produce coherent SH photons. If the size of liposomes is too large to induce any type of spatial confinement in the inner shell, then the bilayer will have the same structural influence as an extended planar bilayer.

Freestanding horizontal planar lipid membranes in water were formed following the Montal-Müller method [310–313]. Two separate lipid monolayer interfaces were formed at the air/water interface and apposed in an 80-120- μ m-sized circular aperture in a 25- μ m thick Teflon film, producing an extended planar bilayer with a radius of ~ 50 microns. This horizontally mounted membrane was imaged with a medium repetition rate, wide field nonlinear SH microscope [317]. Two counter-propagating 190 fs, 1032 nm, 200 kHz pulsed beams with an opening angle of 45° illuminate the membrane interface, such that phase matched SH photons are emitted along the surface normal (Fig. 5.1A, see Ref.[316], and the materials and methods section). Figure 5.1B shows SH images recorded of (i) a pure water, i.e. without an interface, (ii) a symmetric lipid bilayer of identical lipid leaflets composed of 100 % DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), and (iii) an asymmetric bilayer

Chapter 5. The structural 3D confinement of water leads to structural changes involving 10⁷ water molecules

composed of 37.5:37.5:25 mol % 1,2-dipalmitoyl-sn-glycero-3-phosphoserine (DPPS), DOPC, and cholesterol (Chol) on the top leaflet, and 37.5:37.5:25 mol % 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), DOPC, and Chol on the bottom leaflet. Fig. 5.1C shows a bar graph of the integrated counts of the corresponding SH images (i-iii) of bulk water and of the symmetric and asymmetric bilayers from 5.1B, normalized by the SSS response of bulk water [178]. SH Images (i) and (ii) produce a negligible number of photons, while SH image (iii) produces a significantly higher number of SH photons. The lack of a response from the symmetric bilayer confirms that two identical planar leaflets spaced 4 nm away and with opposite orientation cancel each other's SH response. In other words, they do not give rise to a difference in water structure. The coherent SH response of the asymmetric bilayer on the other hand does, because the structure on both leaflets is different. Here the SH signal arises from the charge-dipole interaction between the charged head groups and the dipolar water molecules, which creates a non-random orientational distribution of water dipoles along the surface normal. Next, we investigate whether there are structural differences between water in contact with leaflets on either side of DOPC liposomes, 58-140 nm in diameter. To probe the water structure in contact with these nanometric objects, we switch to second harmonic scattering (SHS) [158, 164, 168–170, 189, 221, 325], as objects below the diffraction limit cannot be SH imaged [326].

Fig. 5.1D shows an illustration of the SHS experiment. Fig. 5.1E shows AR-SHS patterns of 138 nm and 72 nm diameter liposomes made of neutral DOPC lipids. The patterns were recorded by focusing near infrared fs laser pulses in the liposome dispersion and detecting the emitted SH photons with a detector placed on a rotating arm. The emitted SH photons originate from both the liposome interfaces as well as from pure water. The coherent response from the aqueous interface is recorded with all beams polarized along the scattering plane (PPP polarization). It consists of the SH emission from the aqueous liposome dispersion from which the SH response of the neat water is subtracted. Just as with the SH images, this differential signal was then divided by the incoherent isotropic SH scattering of water recorded in the SSS polarization combination [178] (i.e. all beams polarized perpendicular to the scattering plane). The resulting SH intensity values $I_{norm}(\theta)$ are plotted as a function of scattering angle θ . The resulting SH scattering patterns were further normalized to both the number of liposomes in solution as well as to their size [130]. The size distribution is measured with dynamic light scattering. This size distribution is then used to calculate an effective radius $(R_{\rm eff})$ that would generate the same scattering pattern as the distribution of multiple radii. We use R_{eff} to determine the size normalized single liposome SH intensity. The procedure is also listed in the subsections 2.2.2 and 2.2.3 of Chapter 2. The inset shows the total value of $I_{\text{norm}}(\theta)$ integrated from -85 to +85° of scattering angle, for both patterns. Fig. 5.1E shows that SH intensity is visible for both liposome samples, indicating that the inner water structure differs from the outer water structure. The inset shows the angle integrated SH intensities. Since the liposomes are neutral, this indicates that the difference could be caused by spatial confinement. The smaller sized liposomes generate a ~20 times larger SH response per object, indicating that the difference in water structure is bigger for smaller liposomes.



Figure 5.1 – Water structure around curved bilayers (A) Illustration of the SH imaging experiment, by O. B. Tarun. Two counter-propagating beams (190 fs, 1032 nm, ω , red arrows) overlap in space and time to illuminate a lipid bilayer membrane that is freely suspended in water. The bilayer is nearly planar and has a radius of curvature of ~ 50 μ m, as dictated by the opening in which it is located. Non-resonant SH photons (2ω , green arrow) are collected (magnification: 50x, NA = 0.65) in the phase-matched direction. All beams are polarized in the imaging plane. The energy level scheme is shown on the left. (B) SH images of (i) H₂O, (ii) a symmetric membrane composed of DOPC, (iii) an asymmetric membrane composed of 37.5:37.5:25 mol % DPPS:DOPC with cholesterol (top leaflet) and 37.5:37.5:25 mol % of DPPC:DOPC with cholesterol (bottom leaflet). The scale bar (20 μ m) is the same for all images. The acquisition time is 560 ms for each image. The colorbar goes from 0 to 500 counts for images (i) and (ii), and from 0 to 7000 counts for the image (iii). The images were measured by O. B. Tarun. (C) Bar graph of the normalized SH intensity integrated over a 20 μ m x 20 μ m region of interest centered in the images (i), (ii), (iii). In the case of the neat water image, the normalized SH intensity was taken from 1 frame only. The SH intensities from panel (E) are also shown, prior to size normalization, on the right of the bar graph. The SH intensities of the images and scattering patterns were normalized to the SSS response of neat water in the same way. The dashed areas indicate the noise level for each measurement (computed as the standard deviation of the PPP response of neat water) also normalized by the SSS response of neat water. (D) Illustration of the AR-SHS scattering experiment performed on liposomes in solution, by N. Dupertuis and O. B. Tarun. One beam (190-fs, 1032 nm, ω , red arrows) is focused into an aqueous liposome solution, stored in a cylindrical cuvette. SH photons (2ω , green arrow) are collected with a moveable detector in the (x,z) plane to record scattering patterns. All beams are polarized in the (x,z)-scattering plane (P polarization). (E) AR-SHS scattering patterns of DOPC liposomes in pure H₂O (0.5 mg/mL), recorded in PPP polarization combination. The error bar at -45° angle shows the maximum standard deviation over 20 measurements. The data are scaled to correct for differences in size distribution and number density of the scatterers (as described by equation (2.27) in section 2.1.4). The solid lines are fits extracted with the nonlinear light scattering algorithm, scaled accordingly. The inset shows the total value of $I_{\text{norm}}(\theta)$ integrated from -85 to +85° of scattering angle, for both patterns. 67

This shows that confinement effects are already apparent for spheres with diameters as large as 140 nm, which is remarkably larger than previously reported [66, 284, 285, 293], and that a more in-depth size analysis is warranted.

5.3.2 3D confinement restricts the hydrogen bond network

The difference in water structure observed in Fig. 5.1, suggests that further investigation would be informative. We have therefore prepared liposomes of different sizes, spanning radii (R_{eff}) from 29 nm to 70 nm, using neutral (DOPC) as well as charged (DOPS) lipids, prepared in both H₂O and D₂O. This will not only shed light on the spatial extend of the confinement effects but also on the source of the confinement effects. We recorded polarimetric angle resolved SHS patterns in two independent polarization combinations PPP (as in Fig. 5.1D) and PSS (with the SH beam polarized along the horizontal scattering plane, and the other beam along the vertical plane). These patterns were then size and number density-normalized so that the displayed intensities reflect the intensities of the hydration of single liposomes, allowing one to deduce size effects independent of polydispersity. Figure 5.2A shows the size and number density normalized angle integrated intensity $I_{norm}(\theta)$ for 100 % DOPC (red rounds) and DOPS (blue triangles) liposomes. Both types of liposomes display a strong size dependence. To investigate the nature of this size dependence we have fitted the AR-SHS patterns using nonlinear light scattering theory that extracts two unique parameters: $\chi_{s,2}^{(2)}$ and Φ_0 . Φ_0 is the surface potential and represents the spatially integrated electrostatic field from infinity in the aqueous bulk phase to the interface of the liposome. $\chi_{s,2}^{(2)}$ embodies the difference between the anisotropic orientational distributions adjacent to both leaflets. It thus represents the net amount of oriented water, as induced by any non-electrostatic interaction. The nonlinear light scattering theory represent solutions to the Maxwell equations that describe the light-matter interaction of the SHS process. Its application to SHS has been described in detail Ref.[171] and was applied to determine the surface potential of charged liposomes [88, 178], silica [188] and titania [180] particles in aqueous solution. For completeness, the theory and application are provided in the subsection 2.1.2 of the Chapter 2. Here, we only mention the results. Starting with the DOPC liposomes, the fits to the AR-SHS patterns were made using only $\chi_{s2}^{(2)}$ as a fit parameter, since DOPC membranes are neutral and carry no net charge. Figure 5.2B shows the resultant $\chi_{s,2}^{(2)}$ values for DOPC liposomes as a function of the effective radius. The obtained $\chi_{s,2}^{(2)}$ values were used as input (with a factor 2) for the fits to the AR-SHS DOPS patterns since the head group structures of DOPC and DOPS are very similar [178]. Figure 5.2C shows the extracted Φ_0 values for DOPS liposomes as a function of size.

