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Review

Hydration of proteins and nucleic acids: Advances in experiment and theory. A review

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ABSTRACT

Background: Most biological processes involve water, and the interactions of biomolecules with water affect their structure, function and dynamics.
Scope of review: This review summarizes the current knowledge of protein and nucleic acid interactions with water, with a special focus on the biomolecular hydration layer. Recent developments in both experimental and computational methods that can be applied to the study of hydration structure and dynamics are reviewed, including software tools for the prediction and characterization of hydration layer properties.
Major conclusions: In the last decade, important advances have been made in our understanding of the factors that determine how biomolecules and their aqueous environment influence each other. Both experimental and computational methods contributed to the gradually emerging consensus picture of biomolecular hydration.
General significance: An improved knowledge of the structural and thermodynamic properties of the hydration layer will enable a detailed understanding of the various biological processes in which it is involved, with implications for a wide range of applications, including protein-structure prediction and structure-based drug design.

1. Introduction

Water is a ubiquitous element that is indispensable for all organisms. Life evolved in water for billions of years, during which biomolecules "learned" to utilize their aqueous surroundings in both structural and functional terms. Water constitutes ~50–70% of cell content and is therefore not only the native environment in which all biological processes occur but also an integral part of nearly all of biological processes [1–3]. The structural integrity of most bio-macromolecules depends on water, and their mutual interactions determine biomolecular dynamics and function. A certain critical level of hydration, usually estimated to $h \approx 0.2$ (g of water per g of protein) is required for the physiological function of most native proteins [4]. Water is sometimes described as the "twenty-first" amino acid [5], an "integral part of nucleic acids" [6] or a "biological" molecule [7]. However, while water is indispensable for the proper functioning of biological molecules, the functionality dominantly belongs to the biomolecule itself, and thus the term "biological water" should be avoided [8]. Regardless of terminology, it is now clear that water acts not only as a solvent for biological processes, but actively participates in most of them, influencing structure, dynamics and interactions of biomolecules. Thus, it is not surprising that the topic has attracted increasing attention as knowledge of biomolecular systems expands. Several reviews [9–12], books [13] and even special journal issues [14] have been dedicated to this subject. New techniques have been developed, and existing techniques have been improved and applied in novel ways. These developments are encouraging because different techniques can probe different properties and access different time and length scales, and each technique can thus contribute important details to the overall picture of biomolecular hydration.

In this article, we review recent developments in the study of biomolecular hydration. In the first part (Section 2), we provide a brief overview of the biophysical processes in which water is involved, focusing on the properties of surface hydration of native proteins and nucleic acids. Due to the breadth of this topic, many other important aspects of the role of water in biological processes, such as recognition, binding, and catalysis, are mentioned only briefly. The main focus of this review is recent developments in methods that can be applied to the study of hydration structure and dynamics, which are discussed in the second

Abbreviations: MD, molecular dynamics; PMF, potential of mean force; RDF, radial distribution function; SAXS, small-angle X-ray scattering; SANS, small-angle neutron scattering; EINS, elastic incoherent neutron scattering; QENS, quasi-elastic neutron scattering; NMR, nuclear magnetic relaxation; NOE, nuclear Overhauser effect; NMRD, nuclear magnetic relaxation dispersion; ODNP, Overhauser dynamic nuclear polarization; ESEEM, electron-spin echo envelope modulation; DRS, dielectric relaxation spectroscopy; DSC, differential scanning calorimetry; TDFSS, time-dependent fluorescent Stokes shift; QM, quantum mechanics; KITA, kinetic terahertz absorption; H/D, hydrogen/deuterium; 2D-IR, two-dimensional infrared vibrational echo spectroscopy; VSFG, vibrational sum frequency generation spectroscopy; PB/SA, Poisson–Boltzmann/surface area; GB/SA, generalized Born/surface area; IFST, inhomogeneous fluid solvation theory; GIST, grid inhomogeneous solvation theory; 3D-RISM, three-dimensional reference-interaction-site model; GCMC, grand canonical Monte Carlo method; CCG, Chemical Computing Group.

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part (Sections 3 and 4), including X-ray and neutron diffraction and scattering, NMR techniques, time-resolved fluorescence, terahertz spectroscopy, dielectric spectroscopy, and molecular dynamics (MD) simulations. For each method, we critically review its principles and inherent advantages and drawbacks and provide an overview of its contributions to our understanding of the properties of biomolecular hydration. We also compare experimental studies with computer simulations and theoretical models and discuss advances in computational methods and software tools for hydration-site prediction and analysis (Section 5).

2. Water as an indispensable component of biomolecular systems

At the molecular level, the interface at which water contacts macromolecules or molecular aggregates is of greatest interest both biologically and biophysically. Water participates in the folding of the three-dimensional structure of proteins [9,15–18] and nucleic acids [19]. Besides that, water also stabilizes the native structure of proteins [20,21] and nucleic acids [22] and contributes to protein thermal stability [23]. Lowering the relative humidity of DNA duplexes of certain sequences can induce their transition from the most stable B-form to another right-handed form, A, or even to left-handed Z-DNA [24].

The aqueous environment also influences protein binding. Water enhances the interactions between proteins and small ligands [25] as well as other biomolecules [9,26–28] and is involved in protein aggregation [29–31]. Water also mediates DNA-peptide recognition [32], and the interactions between DNA and minor-groove binders [33]. Water molecules often mediate sequence-specific recognition between proteins and DNA sites [34,35]; these interface water molecules are numerous and have specific dynamic properties [36]. Structured water molecules (with long residence times) in the active site are important for ligand recognition and selectivity [37], whereas the dynamics of water molecules determine ligand binding [38] and dissociation [39]. Whereas some water molecules directly mediate binding at the ligand-biomolecule interface, other water molecules are forced to leave the interface to enable ligand access, which contributes significantly to the overall thermodynamics of binding [27,40].

The hydration level influences the dynamics of biomolecules. At low temperatures (below ~200 K), hydration water was reported to suppress protein dynamics, whereas at higher temperatures it facilitates protein fluctuations [41], as shown by neutron scattering measurements of the differences in atomic mean-squared displacements between dry and hydrated proteins. The sharp increase in the mean-squared atomic displacement in proteins at temperatures above ~200–230 K, the so-called dynamic transition, is often ascribed to liquid-glass transition of hydration water [42,43], however, the exact nature of the coupling between these phenomena is still debated. This dynamic crossover has been observed only in hydrated biomolecules (globular proteins [44], intrinsically disordered proteins [45], and RNA [46]) and is absent in dry molecules. The strong influence of solvent translational motion and hydrogen-bonding lifetimes on biomolecule dynamics observed in these studies suggested that the dynamics of biomolecules are controlled by, or "slaved" to, solvent motions [15,47]. However, subsequent experiments revealed significant differences in the dynamics of various proteins, RNA, and DNA and their hydration water [41,48], indicating that the simple picture of slaved dynamics is incomplete. An interdependence between biomolecules and their hydration water is now accepted, but the exact mechanism by which protein and hydration water dynamics are coupled is not yet fully understood [49].

The plasticizing and lubricating effects of hydration water are also an integral component of the biological function of native biomolecules. While some enzymes remain active in organic solvents, they typically retain a hydration layer on the surface, and only a few examples of enzymes that are active below this level of hydration exist [50,51]. Generally, the catalytic function of most enzymes decreases seriously as the hydration level is reduced [4,52,53]. In addition to facilitating the larger conformational transitions necessary for catalysis and allostery [3], water also assists substrate binding [25,54] and is directly involved in the chemical process of hydrolysis reactions. Hydration influences enzyme kinetics [55] and enantioselectivity [56] and affects the electrochemical potential of an enzyme [57]. A hydration funnel of modified solvent dynamics has been proposed to contribute to net enzyme reactivity [58]. Solvent dynamics also plays a role in the catalytic function of ribozyme [59]. The water structure at biomolecular surfaces and the specific interactions of water molecules are key determinants of important cellular processes, such as water transport through aquaporin channels [60], hydrophobic gating of ion permeation in ion channels [61], and mechanogating of the mechanosensitive channel MscL [62].

