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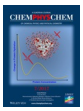

Nonmonotonic Hydration Behavior of Bovine Serum Albumin in Alcohol/Water Binary Mixtures: A Terahertz Spectroscopic Investigation

Dipak Kumar Das,* Debasish Das Mahanta, and Rajib Kumar Mitra*[a]

We report the experimental observation of nonmonotonic changes in the collective hydration of bovine serum albumin (BSA) in the presence of alcohols of varying carbon-chain lengths, that is, ethanol, 2-propanol, and *tert*-butyl alcohol (TBA), by using terahertz (THz) time domain spectroscopy. We measured the THz absorption coefficient (α) of the protein solutions, and it was observed that α fluctuated periodically as a function of alcohol concentration at a fixed protein concentration. For a fixed alcohol concentration, an increase in the protein concentration resulted in nonmonotonic changes in α; thus, it first decreased rapidly and then increased, which was followed by a shallow decrease. An alcohol-induced α helix to random coil transition of the protein secondary structure was revealed by circular dichroism spectroscopy measurements, and the effect was most prominent in TBA. The anomalous change in the hydration was found to be a delicate balance between the various interactions present in the three-component system.

1. Introduction

The addition of small molecules often brings about noticeable changes in the structure and functionality of proteins.[1,2] These cosolvents can preferentially solvate certain segments of proteins, which thereby perturbs the hydration network at the protein surface.[3] The preferential hydration of a protein in the presence of cosolvents may be regarded as a result of the delicate balance of three different contributions: a repulsion and an attraction between the protein and cosolvent and the steric exclusion of the cosolvents acting as classical "crowders". In this regard, water/alcohol binary mixtures have evoked considerable attention in the recent past owing to the various anomalous behaviors of these mixtures, which seem to be dependent on the chain length and the hydrophobicity of the alcohols.[4–6] Anomalous hydration behavior of alcohols has been studied extensively by using various experimental and simulation techniques.[7–19] It has been concluded that the conventional "iceberg" model is not appropriate to explain the experimental results and that the presence of hydrophobic moieties on the alcohol are responsible for the unusual rotational mobility of water.[20]

Alcohol/water mixtures are not homogeneous throughout the entire concentration range;[21] the heterogeneity arises due to incomplete mixing as a result of cluster formation as well as self-aggregation of the alcohol.[15] Given that they are amphiphilic in nature, alcohol molecules can induce opposite effects in water: whereas the hydrophilic part can form favorable H-bonds with water, the hydrophobic part tends to self-aggregate and disrupt the water structure by hydrophobic hydration.[16] These two opposing effects combine together to modify the extensive H-bonding network of water depending on the composition of the binary mixtures.[22] The extent of heterogeneity is dependent on the hydrophobic nature of the alcohol; for example, water/*tert*-butyl alcohol (TBA) mixtures show strong cluster formation at a lower alcohol concentration than water/ethanol and water/methanol mixtures.[16–18] The effect of such inhomogeneous mixing is expected to affect the long-range collective H-bonding dynamics of water, which leaves its imprint in the elusive terahertz (THz) frequency regime.[23] Some recent reports on aqueous solutions of a series of alcohols have indeed identified long-range structural heterogeneity in mixtures.[22,24] A combined THz spectroscopic and molecular dynamics (MD) simulation study from our group also established nonmonotonic dynamics of a water/1,2-dimethoxyethane (DME) mixture.[25]

The inherent heterogeneity of a water/alcohol mixture makes the study of biomolecular hydration in such binary solvents even more complex. There have been a few experimental and simulation reports investigating the effect of different alcohols on the perturbation of protein structures.[26,27] It has been known for a long time that low concentrations of alcohols stabilize proteins as a result of preferential solvation of the alcohol molecules at the protein surface,[28] and the addition of alcohol induces α-helix formation in a certain class of proteins,[29–31] for which the effect is dependent on the length of the carbon chain of the alcohol. The carbon chain promotes the transition according to its length, and the hydroxy group suppresses this transition. At high alcohol concentrations, the