The surface potential increases only a little bit (with an uncertainty of 25 mV). Thus, comparing Fig. 5.2B and 5.2C we see that the main interaction that is responsible for the size dependence in Fig. 5.2A is not electrostatic in origin, but stems from the much stronger size dependence observed in the $\chi_{s,2}^{(2)}$ values. This size dependent increase in orientation anisotropy can be due to either local or non-local interactions: Either the changes in the $\chi_{s,2}^{(2)}$ values are caused by a restricted volume effect, because the limited space in the inside of



Figure 5.2 – Restriction on hydrogen-bond network in H₂O. (A) Integrated normalized intensity $I_{\rm norm}$ as a function of size of the samples for liposomes of DOPC (red, rounds) and DOPS (blue, triangles). The size displayed on the x-axis represents an effective radius, that is, the radius of a fully monodisperse liposome dispersion that produces the dynamic light scattering pattern of the real sample (Ref.[130], see also subsection 2.2.3 of Chapter 2). The error bars correspond to the standard deviation for an acquisition time of 20 × 1 s. Curve fittings are shown for $R_{\rm eff}^{-6}$ dependence for DOPC (red) and DOPS (blue) samples. (B) Absolute value of $\chi_{s,2}^{(2)}$ computed for DOPC samples with our algorithm, as a function of the effective radius. The error bars correspond to the results of the algorithm computation on the AR-SHS patterns that had their standard deviation of the intensity added or substracted. (C) Surface potential computed for DOPS samples, computed with our algorithm using the simulated $\chi_{s,2}^{(2)}$ values found for DOPC, as a function of the effective radius.

the liposome is reducing the 3D degrees of freedom of the hydrogen bond network. Or the changes in the $\chi_{s,2}^{(2)}$ values could be caused by local interfacial interactions. Both effects can be expected to have a certain size dependence. For the non-local interactions, the $\chi_{s,2}^{(2)}$ response should scale with $1/R_{eff}^3$ and the intensity with $1/R_{eff}^6$. For the local interfacial interactions to be dominant, we expect a $1/R_{eff}$ scaling for $\chi_{s,2}^{(2)}$ and a $1/R_{eff}^2$ scaling for the SH intensity: since the leaflet thickness is fixed (to ~4 nm), the difference in area scales with ~ R_{eff} , and therefore the relative (polarization amplitude) intensity between two different sizes is proportional to $(1/R_{eff})$ $1/R_{eff}^2$. The solid lines in Fig. 5.2A represent the scenario of the non-local volume effect $(1/R_{eff}^6)$. Since the line matches the data well, we conclude that the size dependent changes in the AR-SHS intensity arise from changes in the water structure due to a restricted available volume. This means that the reduction in size of the liposomes starts to reduce the number of possible hydrogen bond network configurations which leads to different shapes of the orientational distribution function of water in the inside of the liposome.

Considerations on the electrostatics

It is important to note at this point that the observed dependence on radius could also originate from specific electrostatic phenomena, that might change our conclusions. A first one is that the measured surface potential Φ_0 would be the result of a difference of potentials between the inner and outer leaflets of the liposomes. However, the counterions are neutralizing the inner leaflet according to Gauss's law for closed volumes [327]. Therefore, the surface

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potential values that we get are not differences but the values at the surface of the outer leaflet of the liposomes. Another phenomenon that could impact the radius dependence of the surface potential is a possible change in the electrostatics, more specifically a change in the degree of ionization. Not 100% of the lipids are fully ionized in practice, some of them getting neutralized by the counterions coming from the aqueous solution. One defines the ionization rate as σ_d/σ_0 where σ_d is the actual surface charge density and σ_0 is the maximum surface charge density of a fully ionized liposome (-245 mC/m² in the case of DOPS liposomes). Figure 5.3A shows the linear dependence of the surface charge density on the ionization rate of a surface of DOPS lipids. The key question here is to consider whether the change in size of the spherical vesicles can impact the ionization rate of the lipids at the surface, hence the surface charge density.

To investigate this, we look at the relationship between the surface charge density of a sphere and the potential as described by the nonlinear spherical Poisson-Boltzmann equation. The Ohshima's equation [186] is a solution to this equation for a smooth sphere with a uniform surface charge density σ_d , and provides the potential dependence on the distance from the sphere:

$$\Phi(r) = \frac{2k_BT}{ze} \ln\left[\frac{1 + \tanh\left(ze\Phi_0/4k_BT\right)(R/r)e^{(-\kappa(r-R))}}{1 - \tanh\left(ze\Phi_0/4k_BT\right)(R/r)e^{(-\kappa(r-R))}}\right]$$
(5.1)

as well as the relation between the surface potential Φ_0 and σ_d :

$$\sigma_{d} = \frac{2\varepsilon_{0}\varepsilon_{r}\kappa k_{B}T}{e} \sinh\left(\frac{ze\Phi_{0}}{2k_{B}T}\right) \times \left[1 + \frac{1}{\kappa R}\frac{2}{\cosh\left(ze\Phi_{0}/4k_{B}T\right)^{2}} + \frac{1}{(\kappa R)^{2}}\frac{8\ln\left[\cosh\left(ze\Phi_{0}/4k_{B}T\right)\right]}{\sinh\left(ze\Phi_{0}/2k_{B}T\right)^{2}}\right]^{1/2}$$
(5.2)

with ϵ_0 , ϵ_r , k_B , *T*, *z*, *e*, κ , *R* being the permittivity in vacuum and the relative permittivity, the Boltzmann constant, the temperature, the valency, the elementary charge, the Debye parameter and the radius of the particle. Equation (5.2) is in agreement with exact computer results of the non-linear spherical Poisson-Boltzmann equation (with a relative error of less than 1 %) in the range $0.5 \le \kappa R < \infty$. Fig. 5.3B shows the computed Ohshima's potential in the case of a DOPS liposome with ionic strength fixed at 3.0×10^{-4} M, and for a surface charge density of -2 mC/m^2 (corresponding to a value reported previously for DOPS liposomes [88]). In addition, the dependence of the potential on the ionization rate is shown in Fig. 5.3C and D, at different fixed radii. The radius dependence does not match with the confinement effect that we observe with our samples: according to these computations we should observe a decrease of the surface potential Φ_0 for small vesicles, which is not the case.

The caveat here lies in the fact that we ignored the surface charge density dependence on the radius, that is not obvious in equation (5.2). For a charged sphere in an electrolyte solution, there is a certain charge density above which counterions will collapse onto the surface and prevent further charging. This maximum value is called the critical surface charge density $\sigma_{\rm crit}$, and it depends on the geometry and the ionic strength of the solution. The Manning



Figure 5.3 – Predictions of the Ohshima's equation. (A) Surface charge density of a DOPS liposome as a function of the lipids ionization rate. (B) Surface potential predicted by Ohshima's equation for a DOPS liposome as a function of the vesicle radius, for a fixed ionic strength of 3.0×10^{-4} M and a surface charge density of -2 mC/m^2 . The dashed line shows the criterion of validity of the equation ($0.5 = \kappa R$): the curve is accurate for larger radii. (C) Surface potential predicted by Ohshima's equation for a DOPS liposome as a function of the ionization rate, for a fixed ionic strength of 3.0×10^{-4} M and fixed radii. (D) Zoom on lower ionization range.

condensation theory [328] provides an expression for $\sigma_{\rm crit}$ for different geometries, including the spherical one. For a large sphere (i.e. $\kappa R \ge 1$), the critical surface charge density is defined as:

$$\sigma_{\rm crit} = \frac{e(1+\kappa R)\ln(\kappa l_b)}{2\pi R z l_b}$$
(5.3)

Figure 5.4A shows the computed σ_{crit} as a function of radius, for a DOPS liposome. Its value increases significantly at lower radii, meaning that the constant surface charge density assumption in Fig. 5.3B might not be correct. By assuming that the liposomes are getting charged up to the σ_{crit} density, one can get an estimation of the maximal surface potential allowed by Manning condensation. We introduce this critical charge density value into the Ohshima's equation, and we obtain surface potential values for different radii, shown in figure 5.4B. The most restrictive criterion of validity here is the Manning's one ($\kappa R \ge 1$), meaning that our computations are valid for all radii larger than 25 nm in the case of an ionic strength of 3.0×10^{-4} M. According to this computation, the surface potential is expected to increase

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Figure 5.4 – Consequences of the Manning condensation of counterions. (A) Critical surface charge density of a DOPS liposome as a function of the radius. The inset shows the value of κR as a function of radius. The dashed line shows the criterion of validity of Manning equation ($\kappa R = 1$): the curve is accurate for larger radii. (B) Surface potential predicted by Ohshima's equation for a DOPS liposome with surface charge density reaching the critical value from (A), for a fixed ionic strength of 3.0×10^{-4} M, as a function of the radius of the liposome. The dashed line shows the criterion of validity of Manning's equation ($\kappa R = 1$). (B) Comparison of the curve of (B) with the results of Fig. 5.2C.

slightly at small radii as the increase in surface charge density dominates on the decrease of potential observed in Fig. 5.3B. We also compare in Fig. 5.3C the computed values with the surface potential that we extracted for DOPS liposomes in H_2O . The experimental increase is actually stronger than the predicted change from Manning and Ohshima's equations. Overall, the Manning theory does not seem to impact significantly our conclusions on the confinement effect as it does not predict a larger increase.

There are nevertheless limitations to this electrostatics description. Ohshima's equation is an exact solution for a uniform distribution of charges over a sphere. However SH imaging measurements of planar lipid bilayers shows that the distribution of charges is not homogenous (see Ref. [316], and Fig. 5.1B, image (iii)). Also, Ohshima's equation does not take into account the hydration, the surface chemistry, and the H-bond network of water, but only the surface charge density σ_0 , and the ionic strength. It is therefore only a simple approximation that might not be fully valid for the complex surface of liposomes. We also note that the Φ_0 values extracted with our nonlinear light scattering model are higher than reported in a previous article [178]. There are several reasons for this discrepancy. First, in that previous article, the confinement effect was not taken into account, hence the $\chi_{s,2}^{(2)}$ value was taken for DOPC liposomes of radius 47 nm, and applied for DOPS liposomes of radius 57 nm. Second, and more importantly, the ionic strength postulated for the DOPS sample without any additional salt was lower $(3.0 \times 10^{-4} \text{ M})$. Therefore, the Φ_0 values extracted in that previous article are decreased compared to ours.

In addition, we also measured the ζ potential of liposomes samples. The values are shown in Fig. 5.5. The DOPC values are very low, validating the use of -7 mV as a fixed surface potential value for the DOPC samples in the nonlinear scattering model. The ζ potential values for DOPS liposomes seem to increase slightly at lower radii, but this change remains within the uncertainties range so we cannot conclude directly on a confinement effect from them.



Figure 5.5 – Zeta potential for DOPS and DOPC liposomes at different radii, in H₂O and D₂O.

However, the fact that the surface potential Φ_0 is larger than the ζ potential means that there is a Stern layer around the liposomes, in agreement with the partial ionization of the lipids that we discussed above. Note that our nonlinear light scattering model is insensitive to the shape of the potential $\Phi(r)$ within ~ 3 nm from the particles surface [171], thus insensitive to the presence of the Stern layer.