While playing an important role in the processes mentioned above, the structure and dynamics of water itself are greatly perturbed by the presence of solute biomolecules [11]. This is because of both specific effects, such as the interactions of water molecules with the solute, and nonspecific effects, in which the solute poses boundary restrictions on the H-bonded network of water molecules [3,63]. The structural and dynamic properties of biomolecule-associated water, or water in the hydration layer, thus differ significantly from the properties of bulk water. Nevertheless, the detailed knowledge of these differences continues to be fragmentary, and thus the intensity and the cause of this effect at the molecular level continue to be active topics of investigation [64], as discussed later in this review (particularly in Sections 4.4.1 and 4.4.2). Several factors hinder the characterization of this perturbation: the complicated nature of liquid water, the heterogeneous water/biomolecule interface, and the unclear boundary between bulk water and the hydration shell associated with the biomolecule, as discussed below.

First, although water is a rather simple molecule, comprising only one oxygen and two hydrogens, the structure of water in the liquid phase is surprisingly complicated. Water, due to its small size, tetrahedral shape and capacity to form up to four hydrogen bonds gives rise to a dynamic, three-dimensional network that can readily rearrange in contact with solutes [3,13,65]. Despite great efforts, the multifaceted properties of water structure have not yet been fully described even for pure water. This complicated nature of liquid water confers on it its specific and unique characteristics and undoubtedly contributes to its many anomalous properties, e.g. the most well known density anomaly, in which the liquid phase has higher density than the crystal phase [66], or the vigorously debated liquid water polymorphism [67]. However, the molecular-level origins of these anomalies are not yet fully understood.

Second, water interacting with biomolecules occupies a complex, heterogeneous environment. Some water molecules are buried inside the core of globular proteins, representing an integral component of protein structure by interacting with the unsaturated hydrogen-bonding capacities of main-chain polar groups not involved in secondary structures and of buried polar side-chain atoms [68–70]. Other water molecules are located in confined regions such as internal cavities and active sites. The hydrophobic enclosure of these sites can lead to anomalous entropic and enthalpic penalties of hydration, contributing to ligand binding affinity [25], whereas the distinct dynamic properties of water at the active site have been suggested to contribute to efficient catalysis [71]. The environment of water at the surface of a protein or nucleic acid, which is the main focus of this review, is equally complex. The structure and dynamics of the water molecules in the hydration layer are affected by the complex topography (surface clefts, grooves, pockets and protrusions) [72–74] and varied chemical composition (hydrophobic, polar or charged groups) [64,75] of the biomolecule's solvent-exposed surface. This picture is further complicated by intermolecular interactions with other solvent molecules [9,28,76] and the effect of crowding [77,78].

Third, there is no universal definition of hydration water, and the interpretation of what actually constitutes the hydration layer of a biomolecule typically depends on the nature of the technique used to

probe the structural and dynamic properties of the system [8]. The thickness of the hydration layer is thus a matter of debate, with estimates ranging from one water layer to more than 20 Å [79]. Most methods, particularly those probing structural properties or single-particle dynamics at picosecond or longer time scales, such as X-ray and neutron scattering [80], crystallography [81], and nuclear magnetic relaxation dispersion (NMRD) [82], as well as most MD studies [75] report short-range effects of a biomolecule on hydration water, resulting in a hydration layer thickness of one or two water molecules (~3–5 Å). Other studies, particularly those on collective dynamics and shorter (sub-picosecond) time scales, typically using terahertz [12,83,84] and dielectric spectroscopy [85] as well as some MD studies [86,87], describe a long-range influence and estimate the hydration layer mean thickness as greater than 10 Å, or 3–5 hydration shells. This discrepancy has led to disputes over the correct interpretation of the results of terahertz spectroscopy [88]. However, most of the seemingly contradictory results are gradually being reconciled to a unified picture of the hydration shell. This, we believe, is possible if the fact that different techniques can reveal different aspects of hydration by probing different phenomena at highly varied time scales, is kept in mind [12].

In addition to the question of how many waters are perturbed in the hydration layer, there is the question of the magnitude of this perturbation. An important and, for a long time, controversial question has been the degree by which the reorientation time of water in the hydration layer differs from the one of bulk water (2.5 ps). Early measurements of hydrodynamic radius, dielectric relaxation experiments and NMR-NOE experiments were erroneously interpreted as indicating an extremely slow, almost ice-like hydration layer of biomolecules moving rigidly with the solute at approximately nanosecond timescales [11,82]. Subsequent NMRD measurements [89] and MD simulations [90] reported only moderate (2–3 fold) retardation of rotational dynamics compared to bulk water, whereas several studies of TDFSS measurements [10,91] reported a pronounced slowdown by orders of magnitude relative to bulk water. However, this slow relaxation process (with timescales of hundreds of picoseconds) was later attributed to the motion of the biomolecule itself and not its hydration water [92]. The current picture, based mainly on NMR experiments [89] and MD simulations [64,93], is one of dynamic heterogeneity. With the exception of a few long-lived water molecules buried within the biomolecule's deep clefts, the remaining ~90% of interfacial water molecules are only modestly slowed by the presence of the biomolecule. However, the molecular details of the process, e.g. which properties of the biomolecular surface determine the dynamical perturbation, have only recently started to be deciphered [64], and the study of hydration structure and dynamics thus remains to be a very dynamic field of research.

3. Methods of studying hydration structure

3.1. Crystallography

Macromolecular crystallography enables to gain unparalleled insight into the spatial distribution of water molecules in the vicinity of biomolecular surfaces [94]. The technique is based on the diffraction (elastic scattering) of an X-ray or neutron beam by a protein or nucleic acid crystal and provides information on the mean positions of atoms in the crystal, thus enabling the construction of a molecular model of the biomolecular structure. In this static (or rather time-averaged) structural picture, well-ordered hydration sites are usually observed around polar and charged regions, where the first, directly H-bonded shell of waters is visible, or in confined regions such as active sites, pockets and clefts, where the first two or even three shells can sometimes be resolved. Depending on the crystal, atomic and sometimes sub-atomic (<1 Å) resolutions can be achieved, allowing the determination of the positions of hydrogens and thus hydrogen bonding and protonation states, information that can be critical for understanding macromolecular function. The greatest limitation of this method is the difficulty of obtaining crystals of larger, non-globular or disordered systems. Moreover, even for crystallizable macromolecules, it is unclear how closely the crystal environment, which is influenced by crystal packing and cryogenic temperatures, resembles the biomolecule's native conditions. However, crystallography still provides the most detailed experimental information on the structure of biomolecules and their ordered hydration layer.

Several early studies in the 1980s and 1990s elucidated the distribution of water around proteins and nucleic acids based on crystal structures. For DNA, these studies produced the concepts of "spine of hydration" in B-DNA [95] and "economy of hydration" in A-DNA relative to B-DNA [96]. In proteins, water was observed to be structured around polar and charged groups at the surface and inside clefts and cavities [97]. These internal hydration sites are often conserved among proteins of the same family [98]. Among surface hydration sites, the most conserved are those with the most H-bond partners, both from proteins and from neighboring water molecules [99]. By contrast, diffuse, partially disordered solvent structure is observed around hydrophobic patches [100], and around disordered regions of the macromolecular structure, making the water positions much more difficult to resolve. Problems arising from modeling these disordered regions of the hydration layer as a single structure can be at least partially overcome by ensemble modeling of crystal structures [101].

In addition to providing information on the hydration of individual proteins and nucleic acid structures, the increasingly large volume of available structural data has been used to derive more general insights using data mining approaches, beginning in the late 1980s [102] and 1990s [103]. These studies focused on the water distribution around individual building blocks (amino acid residues, nucleotide bases, and later also phosphates) [104]. Although the number of available crystal structures at the time was low (with low tens of structures used in each study), these studies demonstrated that the water distributions are non-random, creating hydration patterns consistent with the hydrogen-bonding capacity of the polar atoms or at a van der Waals distance from apolar sites (although most such waters are also H-bonded to a nearby polar atom).

These analyses have been recently refined by Nakasako et al. [105], who calculated a series of empirical functions capturing water distribution close to polar atoms of protein main-chain and side-chain groups based on 17,984 crystal structures. The devised functions have direct applications in hydration structure prediction and crystallographic refinement [106]. Similarly, Zheng et al. [107] recently determined the radial distribution functions of water in the proximity of different protein atom types, together with the equivalent potentials of mean force (wPMF), thus enabling the assignment of wPMF scores to particular water molecules in a protein structure and the prediction of potential hydration sites. However, these studies did not consider the local environment of the residue/base. This deficiency was addressed in a recent study that analyzed the conformational dependence of the hydration patterns of amino acid residues in proteins [108] and revealed a strong dependence of the positions and occupancies of the hydration sites on residue conformation.

