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Extended Frequency Range Depolarized Light Scattering Study of *N*-Acetyl-leucine-methylamide−Water Solutions

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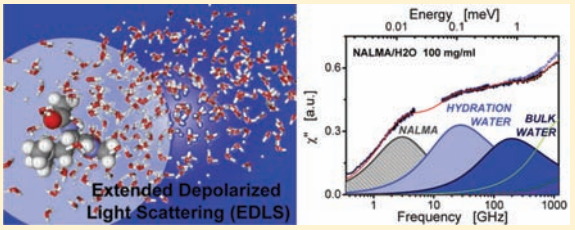
ABSTRACT: We have studied the influence of the amphiphilic model peptide *N*-acetyl-leucine-methylamide (NALMA) on the dynamics of water using extended frequency range depolarized light scattering (EDLS), between 0.3 GHz and 36 THz. This technique allowed us to separate solute from solvent dynamics and bulk from hydration water, providing both characteristic times and relative fractions. In the temperature range 5−65 °C, a retardation factor from 9 to 7 is found for water hydrating NALMA. Moreover, in the same range, a hydration number from 62 to 50 is observed, corresponding to more than two hydration layers. This strong perturbation suggests the existence of a collective effect of amphiphilic molecules on surrounding water molecules.



INTRODUCTION

Water is responsible for most of the characteristic structure, dynamics, and functions of biomolecules, due to its unique hydrogen-bonding capability.[1] It plays a fundamental role in folding of globular proteins, molecular recognition, and enzymatic catalysis processes.[2−5] Moreover, solvent fluctuations are involved in the local and large-scale protein motions that govern biological functions.[6,7] Conversely, the presence of a biomolecule deeply modifies both static and, especially, dynamical properties of hydration water. Despite the large number of previous experimental and theoretical studies,[2,5,8−20] some fundamental issues, such as the characteristic mobility of interfacial water and the spatial extent of the induced perturbation, are strongly debated.[2,5,9−22]

The effect imposed by a biomolecule on water dynamics can be quite complex, due to the variety of polar and nonpolar side chains (energy disorder[14]) together with the existence of surface roughness (topological disorder[23]). As a consequence, the range of possible solvating interfaces is too vast to be experimentally studied in any detail, and, to provide some hint on static and dynamic properties of hydration water, solutions of relatively simple molecules are usually chosen as a model system.[24−31] To this end, great efforts have been devoted to the study of *N*-acetyl-leucine-methylamide (NALMA),[20,24,32−43] an amphiphilic peptide with a hydrophilic backbone and hydrophobic side chain, which is a good prototype for studying the role played by chemical heterogeneity, without the additional effect of topological disorder, typical of protein surfaces. At variance with more hydrophilic peptides, like *N*-acetyl-glycine-methyl-amide (NAGMA), NALMA is known to reproduce the main dynamical anomalies exhibited by hydration water near protein surfaces, suggesting that dynamic signatures near biological interfaces arise from chemical heterogeneity and not just from topological roughness.[34] A retardation effect is generally observed for the dynamics of hydration water that is usually reported in terms of a dynamic perturbation factor[37] or retardation ratio ξ, that is, the ratio between the relaxation time of interfacial and bulk water, which allows one to compare the results obtained by different spectroscopic techniques.

Aqueous solutions of NALMA were studied by neutron scattering,[35,39−42] terahertz (THz) absorption spectroscopy,[32] dielectric spectroscopy,[20] nuclear magnetic resonance (NMR) relaxation,[37,38] and optical Kerr effect,[33] and by molecular dynamics simulations.[34,36] Close to room temperature, all of these studies show the existence of a population of water molecules perturbed by the solute that relaxes slower than pure water. There is, however, a significant disagreement concerning the extension of the perturbation (one or multiple hydration shells) and the entity of the retardation ratio (ξ from 1.5 to 20 or more). Elastic incoherent structure factor data obtained by neutron scattering measurements[39] suggested the existence of very slow water (relaxation time greater than ∼13 ps, corresponding to ξ greater than 10). Quasi-elastic neutron scattering (QENS)[35,39,41] measurements proposed the existence of moderately slow water, characterized by a rotational retardation factor smaller than 3.5 involving just the first hydration shell of NALMA. Conversely, THz spectroscopy indicated a solute-induced retardation effect of the ultrafast water dynamics that substantially exceeds the first hydration layer, extending up to 3−4 shells around the peptide.[32] Dielectric spectroscopy measurements performed on the homologue *N*-acetyl-leucine amide (NALA),[43] and molecular dynamics