5.3.3 The response of the hydrogen bond network

To investigate further the impact of the confinement on the hydrogen bond network of water, we compare light and heavy water. D_2O has a slightly different hydrogen bond network compared to H_2O . Although the net hydrogen bond strength differs by only a few percent at most [12] in strength, the different degrees of freedom that together determine the hydrogen bond network reorganization have different strengths. Breaking and reforming hydrogen bonds occurs through both a stretching and a rotational (or librational) mode [219, 223, 329], as illustrated in the inset of Fig. 5.6A. The stretching mode is stronger for H_2O and weaker for D_2O , while the rotational motion is weaker for H_2O and stronger for D_2O . Overall D_2O has slightly stronger hydrogen bonds, leading to an increase in melting point of 3.8°C at ambient pressure (and 7°C for the temperature of highest density). Therefore, if H_2O and D_2O display a different response to the 3D confinement experiment of Fig. 5.2, the origin of the size dependence arises from three-dimensional hydrogen bond restructuring invoked by a limitation in the spatial degrees of freedom.

Figure 5.6 shows polarimetric AR-SHS measurements of neutral DOPC liposomes dispersed in light (red rounds) and heavy (black squares) water. Figure 5.6A shows the extracted $\chi_{s,2}^{(2)}$ values, together with curves that scale as $1/R_{\text{eff}}^3$ (red line) for H₂O and $1/R_{\text{eff}}$ (dashed black line) for D₂O. The meaning of these curves is explained below. Note that for DOPC, with a

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Figure 5.6 – Influence of nuclear quantum effects. (A) Absolute value of $\chi_{s,2}^{(2)}$ computed for DOPC in H₂O (red, rounds) and D₂O (black, squares), as a function of effective radius with our algorithm. The error bars correspond to the results of the algorithm computation on the AR-SHS patterns that had their standard deviation of the intensity added or substracted. Curve fittings are shown for R_{eff}^{-3} (continuous, red) and R_{eff}^{-1} (dashed, black) dependence respectively for liposomes in H₂O and D₂O. The inset shows two hydrogen-bonded water molecules. The hydrogen bond can be broken by bending or stretching as shown with the black arrows. (B) The integrated SH intensity of neat water in PPP polarization, integrated over the angular range -85° < θ < 85° for H₂O and D₂O, showing that the bulk response of D₂O is bigger than that of H₂O. (C) Sketch of a possible explanation of the extended size range over which confinement effects are observed. Water has been shown to contain structural heterogeneities, such as voids, cavities, wires and density fluctuations. These are here represented as the arbitrary shaped colored structures. The arrows indicate that their interactions might be affected by the 3D confinement. The sketch was reproduced from an original one by S. Roke.

neutral membrane $I_{\text{norm}} \sim \left|\chi_{s,2}^{(2)}\right|^2$, which is why it is not shown here, but in the subsection 5.5.3 of the Appendix (Fig. 5.9). The intensity and extracted $\chi^{(2)}_{s,2}$ values of DOPC liposomes in D_2O have a size dependence that is different from H_2O . Compared to H_2O , the SH intensity and $\left|\chi_{s,2}^{(2)}\right|$ values are smaller. To understand the size dependence, we start by noting that the coherently emitted SH intensity from a liposome in water is determined by two quantities. The first one is the second-order molecular polarizability ($\beta_2^{(2)}$) of water and the second one is the net orientational distribution of water. Spatially averaging $\beta_2^{(2)}$ over the orientational distribution of water molecules results in $\chi_{s,2}^{(2)}$. To determine which parameter is responsible for the result in Fig. 5.6A we need to have an estimation of the relative magnitudes of the hyperpolarizabilities of light and heavy water. This can be obtained by measuring SHS patterns of neat H₂O and D₂O. Fig. 5.6B shows the values of the corresponding integrated scattering patterns recorded in the PPP polarization. The intensity of pure D₂O is 1.95 times bigger than H_2O , which means that D_2O molecules have an on average larger molecular hyperpolarizability than H₂O. Given identical liposome induced changes in the orientational distributions of water molecules in light and heavy water, we would therefore have expected a bigger change in $\chi^{(2)}_{s,2}$ for D₂O than for H₂O. Interestingly, the data in Fig. 5.6A shows exactly the opposite: The SHS response in Fig. 5.6A is bigger for the same size of liposome in H_2O than in D_2O . Thus, confining H₂O results in a much bigger change in the orientational distribution of water molecules than in D₂O. The change in SH response as a function of size reveals another very interesting difference: Confining H₂O results in orientational distribution changes that scale with $1/R_{eff}^3$ whereas the D₂O size dependence is much better described by a $1/R_{eff}$ dependence. The $1/R_{eff}^3$ dependence suggests an inner volume dependence. The $1/R_{eff}^3$ dependence arises purely from a difference in interfacial area with size. Reducing liposome size, while keeping the bilayer thickness constant at ~4 nm [194], increases the relative difference in interfacial area between the inner and outer leaflets for smaller liposomes. This leads to a relatively larger SH intensity per liposome, that scales as a $1/R_{eff}$ dependence. Thus, the D₂O response can be explained simply by a change in relative interfacial area, as one might expect from simple geometrical arguments. For H₂O though, we observe a very different dependence that scales with the available inner liposome volume. Thus, the orientational distribution function of water is more dramatically changed by a restriction in the degrees of freedom of the hydrogen bond network induced by 3D spatial confinement.

In this study the largest liposomes with $R_{\text{eff}} \sim 70$ nm contain $\sim 4 \times 10^7$ molecules, and are ~518 water molecules in diameter. The smallest liposomes with $R_{\text{eff}} \sim 29$ nm contain $\sim 3 \times 10^6$ molecules and span ~215 water molecules in diameter. Our results thus show that spatially confining water to a volume holding >10⁷ water molecules can already considerably impact the configuration of the 3D hydrogen bond network of H₂O. For D₂O, however, the hydrogen bond network is not changing its configuration beyond our detection limit. This means that the spatial extent over which H₂O molecules influence each other is much larger than the extend over which D₂O molecules influence each other. This finding is qualitative in agreement with previous work: H₂O solvates more easily than D₂O [256], the hydrogen bonding network of H₂O is more easily perturbed by electrolytes [59], or temperature changes (see Chapter 3 or Ref. [330]) compared to that of D₂O. Surface tension anomalies induced by salt ions, known as the Jones-Ray effect, occur at lower concentrations in H₂O than in D₂O [153].

5.3.4 The spatial distribution of heterogeneities

The structure of water has been extensively investigated, and a number of studies have pointed out the heterogeneous nature of water: As a mixture of two structural motifs or two states of water [237]; under the form of tetrahedral patches [9, 233]; water clusters [61, 303, 331, 332]; water wires [217, 333–335]; or cavities [252, 336, 337] that can occupy up to ~3 % volume of the liquid. If such substructures are indeed an important part of liquid water the restrictions imposed on the available space involve also the arrangement of wires, cavities or density fluctuations. Although the structures involved are at most 10 - 20 water molecules in diameter (~5-10 % of the diameter of the confined space), their distribution might well be critically restricted, leading to change in the overall hydrogen bond network configuration. In this light, the difference between H_2O and D_2O suggests that, although H_2O and D_2O vary little in their single molecule properties and geometry, the manifestation of cooperative structures such as density fluctuations, wires and cavities is different. This could mean that D_2O has either smaller sized heterogeneities, or a smaller number of them, which would then lead to

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an insignificant confinement effect for the length scales investigated here. This hypothesis is illustrated in Fig. 5.6C. In order to theoretically describe these structural differences between H_2O and D_2O and test our hypothesis it will be necessary to describe this extended confined hydrogen bond network on a quantum chemistry level. Although such computations have been done on a single water molecule embedded in an environment that can be described by a classical force field [151], it will have to be done for a cluster of ~10⁶ water molecules. This would involve computations on a scale that are currently not feasible.

The length scale over which the confinement effects are observed in H₂O in this study are much larger than the confinement length scales that have been previously observed, for example in porous silica [293, 338] (~10 nm), at surfaces with force measurements [295–297] (~4 nm), thin channels [294] (~20 nm), or carbon nanotubes [284, 289] (~2 nm). Based on the above explanation, that involves a restriction in the distribution of heterogeneous substructures, the difference is explained in the available degrees of freedom: a nanopore or a slit is in contact with surrounding bulk liquid, just like the water in a porous material and the water between an AFM tip and a substrate or the two substrates of a surface force apparatus. Given that the reorientation time scale of water and the lifetime of wires and cavities are on the order of picoseconds, there is enough contact with a reservoir of bulk water to enable a relaxation of the restriction. For the 3D liposome shells as investigated here this is not the case, since the hydrogen bond networks of the water molecules inside the shell are not connected to the hydrogen bond network outside the shell.

5.4 Conclusions

Summarizing, the 3D confinement of water was investigated by performing angle resolved polarimetric second harmonic scattering measurements on neutral and charged hollow lipid bilayer shells. These liposomes, having diameters of 58-140 nm contain $\sim 3 \times 10^6$ - 4×10^7 water molecules. We measured the change in water structure between the inside and outside water of the liposomes and compared it to the structural differences found for extended planar lipid membranes. While the latter show no detectable coherent SH response, the liposomes show a size dependent change in the SH intensity for both light and heavy water. The order of water as well as the surface potentials were retrieved from nonlinear light scattering modeling, which showed that 3D confinement manifests itself primarily in the amount of orientational ordering of the hydrogen bond network. For heavy water the shell-size normalized measured change in water ordering matches the change in available interfacial area. For light water, however, the change in orientational order is 8 times larger compared to heavy water and scales with the inner volume of the shells. Our findings demonstrate that 3D structural confinement effects in water can involve up to 10⁷ water molecules. That this has not been found so far is due to two things: the unprecedented sensitivity of second harmonic scattering to longer ranged structural changes [59], and the fact that previous measurements have studied systems in which the confined water was structurally in contact with an open bath [65, 66, 284, 289, 294–297]. The presented data demonstrates the intricate nature of water, and

the need to investigate water-water interactions over longer distances which clearly does play a role in nano- and microscopic structuring of aqueous systems. Biochemical equilibria that determine the aqueous cellular environment typically depend on small changes in hydration structure and energy landscapes. They might well be impacted by the observed confinement effects.

5.5 Appendix

5.5.1 Fitting parameters

For the fitting of the AR-SHS patterns, we used the parameters of Table 5.1. The ionic strength of the solutions was estimated to be 4μ M in the case of DOPC liposomes to account for H⁺ and HCO₃⁻ ions in solution. In the case of DOPS liposomes, it was computed from the counterions concentration coming from salt powder form of the lipids, divided by 2 as half of the counterions are entrapped to ensure electroneutrality inside the vesicles.

In the case of DOPC liposomes, the surface potential was fixed at a value identical for all the samples. On the contrary, the DOPS patterns were fitted with fixed values for $\chi_{s,2}^{(2)}$ and the surface potential left free. For the values for $\chi_{s,2}^{(2)}$, we used the ones from the fit of $\chi_{s,2}^{(2)}$ for DOPC in Fig. 5.6A.