Analogous to proteins, DNA hydration sites organize into networks that depend on the type of DNA structure and on base sequence [109,110], and their spatial properties can be described by an elastic potential [111]. A detailed analysis of the first solvation shell (water and ions) of nucleic acid structural elements and its conformational dependence was subsequently performed by Auffinger and Hashem [112] and is also available as a web service [113]. The localized hydration sites can be used to predict the binding of protein residues to DNA [114] as well as other small-molecule ligands [115]. Another recent study highlighted the importance of water-mediated contacts in protein-DNA binding [35].

Several data mining studies have focused on the differences between water at the hydration layer and in bulk. Chen et al. [81] have computed the radial distribution function (RDF) of water in 105 protein crystal structures at sub-angstrom resolution. The RDF indicates that

hydration-layer water is more similar to liquid water than solid ice. Its first and second maxima (2.75 and 4.5 Å, respectively), are in the same position as those for bulk water, but the peaks in hydration water RDF are sharper, which corresponds to the hydration water being more static than the bulk water. However, the tetrahedral order parameter of water in bulk and near the protein reveals that water is most ordered at a distance from the protein surface between the first and second solvation shells [116]. A powerful new method to assess the resolution of water molecules in PDB structures based on the electron density around individual atoms was recently proposed by Nittinger et al. [117]. The authors applied this method to a large-scale statistical analysis of over 2 million water molecules and obtained a comprehensive delineation of their structural and spatial properties.

3.2. SAXS and SANS

In contrast to X-ray and neutron crystallography, small-angle X-ray and neutron scattering (SAXS and SANS) techniques are based on the elastic scattering of macromolecules in solution. These techniques allow the study of various biological molecules and biomolecular complexes in a wide range of conditions, with no need to crystallize the sample and without the constrains regarding molecular weight which are limiting e.g. in NMR measurements. Compared to crystal diffraction studies, a limitation of these solution techniques is the low resolution of the structural information they provide. On the other hand, SAXS and SANS can be used to investigate biomolecular hydration in a more native-like environment and thus assess the effect of varying solvent compositions. Recent years have witnessed a rapid development of SAXS, both in hardware equipment (radiation sources, detectors) and software for data analysis, as well as a significant increase in studies based on this method [118].

From SAXS measurement, reconstruction of the molecular contours is possible with low resolution. The interpretation of scattering intensities requires accurate predictions based on structural models of the components of the measured system. An important aspect of the fitting process is accounting for the contribution of the solvent to the scattering intensity. This contribution originates from two sources, the solvent-excluded volume and the hydration layer at the surface of the biomolecule. The first hydration layer has an average scattering density ~15% greater than that of bulk solvent, as reported for several proteins based on SAXS and SANS experiments [80]. This increased density was explained by MD simulations in terms of a shorter average distance between water molecules and an increased coordination number due to the disruption of tetrahedral ordering [75,119]. This hydration shell has been traditionally modeled by including a uniform solvent layer of constant thickness around the biomolecule using the program CRYSOL [120]. In reality, however, the hydration shell density (or, the average water-water distance and order parameter) is nonuniform, with angstrom-scale variations due to the different chemical properties of the atoms of the biomolecular interface [121]. Therefore, there has been a collective endeavor to establish more elaborate and physically based representations of the hydration shell. Examples of such recent approaches include the Poisson-Boltzmann-Langevin formalism (AquaSAXS [122]), Zernike polynomial expansions [123], proximal radial distribution functions (HyPred [124]), positioning dummy waters at exposed surfaces (FoXS [125]), and explicit solvent MD (WAXSiS [126]).

Despite these developments in the modeling of the hydration layer, few SAXS studies have focused on analyzing solvent properties in recent years. Stanley and colleagues [127] used SANS coupled with osmotic stress to estimate the amount of water molecules associated (solute-excluded) with lysozyme and guanylate kinase in a solution with polyethylene glycols of varying molecular mass. The calculated thickness of the effective preferential hydration layer surrounding the protein was less than one water layer (3 Å) for all measured solutes, indicating that the water layer surrounding the protein should not be considered to cover the protein surface completely. Albeit indirectly, this interpretation of the solution data supports the general validity of crystallographically observed water molecules.

Similarly, Schneidman-Duhovny and colleagues [128] used contrast variation experiments to validate the hydration layer model in their FoXS program by measuring SAXS profiles at different concentrations of NaCl and KCl. For glucose isomerase, the density of the hydration layer did not vary, whereas for lysozyme, an increase was observed in the hydration layer density at higher salt concentrations. The authors attributed this difference to the more hydrophobic surface of lysozyme, which enables the incorporation of more ions into the first hydration layer with increasing ionic strength. However, they obtained a similarly good fit of SAXS curves by varying the thickness of a layer of explicit water molecules from high-resolution crystal structures. For glucose isomerase, the optimal crystal water layer thickness of 2.8 Å yielded the best fit for all salt concentrations, whereas the optimal thickness varied with salt concentration for lysozyme.

Solution scattering experiments clearly suggest that the properties of the hydration layer differ from those of the bulk, but the thickness and density of this layer remain unclear. To support the interpretation of the SAXS and SANS data, a more explicit model of the hydration layer derived from molecular theory or computer simulations is needed. The recent developments mentioned above hold promise for application in this direction.

4. Methods of studying hydration dynamics

4.1. Neutron scattering in solution: EINS and QENS

4.1.1. Elastic incoherent neutron scattering (EINS)

Among the techniques available to study biomolecular hydration, neutron scattering is notable because of its unique sensitivity to hydrogen atoms. Hydrogens, which are poorly visible via X-ray, have an incoherent neutron scattering cross-section ~40 times larger than any other atom in a biological sample, including deuterium, and incoherent scattering in these systems is almost entirely due to hydrogens in biomolecules or water molecules. The first EINS measurements of protein dynamics were performed by Doster et al. [129] and the field was subsequently advanced most notably by the group of G. Zaccai [130]. A simple way to focus the measurement on the hydration layer is to minimize the signal from the biomolecule by its perdeuteration, i.e., fully replacing its hydrogens with deuterium. EINS on hydrogenated and deuterated proteins hydrated in D_2O and H_2O, respectively, can then be used to calculate the mean-square displacement in the unmasked part of the sample, thus providing information separately on the protein's global flexibility and on water motions on a ps-ns timescale and angstrom length scale. This experiment has been performed by Combet and Zanotti [131], who measured powders of hydrogenated and perdeuterated C-phycocyanin protein and concluded that “the interfacial water is the main ‘driving force’ governing both local and large-scale motions in proteins”. Gallat et al. [45] have reported results complementing these measurements by comparing the coupling between the dynamics of water of hydration and protein atoms in intrinsically disordered proteins and in folded proteins of a similar molecular weight. The authors reported that within a wide range of temperatures the disordered protein tau and its water of hydration have similar atomic mean-square displacements, a sign of a close coupling between their dynamics, whereas the folded proteins displayed only moderate to weak coupling between their atoms and the surrounding water.

4.1.2. Quasi-elastic neutron scattering (QENS)

Elastic scattering techniques enable the study of the structure of biomolecules and their hydration layer and, in the case of EINS, their mean-square displacement. However, to study the dynamics of these systems in detail, we must shift our attention to inelastic scattering. QENS allows the study of nano- to picosecond dynamics at angstrom-length scales [132]. QENS is inelastic scattering that is nearly elastic, centered at

zero energy transfer, and enables to monitor the specific energy exchange between the neutron beam and the sample, which reports on the timescales of motion of its atoms. Quantification of the energy transfer can thus be used to study various relaxation phenomena, such as vibrations, translational diffusion and molecular reorientations. However, to interpret the data from QENS measurements on biomolecular systems, at least qualitative input from theoretical modeling (typically MD simulations) is necessary. The information that is extracted from the measured incoherent scattering function on the various underlying motional modes is thus highly sensitive to the model used. Another limitation of both EINS and QENS is that they require relatively high concentrations of biomolecules in the sample, leading to non-linear effects caused by the water molecules being influenced by several solute molecules.