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(MD) simulations of dielectric response of water−NALMA solutions,[20] gave evidence of a very slow relaxation, located in the nanosecond time scale at room temperature, and a faster one at about 10 ps, close to that of pure water. The fast relaxation was attributed to the collective reorientation of water molecules, which also includes a contribution due to interfacial water whose motion is moderately retarded ($\xi < 1.8$).[43] The slow relaxation was mainly assigned to protein−water dipolar coupling; a contribution arising from strongly retarded water molecules was also suggested by related MD simulation results.[20] ^{2}H NMR relaxation was also used to characterize the rotational dynamics in the hydration shell of NALMA.[37,38] The mean rotational correlation time of perturbed water molecules in diluted solutions was determined within a two-state approximation, with perturbed molecules lying on the first hydration shell and unperturbed ones beyond the first shell. Within this approximation, a moderate retardation factor, $\xi \approx 1.7$, at room temperature was assigned to hydration water. An order of magnitude larger retardation was measured by optical Kerr effect (OKE).[33] The slow dynamics was fitted by a procedure similar to that used for the elaboration of NMR spectra, giving $\xi \approx 12$.

Thus, despite the huge amount of experiments and simulations, it is not yet clear whether it is possible to univocally define the number of water molecules whose dynamics is perturbed by the presence of the peptide together with their retardation factor. Our contribution to a deeper understanding of the dynamics of hydration water comes from extended depolarized light scattering (EDLS) measurements performed in water−NALMA solutions at different concentrations. EDLS is a powerful technique that, combining dispersive and interferometric setups, gives access to the very wide frequency range from ∼0.3 to 3×10^4 GHz. This approach succeeds in providing, with a single measurement, estimates of both the number of water hydration molecules and the retardation factor at picosecond time scales. Previous investigations on water−carbohydrates[44−47] and water−lysozyme[48] solutions demonstrated some peculiarities of different solutes in the perturbation induced on surrounding water. In fact, carbohydrates were found to perturb only a restricted population of water molecules, those implicated in the direct formation of hydrogen bonds with the solute, while the protein was found to extend its perturbation well beyond the second hydration shell. EDLS results of water−NALMA solutions presented here give the opportunity of clarifying whether the strong perturbation induced by proteins is unique or connected with the exposure of hydrophilic and hydrophobic sites, also typical of smaller biomolecules.

EXPERIMENTAL SECTION

NALMA was purchased from Bachem and used without further purification. NALMA aqueous solutions were prepared by weighing and dissolving the peptide in doubly distilled and deionized water, in the 12−100 mg NALMA/mL water concentration range. Dust-free solutions were obtained by filtering the samples through 0.20 μm filters, placed into a 10 mm path optical quartz cell and analyzed using both a 300 mW Ar^+ laser operating on a single mode of the $\lambda = 514.5$ nm line and a 200 mW single mode solid state laser at $\lambda = 532$ nm. No differences were appreciated in the shape of the spectra by use of these two sources. To obtain a wide spectral range from 0.3 to 36 000 GHz, the depolarized scattered radiation I_{HV} was analyzed by means of two different spectrometers. The low frequency region from ∼0.3 to 140 GHz was explored by a Sandercock-type (3 + 3)-pass tandem Fabry−Perot interferometer. To cover the whole frequency range, three different mirror separations

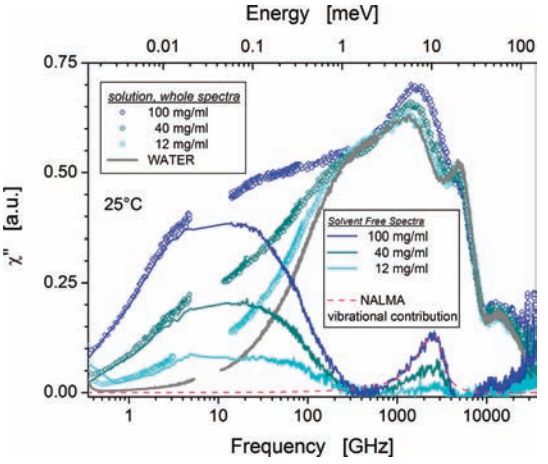

Figure 1. Susceptibility spectra of pure water and of solutions at three different concentrations. Solvent-free spectra are also shown, where the contribution of bulk water has been subtracted to emphasize the contribution of NALMA and of hydration water.