5.5.2 AR-SHS patterns for DOPC and DOPS liposomes

The AR-SHS patterns and their fits are shown in Fig. 5.7 and 5.8.



Figure 5.7 – AR-SHS patterns of DOPC liposomes in H_2O (red, circles) and D_2O (black, circles). Data from -85 to + 85° correspond to the patterns in PPP polarization, while data from 275 to 445° correspond to the PSS polarization. The data were scaled with a factor based on the AR-SHS patterns of neat water measured on the same day, prior to be introduced in our algorithm. The results of the fittings are shown as red and black lines.

Chapter 5. The structural 3D confinement of water leads to structural changes involving 10^7 water molecules

	Φ ₀ (mV)	<i>R</i> _{eff} [nm]	$N_p \; [\mathrm{ml}^{-1}]$	Ionic strength of the solution [M]	Temp. [°C]	n _{liposome} (1028 nm)
DOPC in H ₂ O	-7	69.1778817 50.5388722 39.1332345 69.2279352 50.1151222 39.2573133 62.7365108 46.1574207 35.8107837 29.5165003 62.7365108 46.1574207 35.8107837 29.5165003 62.7365108 46.1574207 35.8107837 29.5165003	$\begin{array}{c} 2.30260 \times 10^{12} \\ 4.55881 \times 10^{12} \\ 8.57811 \times 10^{12} \\ 2.29918 \times 10^{12} \\ 4.63932 \times 10^{12} \\ 8.52124 \times 10^{12} \\ 3.13840 \times 10^{12} \\ 5.96773 \times 10^{12} \\ 7.84747 \times 10^{12} \\ 1.33060 \times 10^{13} \\ 3.13840 \times 10^{12} \\ 5.96773 \times 10^{12} \\ 7.84747 \times 10^{12} \\ 1.33060 \times 10^{13} \end{array}$	4.0×10 ⁻⁶	24	1.4
DOPS in H ₂ O	-100 (left free)	$\begin{array}{r} 23.3165003\\ \hline 63.7216922\\ 57.1684343\\ 43.4654538\\ \hline 63.8344313\\ 56.8135942\\ 44.2003171\\ \hline 61.4375304\\ 53.4733015\\ 40.7632766\\ 35.6922453\\ \hline 60.0674517\\ 54.1556270\\ \hline 41.8246132\\ 35.1964481\\ \end{array}$	$\begin{array}{r} 1.33000\times10 \\ \hline 2.22523\times10^{12} \\ 3.59098\times10^{12} \\ 5.25922\times10^{12} \\ 2.21713\times10^{12} \\ 3.63756\times10^{12} \\ 5.07805\times10^{12} \\ 2.62106\times10^{12} \\ 3.54331\times10^{12} \\ 5.68847\times10^{12} \\ 7.27474\times10^{12} \\ 2.74605\times10^{12} \\ 3.45134\times10^{12} \\ 5.39007\times10^{12} \\ 7.49287\times10^{12} \end{array}$	$\begin{array}{c} 2.72434 \times 10^{-4} \\ 3.51334 \times 10^{-4} \\ 2.90977 \times 10^{-4} \\ 2.72434 \times 10^{-4} \\ 3.51334 \times 10^{-4} \\ 2.90977 \times 10^{-4} \\ 2.97608 \times 10^{-4} \\ 3.01845 \times 10^{-4} \\ 2.66048 \times 10^{-4} \\ 3.01845 \times 10^{-4} \\ 2.97608 \times 10^{-4} \\ 3.01845 \times 10^{-4} \\ 2.66048 \times 10^{-4} \\ 2.66048 \times 10^{-4} \end{array}$	24	1.4
DOPC in D ₂ O	-7	65.8306638 49.4645134 38.3600411 60.1163606 40.7743185 67.1444513 49.5990949 38.7523285 61.6685042 41.660832	$\begin{array}{c} 2.54641\times10^{12}\\ 3.92067\times10^{12}\\ 7.44702\times10^{12}\\ 2.69019\times10^{12}\\ 5.61464\times10^{12}\\ 2.44483\times10^{12}\\ 3.89857\times10^{12}\\ 7.28936\times10^{12}\\ 2.55221\times10^{12}\\ 5.36707\times10^{12}\\ \end{array}$	4.0×10^{-6}	24	1.4

Table 5.1 – Input parameters used in the fit in Figures 5.2 and 5.6. The two samples selected for the patterns of figure 5.1E are highlighted.



Figure 5.8 – AR-SHS patterns of DOPS liposomes in H_2O (red, circles) and D_2O (black, circles). Data from -85 to + 85° correspond to the patterns in PPP polarization, while data from 275 to 445° correspond to the PSS polarization. The data were scaled with a factor based on the AR-SHS patterns of neat water measured on the same day, prior to be introduced in our algorithm. The results of the fittings are shown as red and black lines.

5.5.3 SH response in normalized intensity for DOPC liposomes in H₂O and D₂O

Figure 5.9 shows the integrated normalized intensity I_{norm} as a function of size of the samples for liposomes of DOPC in H₂O and D₂O. The two intensity curves follow very similar trends compared to the $\chi_{s,2}^{(2)}$ curves.

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Figure 5.9 – Integrated normalized intensity $I_{\rm norm}$ as a function of size of the samples for liposomes of DOPC in H₂O (red, rounds) and D₂O (black, squares). The error bars correspond to the standard deviation for an acquisition time of 20 × 1 s. Curve fittings are shown for $R_{\rm eff}^{-6}$ (continuous, red) and $R_{\rm eff}^{-2}$ (dashed, black) dependence respectively for liposomes in H₂O and D₂O.

6 Charge hydration asymmetry at the aqueous interfaces of liposomes and lipid droplets

Water molecules have been reported to hydrate asymmetrically cations and anions. This behavior extends to charged interfaces. Here, we investigate the charge hydration asymmetry by using angle-resolved second harmonic scattering and vibrational sum frequency scattering experiments on lipid droplets and liposomes in deuterated water, made of negative, zwitterionic, and positive lipids. The negative lipid droplets induce the strongest water ordering while the positive ones induce the weakest ordering, due to the electric field contribution from the negatively charged oil. On the other hand, the charge hydration asymmetry is modified around liposomes by the distribution of charges on both sides of the lipid bilayer. Positive liposomes exhibit the strongest transmembrane asymmetry in water orientational ordering. These drastic differences in charge hydration asymmetry highlight the dominance and specific distribution of neutral and negative lipids in cellular membranes.

The work for this chapter was done together with S. Pullanchery, T. Schönfeldovà and S. Roke. N.D. and T.S. performed SHS experiments. S.P. measured the SFS spectra. N.D., S.P., and S.R. interpreted the experimental data. S.R. conceived and supervised the work.

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6.1 Introduction

The dielectric continuum model of water predicts symmetric hydration of oppositely charged ions with similar sizes. Counter to this prediction, various phenomena indicate that positively and negatively charged ions are asymmetrically hydrated: The hydration free energies of anions are more negative compared to cations of similar size [339–341], the reorientation dynamics of water is different around cations and anions [91, 342], anions preferentially adsorb to the air/water interface more than cations [341, 343, 344] and the Hofmeister series of ions that determine solubility and stability of proteins in water is more pronounced for anions compared to cations [48, 345]. A preference of water molecules towards anions indirectly emerged from these observations. A first cause for this asymmetry is the structure of water: with one more negative oxygen atom and two small more positive hydrogen atoms, it is easier for the molecule to get closer to an anion than a cation. A second cause is the possibility for the water molecule of H-bonding with the anions but not with the cations [53, 91, 341, 346].

The direct test for asymmetry in the hydration of opposite charges however requires oppositely charged ions with identical chemical structures. A pair of large hydrophobic ions, tetraphenyl arsonium (TPA⁺) and tetraphenyl borate (TPB⁻), have been extensively studied in this context due to their opposite charges and otherwise identical molecular structure, as well as for their propensity to adsorb at aqueous interfaces [347]. Earlier studies assumed that the two ions have similar hydration energies and used them as standards for thermodynamic solvation scales [348, 349]. We tested this assumption in a previous study by measuring the water response of TPA⁺ and TPB⁻ ions in bulk water as well as at the surface of oil droplets dispersed in water [176]. TPA⁺ and TPB⁻ ions exhibit drastically different hydration behavior both in the bulk and at the interface. The vibrational Raman spectra of water molecules in the hydration shell of tetraphenyl ions revealed that water forms stronger and more abundant π Hbonds with TPB⁻ anions compared to the cations, corresponding to a free energy difference of around 2 kT between the two ions. This asymmetry in the hydration shell structure, along with the inherent asymmetry in water-ion interaction and dispersion interactions dictate the charge hydration asymmetry exhibited by TP ions [176, 350, 351]. Scheu et al. [176] also measured the interfacial water ordering at the surface of oil nanodroplets dispersed in water in the presence of TPA⁺ and TPB⁻ ions. TPB⁻ enhance the interfacial ordering of water molecules next to oil whereas TPA⁺ ions suppress the interfacial water ordering. The TPB⁻ ions form stronger and more abundant π H-bonds with neighboring water molecules, thus orienting more of them with their H atoms pointing towards the oil/water interface. In addition, the adsorption of TPB⁻ ions charges negatively the interface, inducing an electrostatic field that orients the water molecules in the same direction. On the other hand, the weaker π H-bonds between TPA⁺ ions and water molecules orient the water H atoms slightly towards the adsorbed ions, while the electrostatic field induced by the adsorption of cations tends to orient the water molecules in the opposite way, (as shown in Figure 6.1). Therefore, the asymmetry in interfacial water ordering can be understood as the result of a cooperative (for anions) or anti cooperative (for cations) interaction between the electrostatic and hydrogen bonding (H-bonding) effects.



Figure 6.1 – Illustration of the difference in surface hydration between oil nanodroplets with TPA⁺ and TPB⁻ ions, as elucidated by Scheu et al. in Ref. [176]: for negative ions, the hydrogen bonding and electrostatic interactions cooperate (bottom), while for positive ions they do not (top). Note that the fourth phenyl ring of TPA⁺ is pointing into the paper. Picture adapted from a scheme by R. Scheu in the cited article.