The first application of high-resolution QENS to the dynamics of protein hydration was reported by Randall et al. [133]. QENS has since been used to study hydration water in peptides [5], proteins [41,134], nucleic acids [46,48,135] and even inside cells [136–138]. Using neutron scattering, a dynamic transition in the picosecond motions of hydration water can be observed at a temperature of ~220 K, similar to the temperature of the glass-like dynamic transition in proteins [139] and in RNA [140], suggesting a coupling between the dynamics of the biomolecule and its hydration water. The nature of this coupling remains a topic of debate. The transition observed in water dynamics has been suggested by Chen et al. [141] to originate in a liquid–liquid phase transition in the protein hydration water. However, other studies suggest that the dynamical transition originates from kinetic phenomena and is an indication for a liquid-glass transition of hydration water [41,42]. Schirò et al. [142] recently proposed, based on QENS measurements fitted using a rotation–translation model of hydration water motions and supported by MD simulations, that it is the translational component of water diffusion on a protein surface that promotes the large-amplitude anharmonic motions in the protein, which are prerequisite for its physiological function. Similar results were obtained for globular and for intrinsically disordered proteins, suggesting that this connection is independent of the long-range order in the protein molecule, and that instead it is an intrinsic property of the protein dynamics. The same group also reported enhanced water mobility around tau amyloid fibers [31] and proposed that this enhanced water mobility might promote fiber formation through entropic effects.

4.2. NMR spectroscopy

4.2.1. NOE

The field of NMR spectroscopy of biomolecular hydration was pioneered most notably by the group of K. Wüthrich [143] using the intermolecular ^{1}H–^{1}H nuclear Overhauser effect (NOE) technique, which is based on magnetization transfer between water and protein protons. The development of a two-dimensional NOE technique (NOESY) and other technical developments enabled the determination not only of the location of biomolecular hydration sites in solution but also of their residence times. The advantage of the NMR technique is that it employs dilute solutions compatible with the two-state description of the system as hydration water and bulk water.

Early NMR-NOE spectroscopy studies of proteins demonstrated that a small number of water molecules with long residence times were buried in the protein interior in locations identical to those in the crystal structures [143]. However, the water molecules on the protein surface had residence times in the sub-nanosecond range, even in the case of hydration sites that contained well-ordered water in the X-ray crystal structures. The residence times of the surface hydration sites were originally estimated to be 300 ps to 1 ns. These residence times have since been reinterpreted [144] by accounting for long-range dipolar couplings with bulk water and for the exchange of labile hydrogens, resulting in time scales shorter by an order of magnitude. In the case of DNA, the studies indicated [145,146] a parallel between the structure of the first hydration shell in solution and in crystals, at least for the well-ordered waters of the "spine of hydration" in the Drew-Dickerson dodecamer [95]. These NMR studies are important because they demonstrate the principal correspondence between the first hydration shell in solution and crystal in both proteins and DNA and indirectly validate studies of the structure of hydration in crystal structures.

However, the domination of water-protein NOE signal by long-range dipole-dipole couplings to bulk water [144] has hindered the resolution of most of the surface hydration, with the exception of the places where the monitored protein proton is located close to a buried hydration site with a water molecule of sufficiently long residence time. Encapsulation in reverse micelles was recently shown to dramatically slow both the hydration dynamics and hydrogen-exchange kinetics, enabling the spatial resolution of the hydration dynamics of surface water molecules. Using this technique, Nucci et al. observed a wide range of dynamics of hydration water near the surface of the protein ubiquitin, with apparent spatial clustering of hydration sites with similar dynamics [147]. Moreover, the regions of long-lived hydration water correspond to protein surface areas that participate in protein-protein interactions, indicating an evolutionary adaptation in this particular protein that maximizes the contribution of solvent entropy to the free energy of binding [148], but the generality of this observation remains to be explored. It also remains to be established how the results obtained from measurements in reverse micelles relate to the situation in free aqueous solution.

4.2.2. NMRD

Nuclear magnetic relaxation dispersion (NMRD) is an NMR technique based on the measurement of the longitudinal spin relaxation rate of magnetic nuclei, in the case of water of ^{1}H, ^{2}H (deuterium) or ^{17}O. The dispersion, or the frequency dependence of relaxation rates, carries information on the underlying thermal molecular motions, and yields a Fourier transform of orientational time correlation function. The magnet technology that is nowadays available allows to access ^{2}H or ^{17}O frequencies up to hundreds of MHz, corresponding to a time scale of ~1 ns. This means that for long-lived, typically buried, water molecules the NMRD profile can provide information about their number and residence times. For the short-lived water molecules at the protein surface, however, only the average rotational correlation time can be obtained if the number of water molecules influenced by the biomolecule is known - an information usually obtained from MD simulations or simply estimated from the solvent-accessible surface area [82]. NMRD studies of protein hydration have mainly been performed by the Halle group [82]. In combination with MD, their measurements have demonstrated that the protein hydration dynamics is very similar to that of small peptides, except for a limited number of water molecules in protein-specific hydration sites. In this context, hydration can therefore be viewed as a medium-range phenomenon that is determined essentially by the chemical environment of a studied system. Moreover, if the functional form of the distribution of correlation times is known, then the NMRD experiment can be used to determine its parameters. With the input from MD simulations, which indicate that the distribution follows a power law, the measurements reveal that most (about 90%) of the hydration layer waters exhibit merely a two times slower dynamics compared to bulk water, and only a few waters stay trapped in surface cavities for extended intervals of up to ~1 ns [89]. These studies also demonstrated that below certain temperature, activation energy of water rotation around a hydrophobic peptide is decreased compared to bulk water, which signifies that the hydrophobic hydration water rotates faster than in the bulk [149]. This is in accord with the current view that the classical "iceberg" model of hydrophobic hydration, which assumed that hydration layer of hydrocarbons is composed of clathrate water, is misleading. The Halle group has also applied NMRD techniques to measure water dynamics in living cells [150]; ~85% of cell water exhibited bulk-like dynamics, contradicting the results of previous QENS experiments by the Zaccai group [137], which indicated that a significant portion of the

intracellular water manifests substantial retardation. The Zaccai group argues, however, that while NMR measures water re-orientation, QENS can capture both translational and rotational diffusion, and therefore the results from these two experimental methods complement each other instead of being contradictory [138]. The NMRD technique was also applied to study the mechanism of water escape from the internal cavities of myoglobin [151], which suggested that a microsecond escape of trapped water molecules occurs via hydrogen-bonded water chains that temporarily permeate the structure of the protein.

4.2.3. ODNP

In addition to the well-established NMR techniques of NOE and NMRD discussed above, Overhauser dynamic nuclear polarization (ODNP) has recently been applied by the group of Songi Han [152,153] as a magnetic resonance technique that allows highly localized measurements of hydration dynamics via site-directed labeling with a stable radical as an electron spin resonance (ESR) probe that is chemically attached to a macromolecule of interest. The response of nearby water molecules to the excitation of the spin probe ESR transition is then measured by ODNP as an enhancement of their NMR signal. The measurement enables quantification of the diffusional motion of hydration water in the range of tens to hundred of picoseconds in the vicinity (~5–15 Å) of the spin label. The highly amplified NMR signal allows the usage of very low sample volumes and concentrations. The main requirement is a reliable, site-specific incorporation of a stable spin label, typically nitroxide or trityl radical.

This technique has been used to study the hydration dynamics of apomyoglobin in native, molten globular, and unfolded protein states [154]. The translational dynamics of water in the native state is heterogeneous, with the surface hydration shell displaying dynamical retardation of only 2–4 fold relative to bulk water, whereas internal sites interact with dynamically restricted bound water. In the molten globular state, however, the hydration dynamics of the hydrophobic core is only slowed down 4–6 times compared to bulk water, while the unfolded state involves rapidly diffusing water, suggesting that hydration dynamics is strongly affected by local topology of the protein surface. A similar explanation may apply to the anomalously fast translational diffusion of water near exposed backbone positions in DNA duplex reported by the same group [155]. ODNP has also been used in combination with another state-of-the-art magnetic resonance technique, electron-spin echo envelope modulation (ESEEM), to investigate the density and dynamics of water at the surface of free GroES and confined within the cavity after forming the chaperonin GroEL-GroES complex [156]. The results revealed no change in the density and dynamics of water upon complex formation but indicated unusually fast (bulk-like) diffusion dynamics of water near the GroES surface.

