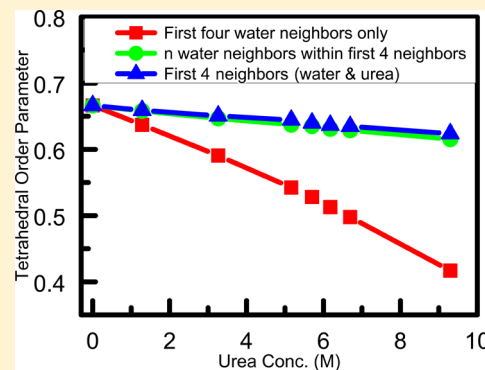


Molecular Dynamics Simulation of Aqueous Urea Solution: Is Urea a Structure Breaker?

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ABSTRACT: An aqueous solution of urea is a very important mixture of biological relevance because of the definitive role of urea as protein denaturant at high concentrations. There has been an extended debate over the years on urea's influence on the structure of water. On the basis of a variety of analysis methods employed, urea has been described as a structure-breaker, a structure-maker, or as neutral toward water structure. Using molecular dynamics simulation and a nearest neighbor approach of analyzing water structure, we present here a detailed analysis of the effect of urea on water structure. By carefully choosing the nearest neighbors, allowing urea also to be a neighbor of a reference water molecule, we have conclusively shown that urea does not break the local tetrahedral structure of water even at high concentrations. A slight change in the distribution of tetrahedral order parameters as a function of urea concentration has been shown to be a result of change in the proportions of *n*-hydrogen-bonded water molecules. The present result thus suggests that urea is able to substitute for water in the hydrogen-bonded network nicely without breaking the tetrahedral, hydrogen-bonded structure of water.



INTRODUCTION

The solution of aqueous urea has received a lot of attention^{1–86} over the last few decades and is still a subject of intense research. There are several reasons behind this intense research. First, an aqueous urea solution at high concentration is widely used to denature proteins^{1,2} and therefore has general biological relevance.³ Apart from that, a urea–water aqueous solution has many interesting properties such as enhancement of solubility of long chain hydrocarbons,^{4,5} prevention of micelle formations,^{6,7} etc. Finally, urea–water systems can be considered as an important test case to understand aqueous solvation of a neutral solute with both hydrophobic and hydrophilic (those providing hydrogen bonding sites) groups.^{8–11} Although it is well-known that a fine balance between water–water, water–urea, and urea–urea interactions regulates the behavior of aqueous solution of urea, fundamental understanding of the correlation between structural aspects of this solution and the above-mentioned properties is still lacking.

In this context, the most important issue that the scientists around the world are trying to understand is how urea denatures a protein? Two important pathways have been proposed to explain the denaturation process. At present, the most widespread view^{12–37} is that urea directly attacks the polar backbone as well as nonpolar residues of a protein helping water to penetrate into the core. In another very popular hypothesis suggested by Frank and Franks³⁸ in the 1960s, it is posited that urea breaks the tetrahedral network structure of water and thus acts as a structure-breaker or chaotrope and indirectly helps in denaturing proteins in water. Since then, many experimental and theoretical studies have been devoted to understand how the structure of water changes in the

presence of urea and have produced disparate results yielding three distinctly different views. In some works, it is claimed that urea disrupts the water structure and thus urea is termed as a structure-breaker or chaotrope. Many others have termed urea as structure-maker or kosmotrope as they find urea to enhance water structure. Both of these two views point to the peculiar behavior of urea in changing the structure of water and suggest an “indirect effect”^{39–44} of urea on protein denaturation. Apart from these two extreme views, another group of findings suggests that there is no or only negligible changes in the structure of water due to the addition of urea. This view suggests urea's action through direct effect; in which urea followed by water penetrates the native protein molecule to denature it.^{45–50} In addition to these views, many researchers also suggest both direct and indirect effects of urea in the denaturation process.