The results from tetraphenyl ions clearly showed that the origin of charge hydration asymmetry cannot be explained on purely electrostatic grounds. Moreover, negative charges seem to stabilize both molecular hydration shells and macroscopic oil/water interfaces. The apparent preference of water for negative ions is relevant for biological membranes which are commonly negatively charged [8]. The lipid composition of biological membranes is indeed dominated by lipids with zwitterionic and negatively charged polar headgroups [70, 77, 84]. The anionic and cationic groups on lipid headgroups are structurally diverse, and are expected to exhibit a more complex asymmetric behavior compared to structurally identical ion pairs. Quantifying charge hydration asymmetry in model lipid membrane requires experimental techniques that can selectively measure the interfacial water structure associated with charged lipid headgroups. Angle resolved [172] second harmonic scattering (SHS) [45, 161, 178] and vibrational sum frequency scattering (SFS) [126, 193, 352] are second order nonlinear scattering techniques that can selectively measure the response from orientationally ordered interfacial molecules at the surface of sub-micron sized particles such as lipid droplets and liposomes [45, 87, 88, 128, 136, 353]. For particles dispersed in water, the non-resonant SHS have been shown to measure predominantly the second order response from oriented interfacial water molecules [162, 185]. The SHS response from water at a charged interface include a fraction of water molecules that are ordered by the structure of the interface and another fraction of molecules that are oriented by the net electrostatic field originating from the surface which in turn report on the surface potential [171, 178, 183]. Along with interfacial water ordering, complementary information about chemical composition and molecular ordering at the interface can be extracted from vibrational SFS by measuring the vibrational resonances of interfacial molecules [87, 128, 130, 136].

Chapter 6. Charge hydration asymmetry at the aqueous interfaces of liposomes and lipid droplets

Herein, we investigate the charge hydration asymmetry of nanoscopic lipid droplets and liposomes formed with oppositely charged lipids and show that the hydration asymmetry is drastically different for lipid monolayer and bilayer systems. Lipids with headgroups containing negatively charged phosphate, positively charged trimethylammonium and zwitterionic groups where both the charges are present were selected to mimic the realistic functional groups present on biological membranes. The interfacial coverage and molecular ordering of lipid monolayers at the surface of oil droplets was measured with vibrational SFS. The negatively charged lipids form the most disordered monolayer on the oil droplet surface. However, the interfacial water ordering as measured by SHS is maximum for the negatively charged lipids, indicating that the electric field contributions by the lipid headgroup and the oil phase act cooperatively with each other. The two electric fields act anti-cooperatively for the positively charged headgroup, thus minimizing the interfacial water ordering. The zwitterionic headgroup showed a trend that was in between the oppositely charged headgroups. The hydration asymmetry is drastically different for liposomes when the charges are present at either side of the hydrophobic barrier. SHS measurements revealed the highest interfacial water order for liposomes containing the positively charged headgroups, followed by the negatively charged headgroup and zwitterionic liposomes ordered the least amount of interfacial water. The key difference between the positive and negatively charged headgroups being the ability of the negative charge to realize hydrogen bonding with water, we find that the hydrogen bonding and electrostatic effects interact anti-cooperatively for the negatively charged lipid across the bilayer membranes. The drastically different charge hydration asymmetry measured for the lipid monolayer and bilayer systems imply that hydration asymmetry of oppositely charged ions is the result of a delicate balance between electrostatic and hydrogen bonding interactions which are further modified when the charges are distributed on either side of the hydrophobic barrier of the cell membrane.

6.2 Materials and methods

6.2.1 Chemicals

Sulfuric acid (95-97%, ISO, Merck), ammonium hydroxide (30%, Sigma-Aldrich), hydrogen peroxide (30%, Reactolab SA), chloroform (Emsure, ACS, ISO, Merck), cholesterol, phosphorus standard solution (0.6 M, Sigma-Aldrich), L-ascorbic acid (ACS,≥99%, Sigma-Aldrich), ammonium molybdate (VI, ACS, 81-83%, Sigma-Aldrich), and d₃₄-hexadecane (C₁6D₃₄, 98% d, Cambridge Isotope) were used as received. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, chloride salt); zwitterionic 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC); negative 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA, sodium salt) were purchased in powder form (>99%) from Avanti Polar Lipids (Alabama, USA) and stored at -20 °C until further use. Ultrapure H₂O was obtained with a Milli-Q UF plus instrument (Millipore, Inc.), with an electrical resistance of 18.2 M Ω cm. D₂O used was 99.8% D atoms with an electrical resistance of >2 M Ω cm (Armar). The chemical structures of the used lipids are presented in Fig.6.2A.

6.2.2 Cleaning procedures

The cleaning procedures were described in section 2.3 of Chapter 2.

6.2.3 Sample preparation

Oil nanodroplets with a lipid monolayer were prepared according to the procedure described in Ref. [115], with 2 vol % d₃₄-hexadecane in D₂O. We mixed the solutions with lipid powders to reach a lipid concentration of 1 mM at a temperature of at least 5 °C above the transition temperature of the phospholipid by using a hand-held homogenizer (TH, OMNI International) for 4 min and an ultrasonic bath (35 kHz, 400 W, Bandelin) for the same duration. The resultant droplet system was used for SFS measurements and was diluted to 0.1 vol % hexadecane with D₂O for SHS measurements. The size distribution of the nanodroplets was measured with dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern). The nanodroplets had a mean hydrodynamic radius in the range of 170-200 nm with a polydispersity index (PDI) of less than 0.25. The hydrodynamic radii were calculated from the intensity autocorrelation function using the optical properties of the liquids (d₃₄-hexadecane and D₂O). The samples were stored and measured in sealed cuvettes. All measurements were performed at 24 °C.

Small unilamellar vesicles were prepared by extrusion according to Ref. [199–201] and section 2.3.2. The liposomes were extruded using a polycarbonate membrane (AvantiPolarLipids, Al) with pore diameter of 200 nm at room temperature. The size and ζ potential distribution of the liposomes were also measured with DLS and electrophoretic measurements at 24 °C (Malvern ZS nanosizer). To determine the size distribution of the vesicles, three subsequent measurements, each 11 runs, were averaged. To determine the ζ potential of the vesicles three subsequent measurements, each 30 runs at automated voltage, were averaged. The liposomes were found to have a mean diameter in the range of 120-145 nm with a polydispersity index (PDI) of less than 0.2. The final lipid concentration was determined using a calorimetric phosphorus assay; for further details see Ref. [202] and the protocol at Ref. [203]. The concentration of the lipids in the sample was 0.5 mg lipids/ ml weight ratio for DLS, ζ potential measurements, and SHS experiments.

6.2.4 AR-SHS measurements

Scattering patterns were recorded from -85° to $+85^{\circ}$ (0° corresponding to the forward direction of the fundamental beam), in 5° steps. The acceptance angle was controlled by an iris and set to 3.4°. Each data point was acquired with an acquisition time of 20×1 s and a gate width of 10 ns.

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6.2.5 Sum Frequency Scattering measurements

The experimental set-up for vibrational sum frequency scattering measurements has been described previously in detail [174, 352, 354]. An infrared (IR) and a visible (vis) laser beams were spatially and temporally overlapped at an angle of 20° in a sample cell with a path length of 200 μ m. The IR pulse was centered at 2900 cm⁻¹ (FWHM = 160 cm⁻¹) and the vis pulses at 12500 cm⁻¹ (FWHM = 15 cm⁻¹) at a repetition rate of 1 kHz. The SFS light was measured at a scattering angle of 55°, collected and collimated by a plano-convex lens (f = 15 mm, Thorlabs LA1540-B). The polarization of the IR beam was controlled by two BaF₂ wire grid polarizers (Thorlabs, WP25H-B), the vis beam by a polarizing beam splitter cube (CVI, PBS-800-050) and a half-wave plate (EKSMA, 460-4215) and the SF beam by a Glan-Taylor prism (Thorlabs, GT15-B). The SF beam was filtered by two short wave pass filters (3rd Millenium, 3RD770SP) before being spectrally dispersed with a monochromator (Acton, SpectraPro 2300i) and detected with an intensified CCD camera (Princeton Instruments, PI-Max3). The gate width was set to 10 ns, and the acquisition time for a single spectrum was 600 s. All SFS spectra were normalized by the infrared profile, measured as the sum frequency signal from a solid film of 100 nm BaTiO₃ particles detected under the same experimental geometry.

6.3 Results and discussion

6.3.1 Oppositely Charged Lipid Droplets

100 nm sized droplets of d_{34} -hexadecane covered with 1 mM of differently charged lipids were formed by the sonication method [87, 115, 136]. Negative, positive and zwitterionic lipid droplets were formed using the lipids 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 1,2dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) respectively. Fig 6.2A shows the structures of the lipids. The phosphate and choline groups present on these lipids are abundantly found in biological membranes [8, 70], providing a realistic model system to study charge hydration asymmetry in membranes. The interfacial water ordering of lipids with phosphate, choline and the phosphocholine headgroups have been extensively studied in Langmuir monolayers formed at the air/water interface [108, 355, 356]. However, water orientation by the same lipids at the oil droplet surface is expected to be different than the air/water interface because of the relative cooperativity between charge-charge, charge-dipole and hydrogen bonding interactions involving the water, oil and the lipid molecules themselves [134, 136]. Therefore, we first characterized the molecular ordering of the three different lipid droplets using vibrational SFS.

Fig. 6.2B shows the SFS spectra in the C-H stretch region measured under SSP (S-sum frequency, S- visible, P-infrared) polarization combination. The peaks around 2850 cm⁻¹, 2880 cm⁻¹, 2905 cm⁻¹, 2930 cm⁻¹, 2945 cm⁻¹ and 2965 cm⁻¹ correspond to the symmetric (s)-CH2 stretch (d^+), (s)-CH3 stretch (r^+), (s)-CH2-Fermi resonance, antisymmetric (as)-CH2 stretch, (s)-CH3-Fermi resonance and the (as)-CH3 stretch respectively. The ratio between



Figure 6.2 – (A) Structures of lipids used in this study. (B) Normalized SFS spectra of DOPC (green), DOPA (blue), and DOTAP (red) nanodroplets in the CH stretching modes region. The positions of the d^+ and r^+ modes are indicated by the dashed lines. The spectra are normalized to the intensity at 2945 cm⁻¹ for comparison. Solid lines are the result of the spectra fitting detailed in the Appendix. The spectra were measured and fitted by S. Pullanchery who also made the graph. (C) Normalized AR-SHS patterns of oil nanodroplets with DOPC (green), DOPA (blue), and DOTAP (red) lipids, recorded in PPP polarization combination. The patterns have been corrected for intensity differences due to differences in the number density and size distribution of the nanodroplets, and the resulting scattered droplet intensity was then normalized to the intensity of bulk water measured in the SSS polarization combination. (D) Illustration of the origin of charge hydration asymmetry for DOPA and DOTAP lipid droplets. For negatively charged lipids (bottom), the electric field contributions from the oil surface and the lipid headgroups act cooperatively to enhance the interfacial water orientation. For positively charged lipids (top), the two electric field contributions act anti cooperatively to suppress interfacial water orientation. The illustration was made by S. Pullanchery and N. Dupertuis.