4.3. Dielectric spectroscopy

Dielectric relaxation spectroscopy (DRS) has long been used to study biomolecular hydration. DRS measures polarization as a response of the sample in aqueous solution to a time-dependent external electric field. The polarization can be investigated either in the time domain or as a function of the frequency of a harmonic field. The frequencies accessible to DRS reach from the far infrared (terahertz) region to megahertz; however, the terahertz range relevant for most molecular motions is only now becoming accessible [157]. The relaxation peaks in the DRS spectrum recorded in the frequency domain reveal the relaxation times of the individual dipole-active processes within the sample. This information enables the measurement of molecular motions such as rotations and re-orientations. However, DRS detects the composite response of all species with a dipole moment within the sample, and thus encompasses many different types of fluctuations, including the collective modes. Thus, the interpretation of the spectra is largely dependent on the molecular model used to interpret the observables in terms model parameters. DRS also requires relatively high protein concentrations, which can lead to non-linear effects on the measurement.

The DRS spectra of biomolecular samples can be decomposed into several processes, with two dominant modes ascribed by consensus to protein tumbling (β-dispersion at ~10 MHz) and to reorientation of bulk water (γ-dispersion at ~20 GHz). Another mode, denoted δ-dispersion, is frequently reported between these modes, the origin of which is disputed in the literature. Traditionally, the presence of a nanosecond timescale process indicated by the δ-dispersion was interpreted as the presence of a large number of very strongly retarded water molecules in the hydration shell. However, this interpretation has been contested [82], and newer experiments suggest that the δ-dispersion originates from slow rearrangements of the biomolecule that then induce changes in the hydration layer [158]. The nature of the mutual influence of the dynamics of the biomolecule and its hydration layer is also a matter of debate. Based on DRS and other experiments, some researchers argue that large-amplitude motions of proteins are “slaved” to bulk solvent fluctuations, determined by its viscosity [47]. Other studies contradict this view by demonstrating that because proteins, DNA and RNA have different relaxation times and temperature dependencies, biomolecular dynamics cannot be entirely slaved to the dynamics of surrounding solvent, and reciprocal influence between biological macromolecules and their hydration water should be considered [48]. The combination of broadband dielectric spectroscopy and differential scanning calorimetry (DSC) has also been used to study the role of water in protein glass transition [43], and enabled to relate the calorimetric glass transition to the relaxation processes observed using DRS.

A recent study focused on elucidating the fluctuations in the orientational and positional structures of protein hydration shells. By combining DRS with depolarized light scattering, the authors demonstrated that the perturbation of the water orientations is long-ranged and propagates three to five hydration layers into the bulk at normal temperature [85]. This structure can be interpreted in terms of dipolar nanodomains in the hydration shell extending 12–15 Å from the protein surface [159].

4.4. Light spectroscopy

4.4.1. TDFSS

One of the most important methods of light spectroscopy applied to the study of biomolecular hydration is the time-dependent fluorescent Stokes shift (TDFSS) pioneered by the Zewail group [91]. TDFSS measures the electric-field dynamics around a chromophore, typically a tryptophan or an attached chemical group. The excitation of the chromophore induces an instantaneous change in its dipole moment, and as the environment relaxes in response, it creates a reaction electric field stabilizing the excited state of the probe and shifting its fluorescence to lower energy. Measuring the time dependence of the fluorescence spectrum thus provides information on the electric field relaxation of the environment surrounding the chromophore. In effect, the TDFSS measurement can be viewed as a localized variant of dielectric spectroscopy. TDFSS was one of the first methods to enable site-specific, time-resolved investigation of hydration dynamics. Another advantage of TDFSS is that it can be performed with diluted samples. However, the chromophore's fluorescence energy is influenced by many processes in the sample, making it difficult to decompose the measurements into individual contributions. These include the collective motions of solvent molecules around the chromophore, counter-ion movement and the motions of the solute itself. Thus, obtaining a molecular description from the dynamics of the electric field is impossible without input from MD simulations or other computational models to aid the interpretation of the results.

The TDFSS measurements demonstrated that in a range of systems, including both protein and DNA, a biomolecule-attached probe has a biphasic distribution of time scales upon excitation [10,91]. The presence of the slower process, with decay times extending from ~10 ps to a few ns, was interpreted as an indication that a portion of the water

molecules in the hydration layer of proteins and nucleic acids are slowed by several orders of magnitude relative to bulk solvent. However, Halle and Nilson [92,160] argued that this interpretation contradicts the picture of water dynamics based on NMR relaxation and MD simulation studies (for details, see the respective sections) and that the long relaxation times measured by TDFSS are associated with slow conformational motions of the biomolecule rather than the fluctuations of water molecules in the hydration layer. Using a simple dielectric model, they demonstrated that the slow decay originates from solvent polarization due to the time-dependent electric field generated by the charges on the protein and the probe. Thus, Halle and Nilsson consider the protein to be the dominant source of the slow decay. Other groups [161,162], however, conclude based on MD simulations that the dynamics of water and protein is strongly correlated, and thus coupled water-protein motion is responsible for the slow dynamics.

The view of Halle and Nilsson was supported by an MD simulation by Furse and Corcelli, who calculated time scales for the relaxation response upon excitation of a probe bound to DNA [163]. The water dynamics around the DNA-bound probe was only retarded by a factor of 2–3, whereas DNA fluctuations corresponds to the slow relaxation term. The results of Furse and Corcelli were contradicted by a study by Sen, Andreata et al. [164], whose simulations suggested that the contribution of the DNA itself to the signal is minor. However, in Furse and Corcelli's study, the probe is farther from the major groove waters, whereas in the system studied by Sen et al., it was in the base stack of the DNA and thus may report on water confined in the grooves of the DNA. Moreover, later simulations of Furse and Corcelli succeeded in attributing the slow TDFSS component to a specific motion in damaged DNA [165].

More recently, Scott and Callis [166] used TDFSS to monitor the effect of the mutation of nearby charged groups on tryptophan fluorescence. Interestingly, the steady-state fluorescence spectral maximum remained virtually unchanged upon the charge-altering mutation of nearby residues. The authors rationalized this observation based on MD and QM calculations and concluded that the contributions from water compensated for the Stokes shift relative to the shifts created by the charged groups. Similarly, Biesso et al. [167] measured the TDFSS of a solvent-exposed tryptophan upon titration and observed constancy of the dipolar dynamics with charge variation. They concluded, based on MD and QM-MM simulations, that the invariance of the slow relaxation component originates in a strong coupling between the protein and its water environment, due to which the alteration of protein's electrostatic properties are counterbalanced by the adjustment of water polarization.

Very recently, a new, simple fluorescence spectroscopy technique was proposed by Amaro et al. [168]. This technique utilizes basic laboratory instruments and is based on the incorporation of an unnatural amino acid as fluorescent probe into the protein structure, which enables site-specificity of the measurement. Analysis of the steady-state fluorescence spectroscopy signal then provides information on the molecular environment of the probe. The measurements, however, report only on the level of hydration of a specific site (number of water molecules within ~5 Å) and not on the hydration dynamics.

4.4.2. Terahertz

Since the turn of the millennium, accessibility of a new generation of sources and detectors of terahertz radiation has opened up the so-called "terahertz gap", i.e., the window between microwaves and infrared frequencies, for biophysical investigations. A key terahertz spectroscopy method is terahertz time domain spectroscopy (THz-TDS), a broadband technique that is usually used for frequency range of 0.1 to 4.0 THz. An alternative technique is based on a p-Ge (p-type germanium) THz laser source, which operates at a single frequency at a time within a more limited frequency range but has the highest output power of the current THz sources [12,84]. Terahertz absorption spectroscopy is important because of the numerous processes that occur in water in this part of the electromagnetic spectrum on the picosecond time scale, including low-frequency collective motions of waters, fluctuating orientations of molecular dipoles and collective intermolecular vibrations. Although it is a label-free technique, terahertz absorption spectroscopy reports not only the collective motions of many water molecules but possibly also the motions of the biomolecule. Thus, MD simulations and theoretical models are necessary to dissect the terahertz spectrum into the contributions of the underlying intermolecular motions.

Advancements in the terahertz technique by the Havenith group have expanded its applications to studies of biomolecular hydration. Based on the concentration dependence of terahertz absorption and an overlapping hydration layer model, they estimated that the dynamically perturbed hydration layer extends very far from the protein surface, ~15–20 Å or even more, corresponding to several layers of hydration water [79,84]. These findings are, however, in stark contrast with studies of protein hydration dynamics by other methods, particularly by NMR [82,89] and MD [169], which indicate that a significant perturbation of water dynamics is limited to the first hydration monolayer. Thus the interpretation of the observations from terahertz absorption measurements of the hydration dynamics of proteins and carbohydrates has been disputed, and some "unphysical assumptions" [88] of the model used for the interpretation of the terahertz measurements have been noted. However, collective modes of extended perturbations of the hydration layer propagating up to 10 Å from the protein surface have been indicated by newer computer simulations [87]. Terahertz and femtosecond infrared spectroscopy were also recently applied to study the hydration of small ions and revealed cooperativity effects and the perturbation of water dynamics extending, in certain cases, well beyond the first hydration layer [170]. Thus, the precise interpretation of the terahertz observations is a matter of active debate.