(d = 13−14 mm, d = 4−3.5 mm, and d = 1 mm), depending on temperature and concentration, were used. The high frequency region from 30 to 36 000 GHz was analyzed by a Jobin−Yvon U1000 double monochromator having 1 m focal length and holographic gratings with two different spectral resolutions: from 30 to 900 GHz with a resolution of about 15 GHz and from 300 to 36 000 GHz with a resolution of about 100 GHz. Temperature was controlled keeping fluctuations within 0.1 K during the measurements. After subtraction of the dark count contribution, low and high frequency spectra were spliced, exploiting an overlap of about one-half a decade in frequency. Subsequently, the imaginary part of the dynamic susceptibility χ'' was calculated according to the relation $\chi''(\nu) = I_{HV}(\nu)/[n_B(\nu) + 1]$, where I_{HV} is the horizontally polarized Raman intensity and $n_B(\nu) = 1/[\exp(h\nu/k_BT) - 1]$ is the Bose−Einstein occupation number.

Shear viscosity measurements were performed with a Ubbelohde model 537-10 capillary in connection with a Schott-Gerate AVS400 viscosimeter.

RESULTS

Susceptibility spectra at different concentrations and temperatures are reported in Figures 1 and 2. Two main regions can be distinguished in the spectra: the one at frequencies higher than ∼300 GHz, where water vibrational modes are localized, and the other at lower frequencies that is mainly affected by relaxation modes. Figure 1 also shows the spectra after normalization and subtraction of water signal, that is, the solvent-free (SF) spectra.[48] SF profiles give evidence of two spectral features increasing with solute concentration: a relaxational contribution, wider than two decades, in the low frequency region and an asymmetric peak in the THz region assigned to internal modes of the peptide, very close to the spectral region of the boson peak in protein solutions.[48] A more detailed analysis of this last feature will be the subject of a further publication. Here, we just mention that position and shape of this peak are almost temperature independent so that it can be subtracted from the spectra, for a more suitable elaboration of the low frequency contributions, by means of the same procedure used for water−lysozyme solutions.[48]

The spectrum from the 100 mg/mL solution at T = 25 °C is shown in Figure 3. After subtraction of the vibrational contribution of NALMA (pink − − −), the difference spectrum (black

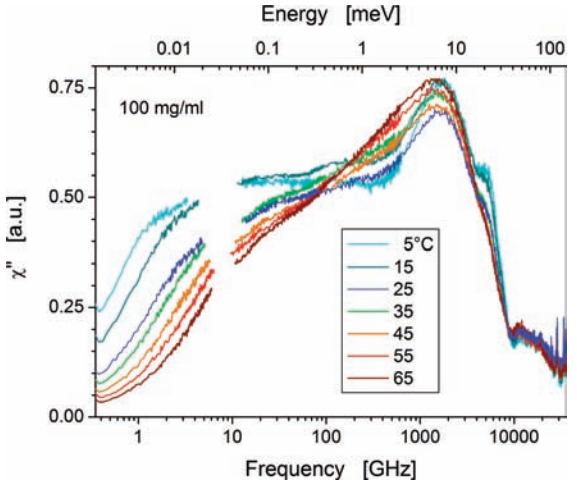

Figure 2. Susceptibility spectra of the 100 mg/mL NALMA−water solutions at different temperatures.

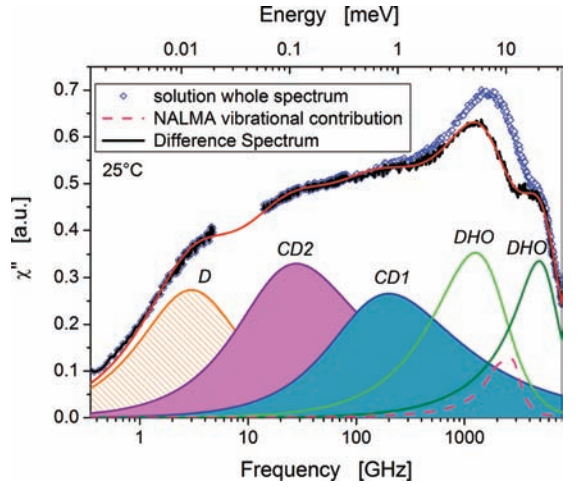

Figure 3. Susceptibility of the 100 mg/mL NALMA−water solution at 25 °C. Experimental data are reported before (blue ○) and after (black line) subtraction of the vibrational contribution of NALMA (pink − − −). The fitting curve (red line) is also reported, together with the single components: rotational diffusion of NALMA (D); relaxation of hydration (CD2) and bulk (CD1) water; and bending and stretching resonant modes of water (DHO).