the amplitudes (s)-CH2 stretch and the (s)-CH3 stretch (d^+/r^+) indicates the degree of tail ordering within the monolayer. $d^+/r^+ > 1$ corresponds to the dominance of gauche defects within the lipid alkyl tails, indicating a disordered monolayer. $d^+/r^+ \ll 1$ indicates a highly ordered monolayer with alkyl trains exhibiting an all-trans conformation [115, 357, 358]. For all three lipid spectra in Fig. 6.2B, $d^+/r^+ > 1$, indicating that all three lipids form disordered monolayers at the oil droplet surface. The d^+/r^+ ratio is 1.9 for DOTAP (Fig. 6.2B, red), 3.6 for DOPC (Fig. 6.2B, green) and 4.0 for DOPA (Fig. 6.2B, blue). The detail of the fitting of the spectra is shown in the Appendix section 6.5.3. Interestingly, for a previously studied system of fully saturated zwitterionic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) lipid, the droplets formed of 1mM lipid had almost no detectable gauche defects, indicating a highly ordered monolayer [115]. The difference between the DPPC and DOPC lipid droplets arises from the difference in phase transition temperatures (T_m): DPPC lipids are in gel phase at room temperature, whereas the unsaturated DO- lipids studied here have T_m well below room temperature, yielding liquid disordered monolayers at room temperature [359, 360]. On the other hand, charged lipids have a remarkably different mechanism of ordering at the

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interface. The degree of disorder previously measured in saturated 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA) monolayer [136] is similar to the degree of disorder measured here for the unsaturated DOPA lipids. Therefore, it is evident that the formation of charged lipid monolayers on the oil droplet surface is predominantly driven by electrostatic interactions. The resemblance between the formation of charged lipid and surfactant monolayers on oil droplet surface was pointed out in Ref. [136]. The similarity in the degree of disorder of DOPA and DPPA monolayers provides further proof for the idea that charged surfactants and lipids adsorb to the oil droplet surface with similar mechanisms. Previous studies on surfactants at the oil droplet surface have shown that negatively charged molecules form dilute monolayers on the oil surface without perturbing the molecular ordering of oil, whereas positively charged molecules penetrate intro the oil phase, forming a more ordered monolayer [175]. Indeed, from Fig. 6.2B, positively charged DOTAP monolayers have the lowest d^+/r^+ ratio which corresponds to a more ordered monolayer than the DOPA and DOPC lipids.

Next, we characterized the hydration asymmetry of the same lipid droplets with SHS. Fig. 6.2C shows the angular SHS patterns of DOTAP, DOPA and DOPC lipid covered droplets under PPP polarization combination. In non-resonant AR-SHS measurements, the SHS intensity reports specifically on orientational ordering of polarizable molecules, i.e. lipid or water, along the surface normal. Under illumination by an electric field $\tilde{E}(\omega)$, the second-order polarization $P^{(2)}$ (i.e. the average second-order response) of the medium is composed by the sum of the induced molecular polarizations $p^{(2)}$ per unit of volume [43]. This is expressed as: $P^{(2)}(2\omega) = \epsilon_0 \chi^{(2)}$: $\tilde{E}(\omega)\tilde{E}(\omega) = N \langle p^{(2)} \rangle$, where $\chi^{(2)}$ is the second-order susceptibility tensor that is characteristic of the material, ϵ_0 is the vacuum permittivity, N is the number density of probed molecules. Under our measurement conditions, water and lipids molecules are non-resonant. Therefore, using the anharmonic oscillator model [43], we can assume that the $\chi^{(2)}$ value is of the same order of magnitude for lipid and water. This means that individual lipid and water molecules generate about the same amount of second harmonic light. As a result, the respective contributions to the total SH intensity from these molecules at the hydrated membrane surface depends on their number densities. In a volume defined by a section of 10 nm² of the lipid interface and extending up to 3 nm from the lipid layer, there are $d \times 10 \text{ nm}^2 \times 3 \text{ nm} \simeq 1000$ water molecules (where $d = 33.2 \text{ nm}^{-3}$ is the number density of heavy water in ambient conditions). Considering the same 10 nm² section of the interface, and an average lipid headgroup area of around 0.66 nm², around 15 lipid molecules are in contact with water. As the SH intensity scales with the square of the number density of scattering molecules, this means that the ratio of intensity from water molecules over intensity from lipid molecules is around $\left(\frac{1000}{15}\right)^2 \sim 4444$. Therefore, SHS intensity emitted by hydrated membranes is originating mainly from water molecules, as shown by Yan et al [162].

Two major effects modify the orientational distribution of water next to lipid interfaces: First, the number and orientation of hydrogen bonds are perturbed by the presence of the lipidwater interface. Second, the interfacial charge causes a non-zero surface potential Φ_0 that induces ordering of water molecules via electrostatic charge-dipole interaction (see equation (6.1) in the appendix section 6.5.1) [171, 183]. From Fig. 6.2C, it is evident that oppositely charged lipids exhibit hydration asymmetry: Negatively charged DOPA lipids (Fig.6.2C, blue) show the maximum ordering of interfacial water whereas the positively charged DOTAP lipids (Fig. 6.2C, red) show the least amount of ordered water at the oil droplet surface. Interestingly, the positively charged DOTAP droplets order even less water compared to zwitterionic DOPC (Fig. 6.2C, green). This is surprising as DOPA and DOTAP droplets have strong absolute ζ potential values, of opposite sign (see Table 6.1). Therefore, one would intuitively expect the positive droplets to induce an interfacial water ordering of the same order of magnitude than the negative droplets. The ζ potential is however very distinct in nature from the surface potential, as it is derived from the mobility of the particles in the solution, and is a measure of the potential at the slipping plane.

ζ potential (mV)	DOPA	DOPC	DOTAP
Droplets	-55.6 ± 8.4	-7.9 ± 9.8	73.4 ± 15.3
Liposomes	-45.0 ± 14.1	-3.2 ± 11.3	56.94 ± 12.2

Table 6.1 – ζ potential values of droplets and liposomes in D₂O.

This behavior observed for droplets is clearly different from lipid monolayers at the air/water interface where charged lipids of either sign order more interfacial water molecules compared to zwitterionic lipids [108, 356]. To understand the origin of this asymmetry next to oil droplets, we first consider the orientation of water molecules next to bare oil droplets. Pure oil droplets carry a net negative charge on their surface [132, 178, 361] which creates an electric field that orders interfacial water molecules (Fig. 6.2D, left, bottom). The addition of negatively charged DOPA lipids also create an interfacial electric field in the same direction which enhances the net ordering of water molecules (Fig. 6.2D, right, bottom). On the other hand, the electric field created by positively charged DOTAP headgroups counteracts the electric field created by the oil surface (Fig. 6.21D, right, top) and effectively cancels out the interfacial water ordering. Based on the higher degree of order exhibited by DOTAP monolayers (Fig. 6.21B) and the near-zero SHS intensity generated by the water molecules (Fig. 6.2C) indicate that DOTAP forms a monolayer that is ordered enough to screen the negative charge originating from the oil molecules. The high degree of disorder exhibited by DOPA (Fig. 6.2B) points to a "patchy" monolayer in which areas of bare oil droplet surface are exposed to water along with the lipid headgroups. In addition to the negative charge, the DOPA headgroups also carry hydrogen bonding sites for water. Therefore, the overall SHS intensity exhibited by the DOPA droplets must originate from combination of water molecules that are oriented via electrostatic field and hydrogen bonding. The zwitterionic DOPC lipids generate ~5 times lower SHS intensity compared to DOPA, but a higher SHS intensity than DOTAP lipids. This intermediate trend is in agreement with the observations that DOPC droplets have small yet negative ζ potentials [87] and PC lipids in Langmuir monolayers order water molecules with their hydrogens pointing towards the lipid headgroup, similar to negatively charged lipids [108, 355, 362]. Therefore the phosphate groups of the PC headgroups play the predominant role in ordering water molecules mostly by hydrogen bonding with water and

В A С 2.5 DOPA DOPC droplets 2.0 Positively liposomes (scaled) DOTAP Integrated $I_{norm}(\theta)$ (nm⁻³) charged 2.0 l_{norm}(θ) [x10⁻³nm⁻³] liposomes 1.5 1.5 1.0 1.0 Negatively 0.5 charged 0.5 liposomes 0.0 0.0 50 DOPC DOPA DOTAP -50 0 Scattering angle 0 (°)

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Figure 6.3 – (A) Normalized AR-SHS patterns of liposomes in pure D₂O made of DOPC (green), DOPA (blue), and DOTAP (red) lipids (0.5 mg/mL), recorded in PPP polarization combination. The patterns have been corrected for intensity differences due to differences in the number density and size distribution of the liposomes, and the resulting scattered intensity was then normalized to the intensity of bulk water measured in the SSS polarization combination. (B) Comparison of the total values of $I_{norm}(\theta)$ from the AR-SHS patterns of figures 6.2C and 6.3A, integrated from -85 to +85° scattering angle. The liposomes values were scaled so that the value of DOPA liposomes match the value of DOPA nanodroplets. (C) Illustration of the origin of hydration asymmetry in liposomes. For positively charged lipids (top), the asymmetry is dictated by the electric field induced ordering alone. For the negatively charged lipids, the electric field induced ordering (bottom left) and the hydrogen bonding effects (bottom right) interact anti cooperatively with each other. The illustration was made by S. Pullanchery and N. Dupertuis.

weak electrostatic ordering effects to a minor extent. For oppositely charged lipids however, the relative cooperativity between different electric field contributions is sufficient to explain the hydration asymmetry as shown in Fig. 6.2D.

6.3.2 Oppositely Charged Liposomes

AR-SHS patterns of liposomes (~110-120 nm diameter) made of DOPA, DOTAP and DOPC lipids are shown in Fig. 6.3A. The intensity was corrected for the radius and the number of liposomes in the suspension. Surprisingly, in contradiction with lipid droplets, the positively charged lipids produced maximum SHS signal (Fig. 6.3A red) in liposomes, followed by DOPA (Fig. 6.3A, blue). Zwitterionic DOPC liposomes produced the least SHS response (Fig. 6.3A, green), generating ~14 times lower SHS intensity compared to DOTAP, and ~7 times lower compared to DOPA lipids.