[a] *Dr. D. K. Das, D. Das Mahanta, Dr. R. K. Mitra*
Department of Chemical, Biological
and Macromolecular Sciences
S. N. Bose National Centre for Basic Sciences
Block JD, Sector III, Salt Lake, Kolkata-700106 (India)
E-mail: chem2007dip@gmail.com
rajib@bose.res.in

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protein becomes destabilized and can even precipitate due to alcohol binding to the active sites of the proteins; also, during self-aggregation of the alcohols some protein molecules might become incorporated.[32–34] Recent fluorescence correlation spectroscopy (FCS) measurements on a model protein lysozyme by the group of Bhattacharyya et al.[26] and MD simulation studies on chicken villin headpiece (HP-36) by the group of Bagchi et al.[27] unambiguously established strong modulation of the protein hydrodynamic radius as a function of ethanol concentration due to the interaction and preferential binding of the alcohol to the active sites of the proteins. The protein structure exhibits a unique oscillating fluctuation due to the different concentration-dependent binding modes of the alcohols with the protein.[26] Most of these earlier studies were concerned with changes in the protein structure, whereas the associated hydration remained mostly unaddressed. It is therefore of interest to investigate whether the aforementioned changes in the protein conformational behavior could also be manifested in the hydration behavior of the proteins. In the present study, we investigated the effect of three different alcohols, that is, ethanol (EtOH), 2-propanol (2-PrOH), and *tert*-butyl alcohol (TBA), on the model protein bovine serum albumin (BSA). The choice of alcohols was determined by their unlimited water solubilities irrespective of the lengths of their carbon chains. The BSA protein is responsible for the transport of various nutrients in animals and is structurally analogous to the human bovine albumin. The crystal structure of this protein is well established,[35] and this protein has been studied extensively as a model for protein stability, folding, and denaturation;[36,37] furthermore, BSA is responsive to the addition of various cosolutes in aqueous solution.[38–40] In a recent study,[38] the effect of ethanol on BSA was found to be bimodal; the abundance of the secondary structural content was found to be dependent on the concentration of ethanol, which contrasts the findings of other proteins such as lysozyme[34] and melittin.[30] This makes a systematic study on the effect of alcohols on BSA interesting. We obtained structural information of BSA in the presence of the alcohols by using circular dichroism (CD) spectroscopy. The hydration dynamics were obtained by using the THz time-domain spectroscopy (TTDS) technique, which is able to probe the rotational and vibrational dynamics of molecules in the frequency range of 0.1 to 4 THz. It covers part of the far-infrared region of the electromagnetic spectrum and probes molecular motions arising from intermolecular interactions, in particular hydrogen bonds.[41–43] THz spectroscopy measurements of aqueous systems provide pivotal information on the various collective vibrational modes of water that occur on the hundreds of femtoseconds to tens of picoseconds timescale. This technique has successfully been employed to extract hydration dynamics in both binary mixtures[22,25,44] and biomolecules.[45–47] In this contribution, we aim to address the issue of how the addition of alcohols modulates the structure and the hydration of a model protein. The results could be helpful in pharmaceutical applications, as alcohols, in many cases, are used as solvents for various drugs, and knowledge of how biomolecules interact with alcohols serves as a prerequisite for that.