line) has been reproduced by the sum of two damped harmonic oscillators (DHO) $\chi''_{DHO} = \mathcal{I}m\{\Delta\omega_0^2[\omega^2 - \omega_0^2 - i\omega\Gamma]^{-1}\}$, with parameters giving position ω_0, width Γ, and amplitude Δ of the peaks as free fitting parameters. A Cole Davidson relaxation $\chi''_{CD} = -\mathcal{I}m\{\Delta[1 + i\omega\tau]^{-\beta}\}$ has been used to model the contribution of bulk water (CD1), where the stretching parameter β has been fixed to the value of pure water $\beta = 0.6$, and the relaxation time τ and amplitude Δ are left free. A second Cole Davidson function (CD2) was used to reproduce the intensity in the range ∼10−100 GHz, which can be reasonably assigned to hydration water, in analogy with what was found in other biosystems.[45,48] Also, in this case, the stretching parameter was fixed to the value $\beta = 0.6$, and τ and Δ were left free. Finally, a Debye function (D), that is, a CD with $\beta = 1$, was used to describe the spectrum below ∼10 GHz originating from the rotational diffusion of NALMA molecules.

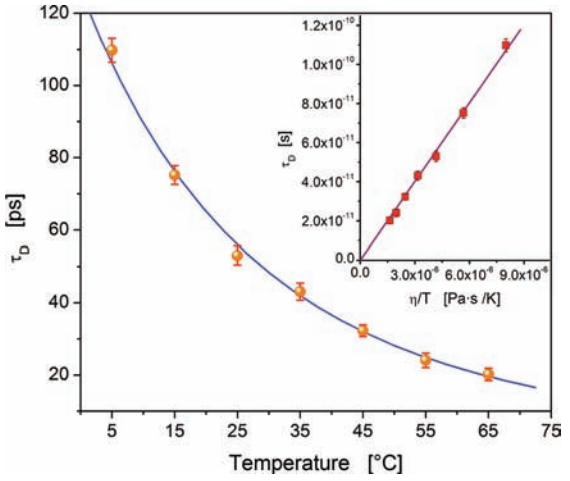

Figure 4. Rotational relaxation time of NALMA as a function of temperature. In the inset, the same relaxation time as a function of shear viscosity η over temperature T.

Figure 3 clearly shows the goodness of the fit. Note that, in the temperature and concentration region here analyzed, the shape of the whole spectrum is scarcely influenced by the stretching parameters so they have been fixed to the values obtained in pure water, as stated above. It is noteworthy that this choice does not influence the temperature and concentration behavior of the relaxation times, giving just a small shift of their absolute values.

■ ROTATIONAL DIFFUSION OF NALMA

The values of the relaxation time of the slow Debye process obtained by the fitting procedure are reported in Figure 4 as a function of temperature. The relaxation time is proportional to η/T, where η is the shear viscosity of the solution, as shown in the inset of Figure 4. Moreover, the amplitude of the relaxation increases linearly with increasing solute concentration (data not shown). These features suggest that the low frequency relaxation may be attributed to the rotational diffusion of NALMA molecules. In fact, in the high dilution limit here explored, where EDLS reveals the single particle dynamics, the relaxation time can be described by the Stokes−Einstein−Debye relationship, $\tau_D = \eta V_h/(k_B T)$, where $V_h = Vf$ is the hydrodynamic volume, V is the volume of the rotating molecule, and f is a rotational friction coefficient dependent on the molecular shape and on the hydrodynamic boundary conditions. If the NALMA molecule is approximated by an oblate spheroid with axial ratio 0.6 and stick boundary conditions are applied, the rotational friction coefficient approaches to unit ($f = 1.04$), and the hydrodynamic volume approximately represents the molecular volume. In fact, from the linear fit in Figure 4, the value $V = (179 \pm 12)$ Å^3 is obtained, close to the van der Waals volume $V = 192$ Å^3 of NALMA. This result supports the idea that the slowest relaxation here reported is due to the rotational diffusion of the solute and also suggests that aggregation phenomena are negligible at this concentration. As a final remark, we notice that the rotational time of NALMA can be fitted by an Arrhenius law (the full line in Figure 4) with activation energy $E_a = 22$ kJ/mol. A further analysis of this temperature dependence is found in the following section.