To understand the effect of urea on the water structure, it is essential to know about the detailed structure of pure water (in the absence of urea). Water is an intriguing liquid because of its many anomalous properties and tetrahedral network structure. However, linking these anomalous properties of water to its structure has been a difficult task. One famous idea about water structure and its relation with different anomalies is that the water is a mixture of two different structural moieties, one of low density tetrahedral liquid phase and the other of high density liquid phase.^{51,52} In recent years, the existence of high-density and low-density structural motifs at ambient water has

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been intensely debated.^{53–56} Water structure is predominantly tetrahedral due to formation of a three-dimensional hydrogen-bonded network, although molecules with fleetingly broken hydrogen bonds contribute⁵⁶ significantly to the nontetrahedral signature depicted by the distribution of tetrahedral order parameters. There is a long-standing debate on the extent and nature of perturbation in the tetrahedral structure of water due to the addition of urea. The cause of this debate is probably due to lack of proper applicability of a suitable framework for quantitatively describing the structure of water in the presence of urea.

Many experimental studies such as NMR, neutron scattering, dielectric relaxation, and thermodynamic measurements have yielded contrasting results on the influence of urea on water structure. The Raman band analysis of Walrafen⁵⁷ and the NMR analysis of Sacco et al.⁵⁸ have indicated urea to be a structure-breaker. From the ultrasonic attenuation measurements⁵⁹ on an aqueous solution of urea and a polymer, it has been observed that, between 2 and 4 M urea concentrations, there is a significant reduction in relaxation time and it has been attributed to cooperative breakdown of the water structure around the polymer. From the recent FTIR spectroscopic study³⁵ on aqueous solution of trimethylamine oxide (TMAO), urea, and guanidine hydrochloride (GdnHCl), it has been observed that the presence of these solutes decreases the strength of the hydrogen bond between water and the infrared probe. It is concluded from this study that although urea and GdnHCl can perturb the hydrogen-bonding property of water, their protein-denaturing ability does not arise from a simple indirect mechanism. Using vibrational sum frequency spectroscopy, Cremer and co-workers⁶⁰ have probed interfacial urea molecules residing at the interface between bovine serum albumin and water and concluded that the urea breaks the water structure. Combining neutron scattering and molecular dynamics simulation, Soper et al.⁶¹ have shown that the first solvation shell of water does not change with the increasing concentration of urea. However, the orientational distribution as calculated in this work has indicated substantial distortion of the tetrahedral structure in the aqueous urea solution.

Similarly, there are many studies that report urea as a noninterfering solute as far as the structure of water is concerned. In this case, urea's ability to denature protein is explained on the basis of direct preferential interaction of urea with polar and nonpolar groups of the protein. Many such experimental and simulation studies^{12,18,46} with almost similar conclusions have been reported. A recent dielectric spectroscopy study⁶² has claimed that urea and water are readily interchangeable in the H-bonding network of water, and therefore urea does not act as a strong structure-breaker of water. A tera-Hertz absorption spectroscopy study⁴⁷ of an aqueous solution of urea has found no evidence of a structure-breaking effect of urea and suggested a direct rather than an indirect effect of urea on protein denaturation. Using polarization-resolved mid-infrared pump–probe spectroscopy, Bakker and co-workers³⁷ have concluded that urea does not change the strength of the hydrogen-bond interactions between water molecules. They have also found that a small fraction of the water molecules strongly immobilized by urea can form two hydrogen bonds with the two sites of the same urea molecule. Very recently, using vibrational spectroscopy and molecular dynamics simulation, Skinner and co-workers⁶³ have observed that all absorption spectra are insensitive to urea concentration suggesting that urea only weakly perturbs the water structure.

The work of Tsai et al.⁶⁴ illustrates that urea molecules distribute uniformly in water networking without disturbing it. This view has been supported by various researchers by analyzing the RDF of water–water and/or urea–water at varying concentration, temperatures, and pressures.^{18,19,40,65–67} By comparing RDFs of water in the urea–water system at various urea concentrations, Wallqvist et al.¹⁴ have shown that urea molecules preferentially get adsorbed into polar residues of the protein and there is no structural distortion of water in the presence of urea. In a very recent, state-of-the-art simulation study based on combined parallel tempering and metadynamics simulation, Parrinello and co-workers²⁵ have investigated the effect of urea on the β -hairpin conformational ensemble and protein denaturation mechanism and have inferred that a preferential direct interaction exists between urea molecules and protein backbone. Pettitt and co-workers,⁴⁵ on the basis of calculated activity coefficients of urea, hydrogen-bond lifetime, and average number of hydrogen bonds have shown that the urea solution is near ideal and thus the water structure is not perturbed. On the basis of the proximity criterion, which provides⁶⁸ detailed local structure around substructures such as atoms, functional groups, etc., Kuharsky et al.⁶⁹ have analyzed an infinitely dilute solution of urea and found no significant structural and energetic perturbation in water due to the presence of urea.