2. Results and Discussion

2.1. CD Measurements

The secondary structure of BSA in the presence of alcohols was investigated by CD spectroscopy in the far-UV region ($\lambda =$ 200–250 nm) (Figure S1 a–c in the Supporting Information). Two negative bands appearing in the far-UV region (at $\lambda = 208$ and 222 nm) typify the predominant secondary ($\approx 60\%$) α-helical structure of BSA.[48, 50] The intensity of the CD signal decreases upon increasing the alcohol concentration. At higher alcohol concentrations, the shape of the CD profile becomes significantly perturbed relative to that in the native state. To investigate the tertiary structure of the protein, we measured the CD profiles in the near-UV region ($\lambda = 250$–350 nm) (Figure S1 d–f). We notice two minima at $\lambda = 261$ and 268 nm along with a shoulder at $\lambda = 287$ nm. These bands correspond to the transitions of the tryptophan residues of the protein and, thus, provide information on the tertiary structure of the protein. We notice that upon increasing the alcohol concentration the intensities of the two minima decrease, and at $X_{alcohol} > 0.1$, these two bands disappear. This indicates considerable perturbation of the protein tertiary structure as a result of binding of the protein with the alcohol. The intensities of these two minima also vary with the hydrophobicity of the alcohols.

For better comprehension of the effect of alcohols on BSA, we plotted the CD signal at $\lambda = 208$ and 222 nm as a function of alcohol concentration (Figure 1 a, b). It is evident that in both cases the CD signal decreases rapidly with the alcohol concentration; at any fixed alcohol concentration, the changes are found to be alcohol specific and follow the sequence TBA > 2-PrOH > EtOH, which also exactly corroborates the hydrophobic contents of the alcohols.

We calculated the relative content of the secondary structure as a function of alcohol concentration (Figure 1 c–e). We found that the abundance of the α helix decreases mostly at the expense of relatively nonstructured random coils. This sug-

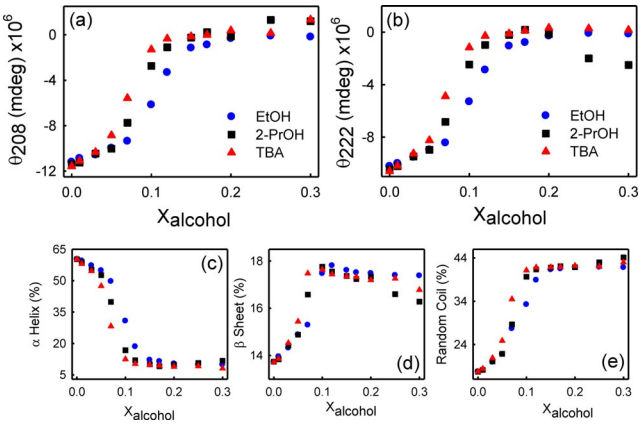

Figure 1. Changes in the CD signal at a) $\lambda = 208$ nm and b) $\lambda = 222$ nm as a function of alcohol concentration for EtOH, 2-PrOH, and TBA. The relative changes in the contents of c) the α helix, d) the β sheet, and e) the random coil are also shown.

gests exposure of the hydrophobic moieties of the protein towards the solvent. Such a decrease in the content of the α helix contrasts that found for other proteins such as lysozyme,[34] β-lactoglobulin,[29] and melittin.[30] The results are indicative of the specific nature of the protein–alcohol interaction; whereas in some cases the alcohol induces volume exclusion to stabilize the helical content, it may also solvate the hydrophobic side chains and destabilize the helical content. As the alcohol molecules are introduced into the aqueous BSA solution, they serve as a good solvent for the protein's hydrophobic milieus.[38] Upon increasing the concentration, the alcohol molecules actively bind to the protein surface and replace the hydrated water molecules; this subsequently produces a strong perturbation in the protein structure, as the hydrophobic residues become exposed to the alcohol molecules. During this process, the more structured helical form is converted into random coils, and the effect is more intense for more hydrophobic alcohols.