A recent technical advancement of terahertz spectroscopy is the development of kinetic terahertz absorption (KITA) spectroscopy [171]. KITA combines terahertz time domain spectrometry with a stopped-flow apparatus, which enables to detect real-time changes in terahertz transmission arising during kinetic processes. This method, in combination with other time-resolved measurements, such as transient kinetic methods and stopped-flow X-ray absorption spectroscopy, allows changes in biomolecular hydration dynamics to be followed in real time during biological processes, e.g., protein folding [171] or enzymatic reactions [54,58].

4.4.3. 2D-IR

The ultrafast two-dimensional infrared (2D-IR) vibrational echo spectroscopy is analogous to 2D NMR, but operates at much shorter time scales. The advantages and limitations of this method were recently reviewed by Koziol et al. [172]. Although IR spectroscopy does not provide high spectral resolution, it enables the study of non-equilibrium processes in a pump-probe fashion due to its sub-picosecond time resolution. The 2D-IR technique is suited for probing dynamics of water hydrogen bonds in a various systems, including the hydration shell of biomolecules. The nonlinear 2D-IR method enables to visualize the dynamic response of water hydrogen bonded to the amide groups a protein or peptide. The dynamics of water near an amide causes fluctuations of its vibrational frequency. Isotopic labeling of specific locations can be used as a direct probe to map the time dependence of these fluctuations and the identification of associated water molecules at chemical bond-scale spatial resolution [173,174]. In DNA oligomers, 2D-IR spectroscopy is capable of resolving the congested stretching absorptions of the NH and OH groups. The femtosecond pump-probe studies at various levels of hydration identified water molecules interacting with specific binding sites around the phosphate groups of DNA backbone, together with another water species exhibiting properties more similar to bulk water [175]. Femtosecond 2D-IR spectroscopy has also revealed reduced structural fluctuations of water in the hydration layer of DNA [176].

Measurements of 2D-IR spectra in combination with H/D exchange enable correlating sensitivity of the amide I frequency to secondary structure with the sensitivity of the amide II band to deuteration. The protein is

dissolved in D_2O, inducing H/D exchange of the amide protons. By observing the 2D-IR cross peaks, water accessibility in the protein can be mapped [177]. Fast 2D-IR measurement also allows monitoring of the kinetics of H/D exchange on the fly [178]. 2D-IR spectroscopy can be employed to measure hydration water dynamics in a site-specific manner by covalently attaching a vibrational probe at the protein surface. These experiments have revealed spatially heterogeneous hydration dynamics, with nearly bulk-like water frequency dynamics next to the exposed, flexible protein groups and more dynamically constrained water due to limited hydrogen bonding network near the more rigid, extended surfaces of a protein [179]. Recent 2D-IR study on the effect of crowding on the dynamics of protein and its hydration water reported a sharp dynamical transition below a certain hydration level. Both the protein dynamics and the hydration dynamics exhibited pronounced slowdown, which the authors attributed to an independent-to-collective hydration transition, with the estimated protein-protein distance at which this phenomenon occurs of 30–40 Å. [78].

4.4.4. VSFG

In vibrational sum frequency generation spectroscopy (VSFG), a visible and an infrared photon are combined to generate a photon at their sum frequency. Symmetry constraints render centrosymmetric media (bulk samples) inaccessible to this technique, making it highly specific to surfaces, thus enabling gas–solid, gas–liquid and liquid–solid interfaces to be probed with high selectivity. The generation of a spectral response is enhanced when the IR frequency is resonant with a vibrational mode of a molecule at the interface [180]. This technique was recently applied to the protein/water interface to probe the ice-binding site of an antifreeze protein AFP-III [181]. The authors observed, even at room temperature and in an aqueous solution, ice-like water layers at the surface of the protein. When the temperature was decreased to the physiological temperature of operation for AFP-III, i.e. between 0 °C to −2 °C, the amount of ice-like ordered water increased. A single-point mutation disrupted completely the water ordering as well as the antifreeze activity of the protein, indicating that these layers of ordered ice-like water molecules are necessary for the recognition and ice-binding activity. However, a different mechanism of antifreeze action was recently observed using the VSFG technique in another antifreeze protein, DAFP-1. In this case, the mechanism depends on short-ranged interaction of an extremely well-ordered array of threonine residues in the ice-binding site with the ice surface [182].

4.5. Theoretical models and simulations

As indicated above, the interpretation of data from many experimental techniques requires a theoretical model. Such a theoretical framework can also help to provide a coherent interpretation of potentially contradictory results from different experimental techniques. Theoretical predictions, by contrast, require experimental validation and testing of the scope of the model. Thus, theory and experiment have complementary roles. Most often, such a theoretical framework is derived from MD simulations. The advantage of MD is that it enables the simulation of any molecular system of choice. It can also provide site-resolved information that is otherwise only accessible by a few experimental techniques, such as fluorescence spectroscopy [10] and special NMR techniques [147]. However, the simulation of larger systems with explicit solvation can become prohibitively time consuming, highlighting the need for less computationally expensive methods.

4.5.1. Single particle properties

Initially, MD studies of biomolecular hydration focused on the structural aspects of hydration and the agreement between experimentally observed hydration sites and the probability distributions obtained from simulation [22,183–185]. These early MD studies generally confirmed experimental results, such as the above-mentioned observation [80] that within the 3 Å of the first hydration layer of proteins, water has 15% higher density than in the bulk [75], or the renowned spine of hydration observed in the minor groove of duplex DNA and the different hydration properties of A- versus B-DNA and of C/G versus A/T base pairs [186]. More recently, the three-dimensional solvent distributions around proteins obtained from MD simulations were compared with the results obtained using integral equation theories and proximal radial distribution method; the agreement between MD and the other two, less computationally demanding approximate methods is encouraging [187,188]. Subsequent MD simulations examined how the proximity of a biomolecule perturbs water dynamics in its hydration shell. Although most of the current experimental data imply a moderately slowed water dynamics in the hydration layer around proteins, the exact degree of this retardation as well as the molecular basis of the phenomenon remained a matter of debate. Water dynamic retardation around proteins was recently explained by local topological factors and hydrogen bonding strength [64,90], showing that most (80%) of the hydration waters manifest only very moderate retardation of their dynamics by a factor of ~2–3, while the dynamics of rest of the hydration water population follows a power-law distribution [89]. Similar conclusions can be drawn from simulation studies of water dynamics around DNA, which demonstrated that water in the vicinity of the DNA backbone is slowed by a factor of ~2–5 [163,189]. However, biomolecular hydration dynamics is spatially heterogeneous, and in certain systems, the specific hydration dynamics near distinct protein surfaces can have a functional role, e.g. facilitating the dimerization and aggregation of insulin [74] or proton transfer and binding in photosystem II [190].

4.5.2. Collective dynamics

Recent theoretical studies have also focused on the collective dynamics of many water molecules in the hydration layer as well as on the coupling between macromolecular motions and the dynamics of the surrounding solvent. Simulation studies of interfacial effects on the dynamics of water have demonstrated that correlated fluctuations of molecules in the vicinity of a solid surface lead to the local viscosity being higher than that predicted based on single particle dynamics [93]. These simulations thus confirm the theoretical model of the effect of local viscosity on the diffusion coefficient of a protein, proposed earlier by Halle [82] and provide an explanation of the molecular origin of the classic observation that the diffusion of proteins and other large solutes in bulk water is considerably slower than predicted. Several studies have indicated that a distinct dynamic coupling occurs between a protein and the surrounding water environment. Heyden and Tobias [87] analyzed the spatial dependence of the correlated motions in the hydration layer of a small globular protein and observed protein-induced long-range collective dynamics with collective vibrations extending as far as 10 Å from the surface of the protein. Conti Nibali et al. [49] studied the coherent collective motions of a protein and its hydration layer, separating the longitudinal and transverse collective modes propagating in the system. Their simulations indicate that protein-water hydrogen bonds rigidify the water network around the solute, i.e. strengthen the interactions in water molecules near the protein surface, and allow protein collective modes to propagate through the nearby solvent. These collective modes could mediate long-range protein-protein interactions, as suggested by the simulations of Kuffel and Zielkiewicz [191]. Schirò et al. [142] proposed, based on simulations and neutron scattering experiments, that the translational diffusion of water is intimately coupled to protein fluctuations both in folded and disordered state, and that the initiation of translational diffusion of the hydration water is independent of its rotational diffusion.