Fig. 6.3B shows a comparison between the total AR-SHS intensities I_{norm} of droplets and liposomes integrated from -85 to +85° scattering angle. For comparison purposes, the liposomes total intensities were scaled with the same factor, so that the value of total intensities of DOPA liposomes and droplets are the same. With this scaling, it appears that the relative total intensity for liposomes and droplets follow a very similar trend between DOPC and DOPA membranes. Strikingly, the main difference resides in the DOTAP case. The SH response is higher for positive liposomes compared zwitterionic and negative ones, while it is dramatically lower in the case of droplets. In the case of liposomes, there are two lipid / water interfaces: on the inner and on the outer leaflets. SH light is generated by water molecules on both sides of the membrane. The non-zero SHS intensity therefore reports on the transmembrane asymmetry of hydration, across the lipid bilayer [130]. The AR-SHS patterns of zwitterionic and negatively charged lipids have been characterized previously [88, 130]. Our measurements match with these previous observations: negatively charged liposomes were found to have higher SHS signal compared to zwitterionic liposomes [130], owing to the higher electric field induced water ordering. Moreover, the electric field at the center of the liposome is expected to be zero based on Gauss' law [327], providing asymmetry in the electrostatic environment in the inside vs outside of the liposome. The absence of electric field in the inside is achieved via the neutralization of the lipid headgroups by counterion pairing on the inner lipid leaflet [285, 325]. Based on electrostatic grounds alone, one would have predicted DOTAP and DOPA liposomes to produce similar SHS intensities, as they carry ζ potential of roughly equal magnitude and opposite signs (see Table 6.1). Yet, clearly, DOTAP liposomes produce roughly twice more signal compared to DOPA, and ~14 times more signal than DOPC liposomes. Therefore, the molecular orientational ordering of water molecules is the most asymmetric across the DOTAP membrane among all the samples. Since the positively charged TAP group of DOTAP cannot form H-bonds with water, this group orders water molecules via the electrostatic field alone (Fig. 6.3C top). The asymmetry in electric field induced orientation across the membrane then generated the SHS response. The difference between hydration asymmetry of DOTAP and DOPA lipids originate from the ability of PA headgroups to hydrogen bond with water. As shown in Fig. 6.3C bottom, in addition to the electric field induced asymmetry, some water molecules are directly hydrogen bonded to the negatively charged lipid headgroups. The H-bonding interaction interacts with the electric field induced asymmetry anti-cooperatively to yield lower SHS intensity for the DOPA liposomes compared to DOPC lipids.

The above observations highlight the need to consider the membrane chemistry in relation with the surrounding water, and in terms of relevant interactions. For both liposomes and nanodroplets, the orientational ordering of water molecules occurs via a combination of: 1) the electrostatic charge-dipole interaction with the charge of the lipid interface, 2) the hydrogen-bonding between lipid headgroups and neighboring water molecules. These two interactions compete or cooperate to modify the amount of water molecular ordering or its transmembrane asymmetry. The electrostatic interaction may well be symmetric with respect to the sign of charge, but the dipolar nature of the water molecule and the directionality of the hydrogen bond [11] induce an hydration of the charged lipid membranes that is overall asymmetric. This balance is further modified when the charges are distributed on either side of the lipid bilayer. The consequence of this balance of interactions is that the water orientational ordering around positive lipid membrane is drastically different from that around zwitterionic or negative membranes. The water orientational ordering around positive lipid droplets is reduced depending on the lipid coverage, while it is strong and asymmetric across the membrane around positive lipid bilayers. These dramatic changes should be put in per-

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spective with the fact that membranes are mostly negative or neutral, and with the biological functions assumed by lipid asymmetry in membranes. Negatively charged lipids, even in small quantities, are distributed very specifically on the different cellular lipid membranes. They play a role in the recruitment of cationic proteins or enzymes, or in phenomena like apoptosis, blood coagulation, or membrane fusion [86, 363]. On the contrary, positive lipids are very scarce, and their excess is often associated to cytotoxicity [364–366].

6.4 Conclusions

In summary, we have investigated the differences in water molecules reorientation around charged and neutral lipid mono- and bilayers. We found that the monolayers of negatively charged lipids are the most disordered, but generate the most orientational water ordering, due to cooperative electric field contributions from the negative headgroups and the oil phase. On the contrary the contributions from lipid and oil act anti-cooperatively around positively charged lipid monolayers, thus reducing greatly the orientational ordering of water molecules. The zwitterionic monolayers follow an intermediate trend. In the case of lipid bilayers, the charge hydration asymmetry is drastically different because of the presence of two lipid/water interfaces on each side of the membrane. The positively charged lipid bilayer induces the strongest asymmetry in interfacial water ordering, followed by the negatively charged lipid bilayer. The zwitterionic bilayers are characterized by a reduced water ordering asymmetry, due to the absence of any electric field induced water ordering. As negatively charged headgroups can hydrogen bond with water molecules, contrarily to positively charged ones, the hydrogen bonding interaction orders water molecules on both sides of the negatively charged membrane thus reducing the water ordering asymmetry on both sides of the membrane. The well-known charge hydration asymmetry around ions in solution is modified at charged lipid interfaces. The charge hydration asymmetry observed between lipid monolayers and bilayers is drastically different and depends on a delicate balance between electrostatic and hydrogen bonding interactions. This charge hydration asymmetry is further modified by the presence of charges on the lipid headgroups on both sides of the bilayers.

This striking difference of charge hydration asymmetry brings thought material to understand the yet elusive balance of interactions between the different cellular organelles, and the peculiar distribution and dynamic exchanges of lipids between them. This might also shed light on the scarcity of positive lipids in biological cells, and on their frequent toxicity.

6.5 Appendix

6.5.1 The molecular sources for SHS from water around charged particles

As explained in section 2.1.2, there are two types of interactions contributing to the ordering of water molecules and thus to the SHS intensity. First, the presence of charges at the surface of the particle generates an electrostatic field. The different responses to this field can be grouped
under an effective third-order susceptibility tensor $\chi^{(3)'}$ as described in Ref.[183] Second, the orientational order induced by any other chemical interaction (e.g. hydrogen bonding) at the surface can be represented by the surface second-order susceptibility tensor $\chi_s^{(2)}$ [367, 368]. These two sources are represented respectively by the effective second-order and effective third-order particle surface susceptibility $\Gamma^{(2)}$ and $\Gamma^{(3)'}$ that depend on R, $\chi_s^{(2)}$, $\chi^{(3)'}$, and on the scattering angle θ . These effective susceptibility quantities capture the scattering geometry of the experiment, the interfacial structure, and the electrostatic field altogether. They are defined in more detail in section 2.1.2 and Refs.[182, 183]. The total SHS intensity coming from the particles in solution is therefore proportional to:

$$I_{2\omega} \propto \left| \Gamma^{(2)}(R, \chi_s^{(2)}, \theta) + \Gamma^{(3)'}(R, \chi^{(3)'}, \theta) \Phi_0 \right|^2$$
(6.1)

6.5.2 Origins of the SHS signal in the case of droplets and liposomes

In the case of oil droplets, the SHS intensity reports on the ordering of water molecules as explained in the section above.

In the case of liposomes, there are two lipid / water interfaces: on the inner and on the outer leaflets. SH light is thus generated by water molecules on both sides of the membrane. While the liposome bilayer is made of two lipid leaflet / water interfaces with same composition and opposite orientation, one would expect the SH signals from these two interfaces to cancel out, as observed in the case of planar lipid bilayers [316]. Therefore, any transmembrane asymmetry in hydration along the surface normal of the membrane will increase the overall SHS intensity. The first contribution comes from the curvature of the liposome membrane. It induces a small amount of transmembrane asymmetry in terms of volumes of ordered water on both sides of the bilayer. Therefore, the destructive interferences due to this partial symmetry between the inner and outer lipid-water interfaces reduce but do not cancel completely the amount of SH light generated. Additional interactions also contribute to increase or decrease the amount of transmembrane asymmetry. Typically, an interaction taking place only on the outer side of the membrane will increase the asymmetry and the overall SHS intensity. On the opposite, an interaction occurring similarly on both sides, might decrease the relative asymmetry, thus the intensity. To summarize, a strong SHS intensity from liposomes requires both a strong water orientational ordering by the lipid membrane, and a strong transmembrane asymmetry of this ordering.

6.5.3 SFS spectral fitting

The SFS intensity I_{SFS} can be described by the following Lorentzian line shape expression [369]:

$$I_{SFS} \propto \left| A_{NR} e^{i\Phi_{NR}} + \sum_{i} \frac{A_{i}}{\omega - \omega_{i} + i\gamma_{i}} \right|^{2}$$
(6.2)

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where A_{NR} is the amplitude of a weakly dispersive ('non-resonant') background, Φ_{NR} is the phase of the background signal relative to that of the resonant signal, A_i is the amplitude of the *i*-th vibrational mode with the resonance frequency ω_i and linewidth γ_i . The strength of the vibrational mode is proportional to A_i ($\omega = \omega_i$). The normalized SFS spectra were fitted using Eq. (6.2), employing IGOR Pro 7 (WaveMetrics) and using Levenberg-Marquardt iterations. The fitted parameters for the SFS spectra are shown in Table 6.2.

	Ai		
	$\omega_i (\mathrm{cm}^{-1})$		
	$\gamma_i (\mathrm{cm}^{-1})$		
mode	DOPC	DOTAP	DOPA
	16	9.3	20.8
d^+	2851	2863	2851
	12	14	14
<i>r</i> +	4.4	4.8	5.2
	2878	2884	2878
	10	12	12
<i>d</i> ⁻	1.1	1.0	2.0
	2906	2906	2906
	8	8	9
	10.1	10.5	11.4
d_{FR}^+	2927	2928	2928
	12	16	14
	5.0	9.0	8.0
r_{FR}^+	2945	2947	2945
	12	16	14
	-3.0	-0.1	-0.1
r ⁻	2965	2960	2960
	12	14	14
A_{NR}	0.1	0.1	-1.7
Φ_{NR}	-40°	-40°	-11°
d^+/r^+	3.6	1.9	4.0

Table 6.2 – Fitted frequency, amplitude and linewidth for the SFS spectrum in the C-H stretch region of the droplets.

7 Conclusion and outlook

7.1 Summary

In this thesis, we studied various aqueous soft matter systems by using second-order nonlinear spectroscopic techniques to probe water molecular orientational ordering. We focused first on pure liquids and electrolyte solutions, where the fluctuations and structuring effects break the centrosymmetry and can be probed with second harmonic scattering. Then, we investigated the hydration around model membranes made of lipid monolayers and bilayers. The results obtained improve the current knowledge of the structure of water, and of the balance of interactions occurring at hydrated lipid interfaces.