2.2. TTDS Measurements

We studied the hydration dynamics of alcohol/water binary mixtures (Figure 2). The frequency-dependent absorption coefficient [$\alpha(\nu)$] in the THz region is correlated to the cooperative

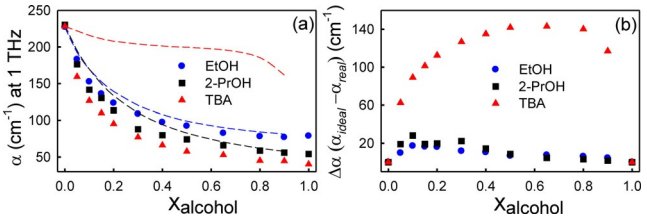

Figure 2. a) The α value measured at 1 THz for alcohol/water binary mixtures as a function of alcohol mole fraction. The dotted lines indicate the corresponding α_{ideal}. The changes in $\Delta\alpha$ ($\alpha_{ideal}-\alpha_{real}$) are shown in panel b.

hydrogen-bonding dynamics of water, as it manifests the density of states of molecules undergoing such motions.[46] The absorbance of water in this frequency region is very high relative to that of other molecules,[22,51,52] and this provides a unique advantage to identify the changes in these parameters and to extract information on the alteration in the hydration dynamics.

In previous studies, we concluded that monovalent salts[53] acted as water-structure breakers, whereas a structure-making trend was more evident in otherwise naive or protein-stabilizing polymers.[54] We measured the THz-frequency-dependent parameters of different alcohol/water mixtures (Figure S2). Figure 2a depicts the absorption coefficient measured at 1 THz for all three binary mixtures. Upon increasing the alcohol concentration, the α value expectedly decreases, and the extent of decrease follows the order $\alpha_{EtOH} > \alpha_{(2\text{-}PrOH)} > \alpha_{TBA}$, that is, the higher the carbon-chain length, the lower the absorption coefficient. The order is anticipated from the increasing size and the increasing hydrophobicity of the molecule. We calculated α_{ideal} by assuming ideal mixing of the components [Eq. (1)]:

$$\alpha_{ideal} = \frac{\rho_{real}}{\rho_{ideal}} \sum_i \phi_i \alpha_i(\nu) \quad (1)$$

in which ϕ_i is the volume fraction of the *i*-th species, ρ_{real} is the measured density of the mixture, and $\rho_{ideal} = \sum_i X_i \rho_i$. The measured values of α deviate strongly from the calculated ones (broken lines in Figure 2a), especially for TBA. A similar nonideal mixing behavior was observed in water/DME[25] and water/dioxane[44] binary mixtures. For better understanding, we plotted the relative change in α ($\Delta\alpha = \alpha_{ideal} - \alpha_{real}$) as a function of alcohol concentration (Figure 2b). We observe decent changes in $\Delta\alpha$ for EtOH and 2-PrOH, but the change is drastic for the TBA/water mixture. In a recent THz study, Li et al.[22] reported a bell-shaped α profile in alcohol/water binary mixtures by using MeOH, EtOH, and PrOH. The observed nonideality emanates either from the formation of defects in the hydrogen-bonding network of water or alcohol–alcohol aggregation and the formation of a clathratelike structure or, more likely, due to a delicate interplay between all these factors. It was previously reported that the entropy of alcohol/water mixtures increased far less than that expected for an ideal solution of randomly mixed molecules;[55] this supports their strong mutual interaction, as also reciprocated in the unusual changes in volume and density.[56–58] The marked deviation in TBA is perhaps rooted in its large size and the presence of hydrophobic butyl groups; this could result in intermolecular aggregation (even at 5% TBA concentration), which would thereby induce incomplete mixing with water.[59]

We measured the frequency-dependent α values of aqueous BSA solutions [at a fixed protein concentration of 1 mg mL^{-1}] in the presence of alcohols (some representative profiles are shown in Figure S3). The protein concentration was low enough to avoid self-aggregation. The protein solutions have lower $\alpha(\nu)$ values than pure water, as determined by replacing high-absorbing water molecules with proteins.[60] We observe that the THz absorption of protein/water/alcohol mixtures does not change linearly, and to obtain better understanding we plotted the relative change in the absorption as a function of alcohol concentration (Figure 3), which is represented by the following equation [Eq. (2)]:

$$\frac{\Delta\alpha}{\alpha_0} = \frac{\alpha_{protein+alcohol+water} - \alpha_{alcohol+water}}{\alpha_{alcohol+water}} \quad (2)$$