Many studies have mentioned that both direct and indirect effects are responsible for the denaturing effect of urea. A recent NMR study of Almaraz et al.⁷⁰ has found that the initial step of urea denaturation is guided by direct interaction. However, they have suggested the breaking of hydrophobic collapse due to an indirect effect as the final steps for protein denaturation. Similarly, the work of Nilsson and co-workers⁴¹ has also suggested both direct and indirect effects of urea on protein denaturation. Bennion et al.⁴⁰ have used MD simulation to show the involvement of both direct and indirect mechanisms in the denaturation process.

In a recent development, Stumpe et al.⁷¹ have shown both average number of hydrogen bonds per water molecule and water–water radial distribution functions to be independent of urea concentration and inferred that a water molecule in the hydrogen-bond network of water is easily substituted by a urea molecule through water–urea hydrogen-bond formation. This work also reveals that a water–water hydrogen bond is stronger than a water–urea hydrogen bond, and this may be the driving force behind self-aggregation of urea in its concentrated solution. Although they are unable to detect any structural change in the water network due to the addition of urea, from the comparison of the hydrogen-bond energy of water–water and water–urea pairs, they have described urea as a structure-maker or “kosmotrope”. From the analyses of Kirkwood–Buff integrals, Chitra et al.⁷² have described urea as a slight structure-maker. In an earlier work, Vanzi et al.⁸ have evaluated the hydration heat capacity of urea and from that concluded that the solute appears to enhance the water structure. In a recent publication, Yamzaki et al.⁷³ also have discovered that water is more structured in urea–water solution as compared to the same in the bulk.

Most of the computational studies are based on the analyses of radial distribution functions (RDFs).⁷⁴ Although the RDF is a central statistical mechanical quantity, which signifies two-particle reduced distribution function describing structure of a homogeneous isotropic liquid,⁷⁴ it cannot quantify angular distortion if any in the hydrogen-bonded, tetrahedral structure

of water. The relative angular arrangement of water molecules around a central water molecule is of prime importance to understand any orientational distortion in the hydrogen-bonded network. In fact, there are some studies in which it is shown that various angular distributions of water in concentrated aqueous urea solution is quite different from the same in pure water. Since water is a tetrahedral liquid, any measurable distortion in this tetrahedral arrangement due to the addition of urea will signify breakdown of water structure. Various order parameters in liquid water have been developed and extensively used to quantify tetrahedral order in pure water as a function of temperature and other thermodynamic parameters. Exactly on this line, Idrissi et al.⁷⁵ have analyzed distortion if any in the tetrahedral local structure of water in the presence of urea. They have calculated various order parameters using a nearest neighbor approach. Their results have indicated considerable distortion in the local structure of water and thus they have described urea as a structure-breaker.

However, in calculating various radial and orientational parameters, they considered only water molecules (not urea) as neighbors of a reference water molecule. Considering the fact that a large number of studies^{14,18,19,37,40,45,47,62–67,69} have shown that urea mixes well with the water and that it is able to substitute⁷¹ for water in the hydrogen-bonded network very well from geometric and steric consideration, it is reasonable to expect that in the solvation shell of a water molecule, some of its nearest water neighbors will be replaced by urea molecules. Thus, while considering the tetrahedral arrangement of the four nearest neighbors around a reference, central water molecule, some of the vertices of the tetrahedron may be occupied by urea sites preserving the tetrahedral structure. In this article, by properly taking into account the nearest-neighbor sites irrespective of whether it is from urea or water, it is shown that the local tetrahedral structure of water is not broken by urea even at high concentrations. The analysis of the orientational structure has been done by carefully analyzing the average tetrahedral order parameter, distributions of tetrahedral order parameters, hydrogen-bonding angles, and angles (triplet angle) extended at a central water molecule by two of its nearest neighbors. Radial arrangements of neighboring water molecules have also been analyzed by calculating average, fluctuation, and distribution of radial distances of n th nearest neighbor.