In Chapter 3 we probed the temperature dependance of intermolecular correlations in water, first in pure light and heavy water, and then in electrolyte solutions. The H-bond network of D₂O is relatively more structured compared to H₂O, because of stronger H-bonding, and exhibits more spherical symmetry. Temperature changes induce significant transformation in the structure of the liquid. First, the molecular orientational ordering of water decreases with increasing temperature. This effect is relatively stronger in H₂O than in D₂O. This goes together with a transition from correlations with non-spherical symmetry towards correlations with more spherical symmetry. Moreover, temperature changes also impact the structure of electrolyte solutions. The long-range orientational ordering effect caused by the addition of salt in water exhibit a shift towards higher concentrations when increasing the temperature. This indicates a corresponding decrease in the influence of the combined electrostatic field in the solution on the water-water correlations, or in other words a larger screening capacity of the liquid. The trend of the concentration shift qualitatively follows the theoretical Debye-Hückel model. However, the unexpected magnitude of the shift reveals that the model fails to grasp the complexity of the water structure, as it neglects H-bonding and nuclear quantum effects. This incompleteness reveals in particular in a relatively higher deviation for D₂O that has a stronger H-bonding network. These results present a challenge for future molecular dynamic simulations to refine the models of water.

This study was followed by an investigation of the source of molecular orientational

correlations in the SHS measurements of liquids, to discriminate between coherent crosscorrelations and incoherent auto-correlations. In Chapter 4, pure H_2O and pure CCl_4 , a model Lennard-Jones liquid, were studied by a combination of SHS measurements and molecular dynamics simulations. These showed that transient voids create heterogeneities in H_2O and CCl_4 , and that charge density fluctuations are present around these cavities in water, leading to coherent emissions of SHS. These coherent emissions relatively increase with temperature. These findings show that water is not a uniform and isotropic liquid, but is subject to nanoscale fluctuations that can live up to hundreds of femtosecond.

This heterogeneous nature of liquid water might well be crucial when considering the confinement of water by interfaces. In Chapter 5, we investigated the effects of the 3D confinement of water within zwitterionic and charged liposomes of different sizes, in terms of the change in water orientational ordering between the inside and the outside of liposomes. The measured change in SH intensity with respect to the size of liposomes was analyzed with a nonlinear scattering model, and separated into two contributions: the interfacial orientational ordering of water and the surface potential. The confinement effects are primarily caused by the interfacial ordering. For heavy water, the measured size-dependent change follows the change in available interfacial area. On the contrary, the change in orientational order in light water is 8 times stronger than in heavy water and matches with the change in inner volume of liposomes. Our results demonstrate that 3D confinement effects can extend to a larger scale than previously reported, and involve up to 10⁷ water molecules. This could be achieved thanks to the unprecedented sensitivity of SHS to the collective intermolecular correlations, and because of the confinement of water without any contact to an external bath.

Finally, we investigated the differences in hydration around neutral and charged lipid membranes, in the form of monolayers (lipid droplets) and bilayers (liposomes). Around the monolayers, the negative lipids generate the most water orientational ordering, while the positive lipids generate the least. This charge hydration asymmetry originates from respectively cooperative and anti-cooperative electric field contributions from the charged lipids and the oil that acts as negatively charged. The charge hydration asymmetry is very different around lipid bilayers made with the same lipids. The negatively charged bilayer generate a strong transmembrane asymmetry in water orientational ordering, but the positively charged bilayer induce an even stronger transmembrane ordering asymmetry. Overall, the charge hydration asymmetry results from a delicate balance between electrostatic and hydrogen bonding interactions, that is further modified by the presence of charges on lipid headgroups on both sides of the bilayer.

7.2 Outlook

These results provide advancements in our understanding of the intricate nature of water, and of the delicate balance of hydration of membranes. It appears crucial to investigate water-water interactions over long distance, and to include the balance of all interactions involved in

the hydration of membrane future studies, with suitable techniques and simulations. This thesis opens several possibilities for future research.

The isotopic differences in the long range interactions reported in Chapters 3 and 5 call for the systematic inclusion of nuclear quantum effects (such as hydrogen bonding) and detailed non-local structures into new models of water. Water is clearly not an isotropic liquid, and its heterogeneous nature should not be neglected in future studies. In Chapter 4, we showed that fs-ESHS can detect the heterogeneities in liquids with a high sensitivity. We observed in particular a deviation of the SHS intensity from theoretical predictions for incoherent HRS intensity, attributed to additional coherent contributions. Following the reflexion around the presence of a coherent contribution only in PPP and PSS polarization combinations, it should be possible to separate the coherent response from the incoherent response in the measured SHS patterns. This can be done by extracting the $\left\langle \left(\beta_{yyy}^{(2)}\right)^2 \right\rangle$ and

 $\left\langle \left(\beta_{yxx}^{(2)} \right)^2 \right\rangle$ from the fully incoherent SSS and SPP patterns. These values can then be used to compute the incoherent contributions to PPP and PSS patterns according to HRS theory. Any excess of SHS intensity in real measurements compared to the theoretical predictions can thus be attributed to a coherent response and the presence of intermolecular correlations. Together with additional MD simulations, this could allow a more accurate characterization of the heterogeneities, bringing further insight into the liquid structure. In addition, the measurements and simulations realized in this chapter will be extended to other liquids with various interactions and structures, such as polar liquids without hydrogen bonding, or polar liquids that form a different hydrogen bond network compared to water. For instance, alcohols are known to usually form 1D networks, formamide a 2D layered network, and so on. Formamide in particular would be interesting to investigate as it has been proposed in several life origin scenarios as an alternative solvent of life. Measurements were and are currently conducted on different liquids by Y. Chen, N. Dupertuis, and T. Schönfeldová. The studies will be brought also by T. Schönfeldová to liquid mixtures such as dimethylsulfoxide (DMSO) in water, used for cryogenic and solubility enhancement purposes.

In terms of lipid biochemistry, the investigations carried out in Chapters 5 and 6 provided a better molecular understanding of water confinement and charge hydration asymmetry around model lipid membranes. The findings of Chapter 5 should be further investigated by molecular dynamics simulations. In terms of applications, a first outcome is that the relative increase of the normalized intensity caused by the confinement of water could be used at our advantage in vibrational SFS measurements of liposomes. Due to the quasi-symmetry of the lipid bilayers, some of the scattering intensities are indistinguishable from noise in these measurements. Using smaller liposomes could be a way to eventually measure these contributions. Another possibility would be to add water channels into the membrane, to verify if this would be enough of contact to an external open bath to reduce the confinement effects. On the other hand, the study of the balance of interactions responsible for the charge hydration asymmetry could be expanded to a variety of lipids with different headgroups, selected to study different balances of interactions. The systems could be made more complex also by adding different ions in the solution to get closer to the physiological conditions, and to observe their impact on the hydration of the membranes. For instance, the lipid membrane investigation could be extended to the label-free permeation studies of ions through the lipid bilayers of liposomes. Preliminary measurements (not shown in this thesis) indicate that the tetraphenyl ions used in the so-called TATB hypothesis do not permeate into neutral liposomes symmetrically with respect to their charge. This kind of study could be pursued in the direction of the growing research field of drug encapsulation in liposomes.

Finally, the observed charge hydration asymmetries around lipid monolayers and bilayers could be put in relation with the dominance of neutral and negative lipids in natural membranes. In particular, negative PS lipids, mostly present on the inner leaflet of cells and organelles, are essential for cell signaling and survival. Their transport to the outer side of the membrane is characteristic of cellular conditions such as apoptosis, cancerous behavior, or Alzheimer disease, and thus essential for the recognition and clearance of such cells. On the contrary, positive lipids are very rare, and often related to toxicity or used for gene transfection experiments. It would be interesting to investigate if the observed reduction of orientational ordering of water around model oil droplets could be linked to an instability for the equivalent organelles present in cells, inducing their aggregation or fusion. Also, as the lipids are displaced through cells compartments via multiple biological processes, it could indeed be unfavorable for a biological cell to contain such positive lipids in general.

Nonlinear spectroscopic techniques coupled with scattering are experimental tools with an exquisite sensitivity to interfaces and heterogeneities. They can be used hand in hand with simulations to address many physical, chemical, and biological but also fundamental questions. The versatility of these techniques allows to study many different systems at the molecular level, ranging from liquids to realistic model systems. Given the efficiency of using light to investigate water and order, it seems that there are many opportunities and a bright future ahead to unravel the mysteries of our universe.

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Education

- 2017–2021 **PhD at the Laboratory of fundamental BioPhotonics**, *EPFL*, *Lausanne (Switzerland)*.
 - 2016 **Master thesis project at the Research Institute in Applied Mechanics**, *Kyushu University (Japan)*.
- 2011–2016 Master of Science in Physics Engineering, EPFL, Lausanne (Switzerland).
- 2011–2016 Bachelor of Science in Physics, EPFL, Lausanne (Switzerland).

Professional experience

- 2020 Start-up foundation, Oryl Photonics Sarl, Lausanne (Switzerland).
- 2017-2021 **Teaching assistant in the Laboratory for fundamental BioPhotonics**, *EPFL*, *Lausanne (Switzerland)*.
- 2015-2016 **Graduation internship on sensors integration in biomedical devices**, *Biosafe SA*, *Eysins (Switzerland)*.

Selected conferences

- 2019 Gordon Research Conference on Chemistry and Physics of Liquids, Holderness, United States
- 2019 CECAM workshop on Water dynamics, Paris, France
- 2017 GSS Summer School Solvation Science, Bochum, Germany

Scholarships and Awards

- 2020 La Forge Award, at Innosuisse Business Concept course
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- 2017 Travel grant for GSS Summer school in Bochum (Germany)
- 2016 Friendship Scholarship of Kyushu University (Japan)

Qualifications and skills

Wet Chemical safety; preparation, analysis & handling of liposomes and oil-in-water nanoelaboratory mulsions; piranha glassware ultrapure cleaning Optics Laser safety; femtosecond laser spectroscopy; second harmonic generation; optical laboratory parametric amplifier; dynamic light scattering

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Extracurricular activities

Associations

EPCEPFL Photonics Chapter : Officer (2021); Treasurer (2020); Officer (2019)NewspaperContributor to EPFL satyrical newspaper "COUAC" (2015-2016)PhysicsSecretary for EPFL physics student association "Les Irrotationnels" (2015)associationSecretary for EPFL physics student association "Les Irrotationnels" (2015)

Interests

Writing Short stories author, participating to writing workshops
History Historical grand strategy games; uchronia
Roleplaying Member of ARNV (Association Rôlistique du Nord Vaudois) since 2017
Sport Badminton, swimming