The $\Delta\alpha/\alpha_0$ parameter bears the signature of protein hydration explicitly.[48] All curves deviate strongly from linearity and show an unusual oscillating trend. A simple three-component model (water/alcohol/protein) does not suffice to explain the observed trend, which was also observed in DMSO/water/lysozyme mixtures.[48] Notably, the oscillating feature is distinct for the different alcohols. For EtOH and 2-PrOH, the parameter oscillates between positive and negative values of $\Delta\alpha$, whereas for TBA, it is always negative. To understand the fluctuations it is important to consider that the overall hydration dynamics are a delicate balance between competing interactions: alco-

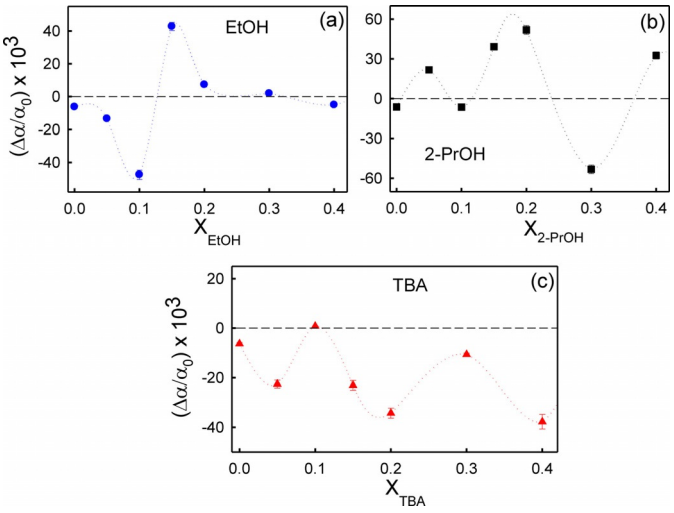

Figure 3. Relative terahertz absorption coefficient (at 1 THz) ($\Delta\alpha/\alpha_0$) in the presence of BSA as a function of alcohol concentration for a) EtOH, b) 2-PrOH, and c) TBA. The dotted lines are guides for the eye.

hols preferentially solvate the protein surface and deplete the hydration layer, which in turn increases the abundance of bulk water. Considering that the absorbances of hydrated and bulk water are different,[46,48] this factor leads to a change in $\Delta\alpha$.

Moreover, with the progressive addition of the alcohol the buried part of the protein becomes exposed (as evidenced from the CD results, wherein we observe an increase in the content of the random coil at the expense of the content of the more structured α helix), which increases the nonpolar solvent accessible surface area (SASA). This facilitates alcohol binding at the protein surface. This process is opposed by possible alcohol–alcohol self-aggregation, which certainly modifies the hydration dynamics in binary mixtures (see Figure 2). An additional factor comes in the form of alcohol-induced size variation of the protein. Recent MD simulation studies by Ghosh et al. indicate that the gyration radius (R_g) of a globular protein chicken villin headpiece (HP-36) exhibits oscillating behavior as a function of ethanol concentration.[27] Similar behavior was experimentally realized by Chattoraj et al., who reported on the oscillating size of lysozyme as a function of EtOH concentration.[26] The fluctuation in the protein size has been explained on the basis of a delicate balance between alcohol–alcohol and alcohol–protein interactions. Interestingly, we also notice an identical fluctuation in the hydration dynamics, and the oscillating pattern varies upon increasing the hydrophobicity of the alcohol. The periodic retardation and acceleration of the hydration dynamics is perhaps caused by counter-interactive interactions of the various factors described earlier. Whereas preferential alcohol accumulation at the protein surface and an increase in protein size decrease the α value, water–alcohol and alcohol–alcohol aggregation and a decrease in protein size increase the α value.