1. MODELS AND METHODS

In the present investigation, we have used the TIP4P/2005⁷⁶ rigid body atomistic model of water having fixed bond lengths and bond angles. This model is a new variant of the TIP4P model and is able to reproduce most of the anomalies of water. For urea, we have used a flexible model with bond, angle, and dihedral terms in the intramolecular potential in addition to usual nonbonded Lennard-Jones and Coulomb interactions. The details of the urea model are given in ref 75. To check the robustness of the results obtained with respect to the urea models, we have performed additional simulations with KBFF⁶⁷ and AMBER⁸⁶ models of urea. The calculated values of order parameter q_4 (see eq 2) as a function of urea concentration, as obtained from different models, show the same general trend and in accordance with the conclusions of this work. Aqueous urea solutions of different concentrations are prepared by considering a pre-equilibrated water box of an appropriate size and then substituting some of the water molecules by urea to achieve a specific concentration. Specific details of the numbers

of urea (N_U) and water (N_W) molecules with the volume of the boxes and molarity of the resulting solutions are given in Table 1. Although we have studied a wide concentration range from 0 to 9 M of aqueous urea solutions, concentration range of 6–8 M is important for protein denaturation studies.

Table 1. Different Systems Simulated in This Work

N_u	N_w	X_u urea mole fraction	volume (nm^{-3})	molarity (M)
0	512	0.0	15.37	0
20	812	0.024	25.81	1.29
60	872	0.064	30.507	3.27
120	993	0.114	38.60	5.16
120	873	0.121	34.95	5.70
120	783	0.133	32.26	6.18
120	699	0.147	29.76	6.70
180	631	0.2219	32.14	9.30

All the simulations were performed in NPT ensemble⁷⁷ using the molecular dynamics extended system approach⁷⁸ of Parrinello and Rahman to fix pressure and the Berendsen algorithm⁷⁹ to fix temperature. Periodic boundary conditions and minimum image conventions were used in all three directions. For water, the bonds and the angle of a water molecule were constrained by LINCS algorithm⁸⁰ and particle-mesh Ewald (PME) method^{81,82} was adopted for treating electrostatic interactions. Equations of motions were integrated using a leap-frog algorithm⁷⁷ with a time step of 0.5 fs. All the simulations were carried out at a target pressure of 1 atm and a target temperature of 300 K using GROMACS⁸³ simulation package. All the analyses were performed using our home-grown software. For all simulations, trajectories for the first 10 ns were discarded for equilibration and the same for the next 4 ns have been stored for analyses.

Local Orders. In the case of a perfectly tetrahedral structure, there are four nearest neighbors in the first solvation shell and another 12 neighbors in the second solvation shell around the central water molecule. Radial distribution functions depict radial arrangements of neighboring water around a reference, central water molecule, but cannot provide any information about the angular preferences of the neighboring molecules. Therefore, to measure the extent of oriental orders of the water molecules in the first solvation shell of a reference water molecule, two different orientational order parameters^{84,85} have been used here.

Tetrahedral Order Parameter and Triplet Angle. In this approach, a tetrahedral order parameter q_i is associated with each of the water molecules in the system. The parameter q_i measures the degree of tetrahedrality of the first solvation shell by using four nearest neighbors:

$$q_i = 1 - \frac{3}{8} \sum_{j=1}^3 \sum_{k=j+1}^4 \left[\cos \theta_{jik} + \frac{1}{3} \right]^2 \quad (1)$$

where “ i ” is the central molecule and θ_{jik} is angle formed by neighbors j and k at the reference, central water molecule i . The value of this quantity is 1 for a perfect tetrahedral structure and the normalization factor of $3/8$ before the summation ensures the value of q_i to be zero for a perfectly disordered system. Since there are four neighbors, there are six angle terms in the summation and each term contributes $4/9$ because of angular averaging. To get a q_i value of zero for the random uncorrelated orientations, therefore, the normalization constant of $3/8$ arises

from the contributions of six angles as $1/(6 \times 4/9)$ as used by Errington and Debenedetti.⁸⁴ In the present investigation, apart from using four neighbors, we have considered central molecules with two and three neighbors as well (see Result and Discussion). For calculating the tetrahedral order parameter in those cases we have used proper normalization. Details of how these normalization constants have been calculated are given in ref 56. The average value q_4 , the tetrahedral order parameter, is calculated by averaging over all the N molecules and over the ensemble using the following equation

$$q_4 = \frac{1}{N} \left\langle \sum_{i=1}^N q_i \right\rangle \quad (2)$$

The angle $\cos \theta_{ijk}$ (see eq 1) which is also known as a triplet angle is itself a very sensitive order parameter to gauge the local tetrahedral orientation of any two nearest neighbors of a central molecule, and hence we have used the distribution of this triplet angle also in our present analysis.