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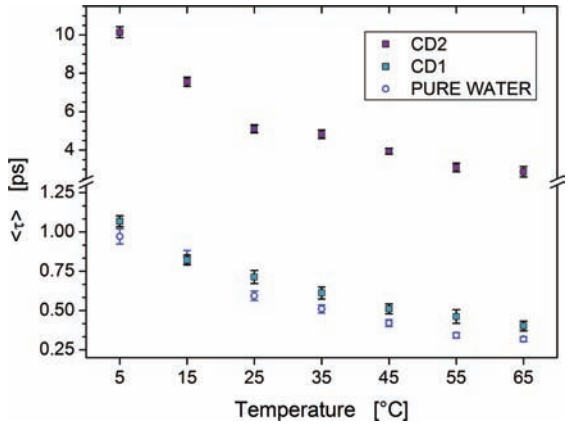

Figure 5. Relaxation time of bulk (CD2) and hydration (CD1) water together with relaxation time of pure water as a function of temperature.

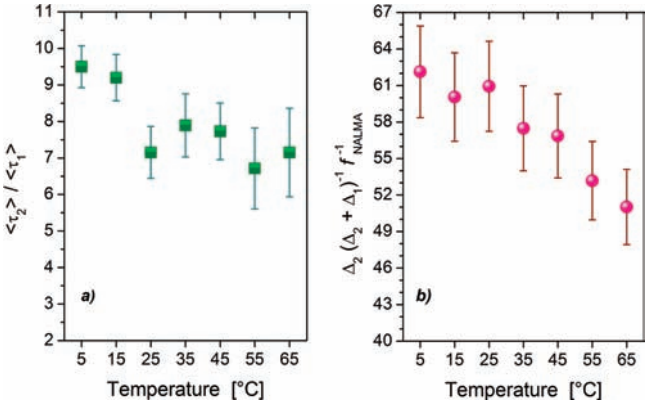

Figure 6. (a) Retardation factor of hydration water. (b) Average number of water molecules dynamically perturbed by one NALMA molecule derived considering the amplitude of the hydrating (Δ_2) and bulk (Δ_1) water relaxation processes as resulting from the adopted fitting procedure.

HYDRATION AND BULK WATER

Figure 5 shows the temperature dependence of the average relaxation time of bulk and hydration water obtained from the CD relaxation parameters by the relation $\langle\tau\rangle = \beta\tau$. The good agreement between the relaxation times obtained for the bulk component (CD1) and those measured in pure water substantiates the adopted elaboration procedure, supporting the proposed dynamical scenario in terms of two water components. Concerning hydration water (CD2), its relaxation time is well separated from that of bulk water in the whole temperature range, with a retardation ratio ξ slightly temperature dependent as evidenced in Figure 6.

From the Arrhenius fit of data reported in Figure 5, it is possible to estimate the relevant activation energies. A value of 16 kJ/mol was obtained for the hydration component, to be compared to the value of 15 kJ/mol of bulk water. The small difference in activation energy of bulk and hydration water is responsible for the small temperature dependence of ξ in Figure 6. We recall that the relaxation dynamics probed by our EDLS experiments is connected with fast density fluctuations, mainly associated with the hydrogen-bonding rearrangements of the water network,[46,49] and the obtained activation energies support this interpretation.[50] We also notice that this activation energy is remarkably lower than that reported here for the rotation of NALMA. This is also evidenced by the increased spectral separation between the D and CD2 contributions on decreasing temperature, facilitating the unambiguous determination of the relaxation parameters of both solute and solvent.