To investigate the effect of alcohol on protein hydration further, we studied protein-concentration-dependent THz absorption in EtOH/water and TBA/water mixtures keeping the alcohol concentration fixed at 5 mol%. The concentration-dependent α values of BSA in these mixtures (averages in the 0.95–1.05 THz range, in this frequency window water does not show any characteristic vibrational signature and α increases monotonically) are shown in Figure 4. We observe an intriguing non-

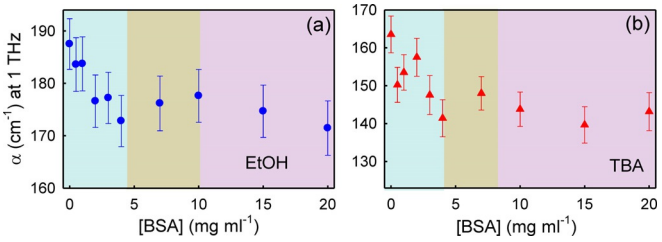

Figure 4. The α value measured at 1 THz as a function of BSA concentration in a) 5 mol% EtOH and b) 5 mol% TBA.

monotonic change in α as a function of alcohol concentration. Notably, THz absorption of BSA in water was previously performed by Xu et al.[61] and Bye et al.;[62] in both of these studies the authors reported a monotonic decrease in α with protein concentration. According to previous reports, a three-component model is a better approach to explain protein hydration, as it takes into consideration the THz-accessible extended-hydration sheathe around the biomolecules.[63] However, in this present study, the system is more complex due to the presence of alcohol as a third component. The α versus protein concentration profile can be divided into three distinct segments (Figure 4): in the low alcohol concentration region, α decreases rapidly (region 1); it then increases modestly (region 2), which is followed by a shallow decrease (region 3) after passing through a maximum.

The profile in the high-protein-concentration region resembles the concentration-dependent THz profile reported earlier. In the case of TBA, the pattern remains almost the same but with a distinct rise at a BSA concentration of about 2 mg mL^{-1} after a sharp fall (Figure 4b). Simple replacement of polar solvents with protein molecules only provides a linear decrease in α and is, therefore, not sufficient to explain the complex nature of the curves. To describe the observed unusual non-monotonic nature of the profile, we need to consider the various interactions involved between the different components. Notably, the α value of a water/alcohol mixture is lower than that of pure water, and the difference increases upon increasing the length of the carbon chain of the alcohols (see Figure 2). Also at this low alcohol concentration (5 mol%), the protein is modestly unfolded with α-helix contents of 55 and 47% in water mixtures with EtOH and 2-PrOH compared to 60% in pure water, with a proportionate increase in the random-coil content. This leads the protein to expose a fraction of its otherwise-buried hydrophobic residues towards the solvent.

The initial steep decrease (region 1) is attributed to the replacement of high-absorbing polar binary solvent with protein molecules. As protein is introduced into the water/alcohol binary mixture, the preferential binding of alcohol at the protein surface disrupts the apparently present water–alcohol and

alcohol–alcohol clusters in the mixture. The effect is more prominent in TBA due to its higher affinity to bind the alcohol-induced exposed hydrophobic surface (higher content of random coil, Figure 1) as well as the nonpolar SASA of the protein.[25] An optimizing effect between these two opposing contributions brings about the increased α value at about 2 mg mL^{-1} protein in TBA. Given that the total alcohol concentration is low, beyond a certain protein concentration the protein surface becomes predominantly solvated by water instead of alcohol and eventually the α value starts to increase[48] (region 2). This increase is initiated at a slightly lower concentration of protein in TBA than in EtOH. As the protein concentration is increased sufficiently, the hydration layers of the proteins start to overlap and α starts to decrease slowly[64] (region 3). The onset of this decrease occurs at a lower concentration in TBA than in EtOH, as the protein molecule is more unfolded and enlarged in TBA than in 5% EtOH (Figure 1). At even higher protein concentrations, the protein molecules aggregate and precipitate out.