Hydrogen Bond. Since water is a hydrogen-bonded, network forming liquid, the average number ($\langle n_{HB} \rangle$) of hydrogen bonds (HB) and their distribution are two useful quantities for the measurement of any structural changes in water. To identify a HB, we have used a standard geometric criterion, according to which two water molecules are considered to be H-bonded only if (i) the interoxygen distance is less than 3.5 Å, (ii) the hydrogen–oxygen (H-bonded) distance is less than 2.45 Å, and (iii) the H–O...O angle is less than 30°. Water–urea hydrogen bonds are also calculated using the same criteria. In this case NH_2 and carbonyl oxygen atom sites of urea are considered for hydrogen bonding.

2. RESULT AND DISCUSSION

In this study we are concerned about the local structure of water. The local structure of water is generally described by both radial and orientational arrangements of molecular sites around a central molecule. One such analysis is performed by calculating intermolecular distance distribution, often expressed as the radial distribution function (RDF). In the case of homogeneous spherically symmetric systems, RDF is a very useful quantity to study liquid structure. But in the case of a liquid like water, where directional bonding leads to a tetrahedral network structure, angular distribution of neighbors around a central molecule is also of prime importance. Assuming the water structure to be nearly tetrahedral, the tetrahedral order parameter (see eqs 1 and 2) provides a good measure of angular arrangements of the nearest neighbors around a central molecule. Any deviation of this order parameter in the presence of urea from its pure water value indicates angular distortion in the water structure. However, in such calculations of radial and orientational distributions, it is very important to choose nearest neighbors properly. Unlike pure water, in which all the neighbors of a central water molecule are water only, in a binary mixture of urea and water, first four neighbors (distance wise) can be either water or urea molecules. In this analysis, we first examine how tetrahedral order parameter and its distribution, calculated on the basis of all the four nearest *water* neighbors (neglecting urea as a neighbor), change as a function of urea concentration. In Figure 1a, we have shown the average value of the q_4^w , calculated on the basis of four nearest *water* neighbors only, as a function of urea concentration. The superscript w indicates that the value

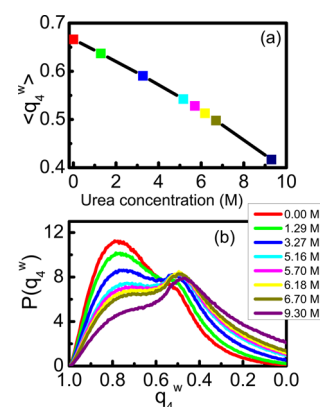


Figure 1. (a) Average tetrahedral order parameter $\langle q_4^w \rangle$ considering all four *water* neighbors (no urea molecule is considered as a neighbor) of a reference water molecule as a function of molar concentration of urea and (b) distributions $P(q_4^w)$ of tetrahedral order parameters. Different colors correspond to different urea concentration as shown in the legend.

of q_4 is calculated by considering all four neighbors as water (without considering urea). A steady decrease of the average value $\langle q_4^w \rangle$ is observed as the concentration of urea increases from 0 M to around 9 M, indicating a clear disruption of the tetrahedral structure of water. The distributions ($P(q_4^w)$) of the tetrahedral order parameter, q_4^w , also show (see Figure 1b) marked variation as the concentration of urea is increased. The distribution $P(q_4^w)$ for pure water (red line) has one major peak at a q_4 value of around 0.8 corresponding to the tetrahedral structure of water (for a perfectly tetrahedral arrangement q_4 should be 1) and another small peak at around 0.5. There was no clear explanation about the origin of this nontetrahedral peak at around $q_4 = 0.5$. Recently, this nontetrahedral peak has been shown⁵⁶ to originate from those neighboring molecules that are *not* hydrogen bonded to the central, reference water molecule. As the concentration of urea is increased, the tetrahedral peak in the distribution decreases and the nontetrahedral peak increases. Similar trend is observed by Idrissi et al.⁷⁵ who concluded that this change is due to the breaking of the tetrahedral structure of water by urea.