To conclude the analysis of results, let us stress the ability of our technique to derive the average number of water molecules dynamically perturbed by a single peptide molecule from the amplitude of the relaxation process of bulk (Δ_1) and hydration (Δ_2) water, and the NALMA/water molar ration f_{NALMA} according to the procedure already discussed in previous papers.[45,48] The hydration numbers obtained from the relationship $\Delta_2(\Delta_2 + \Delta_1)^{-1} f_{NALMA}^{-1}$ and reported in Figure 6 vary from 62 to 50 in the 5−65 °C temperature range, suggesting that the perturbation, slightly increasing for decreasing temperature, extends up to more than two hydration layer. In fact, MD simulations report the number of water molecules in the first hydration shell of NALMA to be ∼23.[20]

DISCUSSION

The extension and intensity of the perturbation induced by the small NALMA molecule on the surrounding water molecules, a retardation factor of about 8 affecting 50−60 molecules, is a quite peculiar behavior of this amphiphilic system, an effect much stronger than that induced by single hydrophilic or hydrophobic molecules of comparable dimension. In fact, it has been shown that small hydrophilic molecules, such as mono- and disaccharides, produce a retardation factor of about 5−6 on a small number of water molecules, about 8−9 for each glucose ring.[45,46] On the other hand, small hydrophobic molecules, such as alcohols, induce a retardation factor of about 2 into the first hydration layer.[51] Thus, the entity of the perturbation induced by NALMA on the surrounding water is surprisingly high and cannot be treated as a trivial combination of hydrophilic and hydrophobic effects.

The comparable values of activation energies of bulk and hydration water, reflected in the small temperature dependence of ξ in Figure 6, suggests a similar restructuring mechanism for both bulk and hydration water. This finding is in general agreement with the picture derived by MD simulations on the reorientation dynamics of water around model solutes.[27,29] Also, NMR data suggest a rather limited increment for the apparent activation energy of the rotational relaxation going from the bulk (∼20 kJ/mol) to the NALMA shell (∼25 kJ/mol) at around room temperature.[37] Conversely, a large increase of the rotational activation energy (from 17 to 29 kJ/mol) was initially derived from ultrafast time-resolved IR studies on water hydrating *tert*-butyl alcohol (TBA) and tetramethyl urea (TMU).[26] Recently, however, a dielectric spectroscopy investigation of this latter system assigned that apparent variation to a progressive reduction of the number of perturbed water molecules upon temperature increase, thus giving an activation energy of about 12 kJ/mol for both hydration and bulk water.[31] A rationale to this effect has been recently given, proposing that in liquid water the reorientation of an OH group proceeds through large-amplitude angular jumps.[52] The slow reorientation of water in the hydration shell close to hydrophobic groups ($\xi \leq 2$) was explained in terms of an entropic effect related to excluded volume.[27] However, here we have two pieces of experimental evidence of a more complex scenario in the hydration of NALMA: the value of $\xi \approx 8$ is much

higher than that expected from the excluded volume effect, and the perturbation extends by more than two hydration layers. To this respect, the effect of NALMA on water is remarkably similar to that produced by much larger biomolecules, such as lysozyme, recently investigated by the same EDLS technique, where a retardation factor of about 6−7 was found, involving at least the first two water layers.[48] As a whole, it seems that the retardation originates from a collective effect, possibly related to the frustration of the structural dynamics of the hydrogen-bond network, induced by the presence of hydrophobic and hydrophilic molecular groups. In large biomolecules, such a collective effect has been recently recognized and related to the existence of large hydrophobic regions. MD simulations have shown that hydrophobic regions significantly affect the interfacial water structure and that unsatisfied hydrogen bonds at the water−solute interface cause an energetic push for in-plane orientations of the water dipoles, promoting the development of ferroelectric hydration shells around proteins, propagating up to 3−5 water diameters into the bulk.[53] In this picture, the fluctuations of a large polarized cluster, comparable in size to the protein, can slave its conformational dynamics, giving an important contribution to protein energetics.[54]

The challenge posed by the present interpretation of water−NALMA dynamics is now more evident. Hydration water around this small amphiphilic molecule shares the large retardation value and the large number of retarded water molecules with proteins, but the single peptide molecule is probably too small to trigger ferroelectric domains around its hydrophobic region. The nature of this collective effect thus remains to be fully explained.

It is worth noting that, although EDLS has given the first unambiguous evaluation of both the number and the retardation ξ of hydration molecules around NALMA, previous spectroscopic investigations also gave some important clue.