3. Conclusions

We reported the hydration behavior of the model protein bovine serum albumin (BSA) in various alcohol/water binary mixtures. Circular dichroism indicated that the contents of the different secondary structures of BSA changed as a function of alcohol concentration and increased carbon-chain length. Terahertz time-domain spectroscopy studies of the alcohol/water binary mixtures indicated a strong deviation in the frequency-dependent absorption coefficient from ideality, which was a manifestation of the making and breaking of H-bonds, water–alcohol cluster formation, and alcohol–alcohol aggregation. We observed a fluctuating change in protein hydration as a function of alcohol. A concentration-dependent study of a protein solution in 5 mol% EtOH/water and *tert*-butyl alcohol/water solutions indicated nonmonotonic behavior of protein hydration. The experimentally observed nonmonotonicity in protein hydration is intriguing, and the inherent complexity of the water/alcohol mixture makes overall understanding of the phenomenon difficult. A detailed simulation considering all the pairwise interactions is certainly necessary.

Experimental Section

Bovine serum albumin (BSA, lyophilized powder, >98% purity) and HPLC-grade alcohols, namely, ethanol (EtOH), 2-propanol (2-PrOH), and *tert*-butyl alcohol (TBA), were procured from Sigma–Aldrich and were used as received. A 1 mg mL^{-1} aqueous BSA solution was prepared in Milli-Q water. For the protein-concentration-dependent study, we fixed the alcohol concentration (alcohol 0.05 mol%), prepared a 30 mg mL^{-1} stock solution of the protein, and the desired concentrations were achieved through dilution. For all of these measurements, only freshly prepared water/BSA/alcohol solutions were used, and the measurements were performed at room temperature (≈293 K).

Circular dichroism (CD) measurements in the far-UV (λ = 200–250 nm) and near-UV (λ = 250–350 nm) regions were recorded with a JASCO J-815 spectropolarimeter by using quartz cuvettes with path lengths of 1 and 10 mm, respectively. The sample scan speed was kept at 50 nm min^{-1} with a response time of 2 s. Three CD spectra were recorded in continuous mode and averaged for each CD experiment. The secondary structural analysis of the CD spectra was done by using CDNN software.

THz time-domain spectroscopy (TTDS) measurements were performed with a commercial THz spectrophotometer (TERA K8, Menlo Systems) and a detailed description of this spectrophotometer can be found in our earlier publications.[48,49] In brief, a 780 nm Er-doped fiber laser [<100 fs pulse width (FWHM), 100 MHz repetition rate] was split into pump and probe beams of nearly equal power (≈10 mW) by using a polarizing beam splitter. The pump beam excited the THz emitter antenna and produced THz radiation having a bandwidth of about 3.0 THz (>60 dB). This THz radiation after transmitting through the sample was focused on a THz detector antenna that was gated by the probe laser beam. The THz antennas were gold dipoles with a dipole gap of 5 μm deposited on LTG-Ga-As substrate. To avoid water-vapor absorption, all measurements were performed under an ultrapure dry nitrogen atmosphere with a controlled humidity of <10% in a liquid cell (Bruker, model A-145) by using *z*-cut quartz windows and a Teflon spacer of 100 μm thickness. The samples were reloaded five times in the sample cell, and nine full scans were averaged together to minimize the error in the results. By varying the time delay between the probe and the pump beam the amplitude and phase of the THz electric field were measured as a function of time. The frequency-dependent power and phase of the transmitted pulse was obtained by using Fourier analysis of the measured electric field amplitude ETHz(t). TTDS involves a coherent detection mechanism and can thus measure both the amplitude and the phase of radiation in a single measurement and can provide information on frequency-dependent optical parameters of the system. Subsequently, the frequency-dependent absorption coefficient [$\alpha(\nu)$](power attenuation) and index of refraction $n(\nu)$ (delay of the THz pulse) can be obtained.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: absorption · circular dichroism · hydration · preferential binding · time-domain spectroscopy

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