Water has a hydrogen-bonded network structure. Thus, the distribution of the hydrogen-bonded angle, θ_{HB} , formed between the line joining the oxygen atom of the central molecule and the oxygen atom of one of its neighbors and the O–H bond of that neighbor, provides a geometric picture of the orientational preference of the hydrogen-bond network. Comparison of the distributions of pure water with the same for binary water–urea solutions of different concentrations may give us an idea about orientational distortion, if any, in the hydrogen-bond network of water. In Figure 2a, we have shown the distribution, $P(\theta_{HB})$ of the *first* water neighbor for pure water as well as for urea–water solutions with different concentrations. We find no significant changes in peak positions and intensities as the concentration of urea is increased. In case of the *second* water neighbor also (see Figure 2b), no significant changes in the distribution as a function of urea concentration is observed. However, if we consider the third (see Figure 2c) and the fourth (see Figure 2d) water neighbors, significant changes in the distributions as a function of urea concentration have been observed. Similar observation has been made by Idrissi et al. (ref 75), and they have

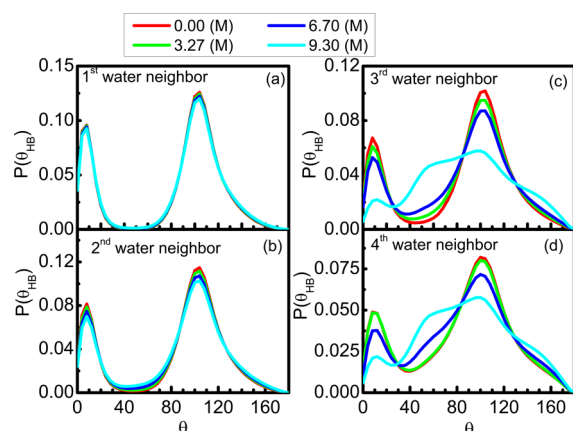


Figure 2. Distributions $P(\theta_{\text{HB}})$ of the hydrogen bonding angle formed by a reference water molecule and one of its neighbors. The hydrogen bonding angle θ_{HB} is the angle formed by the line joining the two oxygen atoms and the OH bond vector. In panels a–d distributions for four nearest *water* neighbors are shown. In this case urea is not considered as a neighbor.

concluded it to be due to the breaking of the hydrogen-bonded network structure of water in the presence of urea.

It is suggested and shown by Idrissi et al.⁷⁵ that the effects of urea concentration on average value and its fluctuation of the distance, r_n , of the n th *water* neighbor from a reference, central water molecule are significant. Here too, with the TIP4P-2005 model of water and by considering only water as neighbors, we find a change in average value, $\langle r_n^w \rangle$ for $n \geq 3$ (see Figure 3a) as

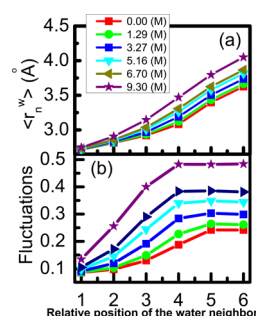


Figure 3. (a) Average oxygen–oxygen distance of the n th *water* neighbor from a reference water molecule for first six *water* neighbors; (b) fluctuations in the above-mentioned quantities.

a function of urea concentration. Corresponding fluctuations are shown in Figure 3b, and a more pronounced change in this quantity as a function of urea concentration is observed. Thus, the present result also seems to reinforce the idea that the water structure is broken in the presence of urea. It is important to emphasize at this point that in all the above cases, we have considered only *water* neighbors, that is, while choosing nearest neighbors we have selected (distance-wise) the first four water molecules without considering urea as a neighbor. As shown in many earlier works⁷¹ that urea readily replaces water in the hydrogen-bonding network of water, and therefore it may so happen that the water molecules, which were third or fourth nearest neighbors of a central water molecule in bulk water, are now replaced by one or more (sites of) urea molecules in concentrated urea solution. In other word, these third or fourth nearest *water* neighbors may not always reside within the so-called first solvation shell (which we considered to be of radius