4.5.3. Thermodynamics

Theoretical studies have also enabled investigations of the thermodynamic parameters of various processes such as folding, binding and aggregation by dissecting the individual enthalpic and entropic contributions. Because direct experimental evaluation of the hydration free energy remains elusive, computational approaches present an attractive

alternative. The most accurate, "benchmark" methods which calculate relative free energy differences, ΔG, between two equilibrium states in any system, including free energy of an alchemical transformation, are free energy perturbation (FEP) and thermodynamic integration (TI). Their usage for the study of interfacial water is described elsewhere [27]. However, both methods are limited to "small" perturbations, such as water molecule binding at a given location. The cost for their accuracy is that they require a number of simulations and an extensive sampling in explicit solvent, which makes the computation too expensive for many applications.

In contrast, implicit solvent models such as the Poisson–Boltzmann/surface area (PB/SA) or the generalized Born/surface area (GB/SA) model provide a rapid and, for many applications sufficiently accurate (compared to other sources of error [192]) estimate of the hydration free energy. These dielectric continuum models render a good approximation of the bulk, isotropic solvent by capturing the consequences of the high dielectric constant of water and the hydrophobic effect. However, they cannot describe specific phenomena such as directionality of hydrogen bonds, their fluctuations, water molecule reorientation as well as bridging water molecules, and thus their usability in studying the hydration layer is significantly limited. This limitation is in part overcome by implicit solvation models such as AGBNP2 that have been developed to incorporate the granularity of water and short-range solute-water interactions [193]. These implicit solvation models can further be tuned based on binding affinity data to cover the first solvation shell effects, such as bridging waters and the expulsion of thermodynamically unfavorable water molecules upon ligand binding [194]. Application of implicit models of solvation in biomolecular modeling has been reviewed recently [195] and will not be covered here in detail. Instead, we will focus on two approximative methods, inhomogeneous fluid solvation theory (IFST) and integral-equation theory of liquids, which have gained popularity in recent years especially in the drug discovery community due to their favorable accuracy/efficiency ratio.

IFST, developed by Lazaridis [196] is a statistical mechanical method that calculates solvation free energies by quantifying the perturbation of a solute on the surrounding solvent relative to its bulk state. The entropic contribution is calculated as the sum of the solute–solvent and solvent–solvent orientational and translational particle correlation functions. IFST can be used to extract local thermodynamic information from MD trajectories and evaluate the entropy of the waters occupying specific hydration sites, along with their mean energetic interaction. A significant advantage of this approach is that the studied system is spatially decomposed, making it possible to evaluate the contributions of particular areas to the overall solvation free energy. However, this method suffers from sampling issues inherent in all MD-based approaches. Another limitation is its sensitivity to the reference interaction energy of a water molecule in bulk. In addition to applications to small molecules and peptides, this method has been used mainly in studies of protein-ligand [25,197] and protein-protein interfaces [27,198] and has found important application in pharmaceutical industry through WaterMap [25] software from Schrodinger, as discussed below. The accuracy of IFST against experimental data and hydration free energies calculated by FEP was recently assessed by Huggins and Payne [199], who reported that although the entropic contributions are slightly overestimated by IFST, the overall agreement between the hydration free energy predictions is encouraging. Another recent work introduced grid inhomogeneous solvation theory (GIST), a development of IFST that discretizes the equations on a Cartesian grid, thus avoiding the need to define hydration sites [200]. A subsequent study compared GIST with the hydration-site approach in a protein system and reported comparable results [201].

In the integral-equation theory of liquids, the solvent is considered via a distribution function. Both solute–solvent electrostatic and van der Waals interactions are fully included in this method, and solvent packing is considered. This approach makes it possible to obtain equilibrium solvent distributions rapidly without sampling and thus is more efficient than explicit solvent MD simulations but consequently does not offer any insight into its dynamics. However, analyzing the distributions provides preferred hydration sites together with localized entropies, enthalpies, and solvation free energies. Developments of this method in terms of the three-dimensional reference-interaction-site-model (3D-RISM) [202,203] allowed to apply the method to large biological systems, such as the hydration water at the protein surface as well as in cavities [204] and around DNA [205] and to analyze various free energy components (e.g. protein's intramolecular energy, hydration energy, hydration entropy) during protein folding [16] protein self-assembly [86] and aggregation [30]. A recent development of the method combines 3D-RISM with elements of IFST [206]. A graphical user interface for solvent analysis with 3D-RISM has been implemented in MOE software by Chemical Computing Group (CCG) [207].

5. Software tools

The software tools for the prediction of the properties of water molecules in the vicinity of biomolecular solutes concern two broad groups. The first one comprises tools that focus primarily on the prediction of structural properties (hydration site positions); the methods in this group are mostly force field-based or knowledge-based, and although some of them also enable to estimate how "favorable" the predicted hydration sites are, the thermodynamic profile can only be approximated. To gain a more precise picture of the hydration free energy, it is necessary to turn to the second group, encompassing methods that are directly aimed at the prediction of hydration thermodynamics, and which include sampling of the system, typically through MD simulation or the Monte Carlo method. To our knowledge, no software package dedicated to calculating dynamical properties of hydration has been released so far. This is because dynamical aspects of hydration, be it single particle properties [64,74,142] (e.g. residence times, reorientation, translational diffusion, HB lifetimes) or collective modes [87,93,163] involved in dielectric relaxation and vibrational motions are typically studied computationally by analyzing standard MD simulations. Thus, only the two groups of software delineated above will be discussed in detail in this section.

5.1. Prediction of structural properties

A number of software tools have been developed for hydration site prediction and analysis based on various theoretical approaches. Many of the scoring and classification approaches, such as Consolv, HINT, and WaterScore, and the treatment of water in molecular docking algorithms such as AutoDock and GLIDE in computational drug design have been reviewed by de Beer et al. [208] and will not be covered here. One of the first computational techniques for water mapping was Goodford's **GRID** [209], which locates energetically favorable positions using a probe that mimics the chemical properties of water or a range of other functional groups. The advantage of GRID and other probe-based methods lies in their ability to accurately locate hydration sites at a low computational cost compared to simulation-based approaches. Their limitation, however, is that the relative importance of these sites is difficult to assess because the associated entropy component is estimated only indirectly, e.g. by evaluating the hydrophobic effect contribution using a "dry" probe. A similar force field-based approach is **Fold-X** [210], which enables to predict water and ion sites within biomolecular structures. A docking approach is adopted by **WaterDock** [211], which uses the free AutoDock Vina software for the prediction of the positions of ordered water molecules in ligand binding sites. GRID-based water prediction is also implemented in the **WaterFlap** tool by Molecular Discovery [212]. An interesting semi-continuum approach is employed in **SZMAP** by OpenEye [213,214]. Sampling of contact positions using one explicit water molecule as a probe is combined

with a Poisson-Boltzmann continuum model of the solvent and a polarizable solute.

Another early technique is the knowledge-based approach adopted by the program **AQUARIUS** [215,216], which predicts the most probable hydration site positions within the protein's first hydration layer. The prediction is based on the distribution of water molecules around the 20 standard amino acids in proteins, as derived from a number of crystal structures. A very similar approach was adopted recently by Matsuoka and Nakasako [217], but in this case, the hydration site prediction is based on an analysis of a much higher number of crystallographic structures that have since become available. A recently developed knowledge-based method is **AcquaAlta** [218], which predicts the position of waters bridging protein-ligand polar interactions based on water geometries derived using data from CSD and from ab initio calculations. **WatCH** is a program for the identification of conserved water molecules in a set of related crystallographic structures. In this method, the structures are superimposed, and a complete linkage clustering algorithm is used to resolve the overlapping water positions into an array of microclusters of maximum density, without relying on any one structure as a reference [98,219].