Our results are consistent with those recently reported by OKE,[33] although those depolarized spectra were recorded in a shorter dynamical range, where only a fit by an average stretched exponential process was possible. Considering the average relaxation time as a population-weighted value over bulk and hydration environments, and assuming 38 as the number of water molecules solvating the peptide, a retardation factor of ∼12 was estimated. If the OKE data are reanalyzed using 60 for the hydration number, a retardation factor even closer to our one can be obtained.

Quasielastic neutron scattering and elastic incoherent structure factor studies[35,39,41] performed at room temperature and concentrations higher than 0.5 M suggested the existence of both weakly and strongly perturbed water reorientations, having $\xi \approx 3.5$ and greater than 10, respectively.[39] These conclusions have been recently revised considering the results of molecular dynamics simulations, which suggested that the model used to fit the spectra was oversimplified.[34,36] To this respect, it should be noticed that the spectra of Figure 3 give evidence of a complex relaxation pattern of water, extending through an energy range of more than three decades; this suggests that a more reliable interpretation of neutron scattering results would require the combination of spectra obtained by different spectrometers operating in complementary spectral regions.

Dielectric spectroscopy[43] and MD simulations,[20] which conversely extend over a very wide spectral region, also reported the existence of an ultraslow (nanosecond) spectral contribution involving water molecules, but only in concentrated solutions of amphiphilic peptides. On the other hand, in diluted solutions, that is, in the range explored here, dielectric spectroscopy can hardly distinguish the existence of two relaxation process for bulk and hydration water.[43] This can be attributed to the continuous exchange of bulk and hydration molecules, which strongly influences both the apparent relaxation time and the amplitude of the two components. In fact, it is known that the exchange rate of water molecules solvating hydrophobic solutes is about 4 GHz,[55] very close to the relaxation rate of bulk and hydration water molecules. Dielectric spectra are thus at intermediate condition between slow exchange, where the two relaxations would be well separated, and fast exchange, where they collapse into a single peak at intermediate frequency. In this condition, a broad non-exponential spectrum is expected, as revealed by experiments.[43] Conversely, this problem does not affect EDLS spectra, because the exchange is an order of magnitude slower than the relaxation time of water.

It is noteworthy that a value of ξ close to that reported here was previously found by NMR investigation of large proteins;[38] on the contrary, NMR applied to NALMA solutions[37,38] gave a retardation of about 1.7, close to that obtained for small hydrophobic molecules, like TBA and TMAO. This result was found assuming that the solute-induced perturbation is short-ranged, affecting only the primary hydration shell, whereas our results suggest the perturbation induced by NALMA to extend over the second hydration layer. This does not help in reconciling the discrepancies between the two techniques because using a larger hydration number in the elaboration of NMR spectra produces a further reduction of the estimated retardation factor. The reason the dynamics of water measured by NMR is much less perturbed by NALMA than that measured by EDLS remains an open question.

■ CONCLUSIONS

In this work, we have exploited the capabilities of EDLS to reveal the extension and entity of the retardation produced by the amphiphilic peptide NALMA on hydrating water molecules.

EDLS has been demonstrated to be unique in encompassing the major problems that affect other traditional techniques. In fact, with respect to INS and OKE, it explores a spectral region wide enough to cover the dynamics of both solute and solvent, and, with respect to NMR and DS, it is sensitive to a hydrogen-bond dynamics that is faster than the exchange rate of water molecules, allowing one to simultaneously measure both the number and the retardation factor of hydration water. Moreover, with respect to DS, it allows one to decouple the dynamics of water from the rotation of the solute, because water molecules rearrange 7−10 times faster than NALMA. By means of this technique, we obtained a retardation factor of about 8, involving more than two hydration layers around each solute molecule, very close to values typical of protein solutions.[48]

On one hand, this similarity corroborates the choice of this simple peptide as the prototype of larger and complex biomolecules, reinforcing the notion that the anomalous dynamics observed in hydrated proteins arises from the chemical heterogeneity, that is, from the existence of interfaces between hydrophilic and hydrophobic domains on the protein surface.[20] On the other hand, the collective nature of this effect, far from being the mere superposition of hydrophilic and hydrophobic effects, is quite unexpected in such a small molecule and reveals a surprisingly strong influence of small biomolecules on surrounding water that still claims a full theoretical explanation.

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