5.2. Prediction of thermodynamics

To predict the thermodynamic profile of hydration sites, the entropic component has to be included. For this, configurations of the system have to be sampled, typically using MD or Monte Carlo simulations. Several software tools are based on IFST analysis of MD simulation data, the most popular arguably being Schrödinger's **WaterMap** [25,220] software, which has found notable popularity in drug development applications. IFST has also been incorporated in the Solvation Thermodynamics of Ordered Water (**STOW**) package [221]. Both programs perform IFST, providing the mean energetic interaction of water occupying specific hydration sites with the rest of the system, and calculate the entropic penalty due to restrictions of bound water molecule's translation and rotation caused by the surroundings. This approach provides valuable insight into the role of specific water sites in molecular recognition and enzyme selectivity and enables the prediction of ligand binding affinities in drug discovery research, lead optimization and structure–activity relationship (SAR) studies.

Several other tools based on MD trajectory analysis have been developed. While they also allow free energy estimation, they are more approximative than IFST. **WATsite** [222] is based on clustering the positions of water molecules sampled by explicit water MD simulations. Similarly to IFST, the free energy of specific hydration sites can be calculated, with the entropy contribution estimated from the probability density of the translations and rotations of the water molecules in the given region. However, the higher-order water–water correlations are not included in this method. A different approach to evaluating the entropic contribution from an MD simulation is adopted by **SPAM** [223]. Instead of extracting the entropy of solvation from the distribution of translational and rotational degrees of freedom, the free energy at a specific site is computed via a partition function, based on the local distribution of interaction energies of water molecules with their environment and the perturbation to this distribution relative to the bulk. Again, the computation is enabled by neglecting the correlations between the motions of different water molecules. In the **Grid Cell Theory** (**GCT**) method [224], the thermodynamic properties of water are calculated for a specified region using the solute–water and water–water interaction energies, forces and torques exerted on the water molecule and from its orientational entropy.

A sophisticated statistical thermodynamics methodology based on λ-dynamics is **Just Add Water Molecules** (**JAWS**) [225]. JAWS performs conformational sampling of the protein chain while simultaneously allowing water molecules to appear and disappear on a grid. This method is implemented by multiplying the interaction energy between each water molecule and its environment by a scaling factor which is allowed to vary between 0 and 1. This enables to calculate hydration site occupancies and to estimate absolute binding free energies. A similar approach is adopted in the **Grand Canonical Monte Carlo method** (**GCMC**) method, in which the number of particles is allowed to fluctuate depending on a constant chemical potential. Relating the binding free energy of water to the chemical potential the binding free energy permits the determination of the affinity of a water molecule at a specific location [226].

5.3. Applications

The predictions and analyses of the properties of the hydration layer obtained using the software tools discussed above have a broad range of applications. As we have seen, the interpretation of the results of many, if not all, experimental methods depends on the model of the hydration layer used. Thus, the development of more sophisticated and physically realistic hydration layer models holds promise for more precise data interpretation. In crystallography, for example, the hydration water distribution function derived from the analysis of crystal structures can in turn be used to aid crystallographic structure refinement by identifying density peaks that are likely candidates for the placement of hydration water molecules [106]. Theoretical studies also benefit from a more accurate modeling of hydration layers. For example, a carefully modeled water network in the initial structure improves the stability of MD simulations [227] and the accuracy of free energy calculations [228].

The area in which water modeling has received greatest interest in recent years is ligand docking and structure-based drug design. Traditionally, the hydration state of the binding cavity was not considered in docking studies, and ligands were docked into “dry” binding sites. However, as the role of water in ligand binding has been gradually recognized, a number of protocols have been developed to account for conserved water molecules [208]. Nevertheless, water contributes to the thermodynamics of binding not only by mediating protein-ligand interactions but also via the free-energy penalty for displacing ordered water molecules from the binding pocket into the bulk. In structure-based drug design, it is therefore crucial to evaluate how much the entropic penalty of water ordering inside the binding site is outweighed by the favorable enthalpy of its interactions. In addition to the displaced water molecules, the potential entrapment of “unhappy” water molecules inside the binding pocket, i.e., water molecules in an entropically or enthalpically unfavorable state, must be considered [229,230]. The magnitude of these effects can be significant. In fact, recent studies have provided evidence that for the determination of binding thermodynamics in biomolecular recognition, the contribution of water is as important as the contribution of the direct protein-ligand interactions [40,231,232]. It is therefore unsurprising that a number of computational methodologies and software tools have been developed to address this topic, as discussed in detail above. Some of the commercial solutions include WaterMap by Schrödinger, SZMAP by OpenEye, 3D-RISM by CCG and WaterFlap by Molecular Discovery.

Moreover, water can affect not only the thermodynamics of ligand binding but also its kinetics. The importance of the kinetic perspective is increasingly recognized in drug development because the residence time of a ligand in the bound state is a crucial aspect of its biological effect in vivo: the longer the ligand remains bound to its receptor, the longer the biological effect endures. The prediction of the kinetic parameters (k_{on} and k_{off} rates) remains challenging for current computational methods, although several promising approaches are being developed [233]. The structural factors responsible for the binding kinetics are not fully understood, although some components have been identified. For example, hydrogen bonds between protein and ligand that are shielded from the access of water are exchanging at slower rates due to higher transition state energy barriers [234]. Desolvation and resolvation processes have been proposed to constitute the rate-limiting steps in protein–ligand binding and unbinding, respectively, in many cases [235]. Unstable or “unhappy” waters trapped between receptor and

ligand in the bound state can contribute to fast off-rates and short residence times [236]. By contrast, for destabilizing the transition state, the presence of these waters could lead to faster binding site desolvation and increased k_{on} values.

The consideration of water-mediated interactions has proven useful not only in predicting protein-ligand interactions but also in many other areas of computational structural biology. Water-mediated interactions are a ubiquitous aspect of biomolecular recognition, and their inclusion improves protein-structure prediction [20] and the discrimination of native protein-protein and protein-nucleic acid interactions [237,238]. Solvated protein-protein docking yielded more precise modeling of biomolecular complexes in the CAPRI experiment [239]. However, docking methods that account for protein–water interactions are still an exception [240,241], and the prediction of water positions at the interface remains challenging [242].

6. Conclusions

In the last decade or so, biomolecular hydration has received increased interest from the scientific community, fueled by the recognition of the key role of the interactions between water and proteins or nucleic acids in determining biomolecular structure, dynamics and function. Methodological developments in both experimental techniques and computation have enabled the study of hydration-related phenomena from new perspectives and with an unprecedented level of detail. This expanded knowledge of hydration increasingly indicates that water is an inseparable part of biological macromolecules. A complete, molecular-level description of the biological processes in which proteins and nucleic acids are involved therefore requires a thorough understanding of the mutual influence between the structure and dynamics of the biomolecule and the water at the biomolecular interface.

In this review, we aimed to integrate recent developments in experimental, computational, and software tools that have contributed to the current view of the properties of water in the vicinity of biomolecules. After several decades of uncertainty about the correct interpretation of experimental observations, the various methods appear to be converging toward a consensus picture of the structure and dynamics of biomolecular hydration. The main structural features of the hydration layer around proteins and nucleic acids are limited to 1–2 layers of water molecules; these first two layers are dynamically heterogeneous, with the majority of the water molecules only moderately slowed compared to bulk. A smaller portion of the surface-restricted water molecules exhibit more pronounced slowing, with a power law distribution of the retardation factor. The molecules most retarded in their motion are typically those in deep surface pockets and grooves. A range of software tools have been developed for the prediction of the location and thermodynamic properties of water near a biomolecule. While the exact thermodynamic parameters of the hydration shell are still relatively challenging to determine, the main principles by which it is governed—i.e., the decisive role of the surrounding biomolecular topology and especially its hydrogen bonding potential—are well established. This consensus view has been challenged by a few recent experiments indicating that several more hydration layers are directly influenced by the interacting biomolecule. The single-particle dynamics of these more distant waters are comparable to the bulk, but the dynamics is perturbed in terms of long-range orientational order and collective vibrational modes. Although the interpretation of these observations continues to be debated, if confirmed, they could have far-reaching implications for the mechanisms of biomolecular recognition.

More research is clearly needed to elucidate the principles governing subtle but important hydration phenomena and to extend the current models to describe the outer hydration layers. However, if applied together, the available experimental techniques and computational tools hold promise to provide a detailed and coherent description of the properties of the entire hydration layer around the very complex systems of biomolecules. Undoubtedly, future, more revealing studies will validate the currently accepted view of biomolecular hydration and expand it using newly developed experimental techniques and theoretical methods.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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