3.15 Å) because of their replacement by (site/s of) urea molecules. To get an idea about how these *water* neighbors are radially distributed around a central water molecule, we have shown, in Figure 4, the distribution of the distances (r_n) of the

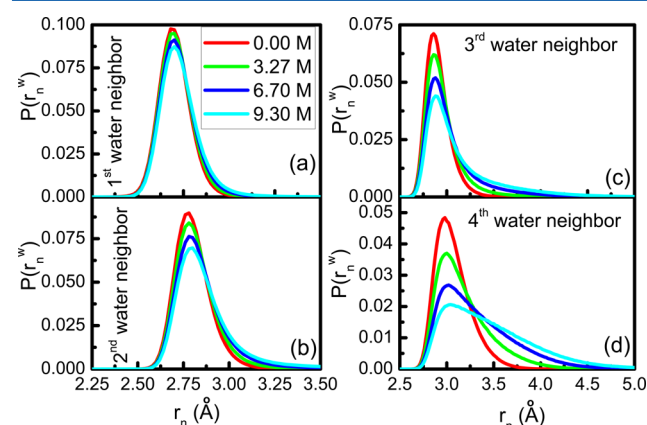


Figure 4. Distributions $P(r_n)$ of the distance r_n of the n th *water* neighbor from a reference water molecule for $n = 1$ –4 are shown in panels a–d.

n th ($n = 1$ –4) *water* neighbor from the central water molecule. It is clearly visible (see Figure 4d) that in a concentrated urea solution, the distributions of the third and fourth nearest *water* molecules extend well beyond 3.15 Å. Therefore, there is a significant chance that these *water* neighbors are now replaced by one or more sites of the urea molecules in the concentrated aqueous urea solution. To get that insight, we have calculated the probability, $P(n, U)$ that the n th neighbor (distance-wise from a central water molecule) is urea as follows:

$$P(n, U) = \frac{(N_U^n)}{(N_T^n)} \quad (3)$$

where N_U^n is the number of occurrences that (site/s of) urea (U) occupies the n th nearest neighbor position and N_T^n is total occurrences that urea and water occupy the n th nearest neighbor position. Therefore, the probability $P(n, W)$ that the n th neighbor (distance-wise from a central water molecule) is water is given by $(1 - P(n, U))$. In Figure 5, we have shown both the probabilities (urea as well as water). It is seen that the probability of third or fourth neighboring position of a reference water molecule being occupied by a urea molecule is increasing (see lines with triangles) steadily with increasing urea concentration. Consequently, the probability that the n th

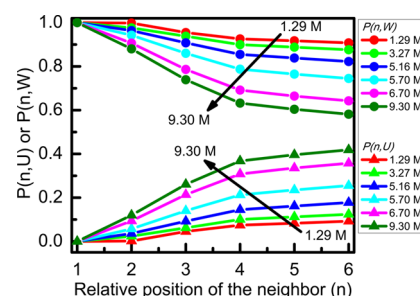


Figure 5. Probability $P(n, U/W)$ that the n th neighbor of a reference water molecule is a urea/water site as a function of urea concentration. $P(n, W)$ data are shown by lines with filled circles and $P(n, U)$ data are shown by lines with filled triangles.

position is occupied by water is decreasing as shown by lines with circles. For example, the probability of the fourth neighbor being a urea molecule at the urea concentration $C_U = 9.00$ M is about 37%. Therefore, if we consider all the four neighbors around a central water molecule as *water*, there is a 37% chance that the fourth nearest neighbor is urea and not water; and therefore if we consider the fourth nearest *water* molecule as one of the neighbors in the first solvation shell to calculate radial and orientational order parameters, there is a high probability that these order parameters will show significant deviations from their respective bulk water value, as these water molecules are now in the second solvation shell. While calculating any order parameter based on nearest neighbors, it is, therefore, desirable to consider both urea and water sites while choosing the first four (distance-wise nearest) neighbors around a central water molecule.

Now, let us recalculate our previously calculated quantities (as shown in Figures 1–4) by considering n nearest neighbors irrespective of whether the neighbors are urea or water. In Figure 6, we have shown the distributions of positions of the

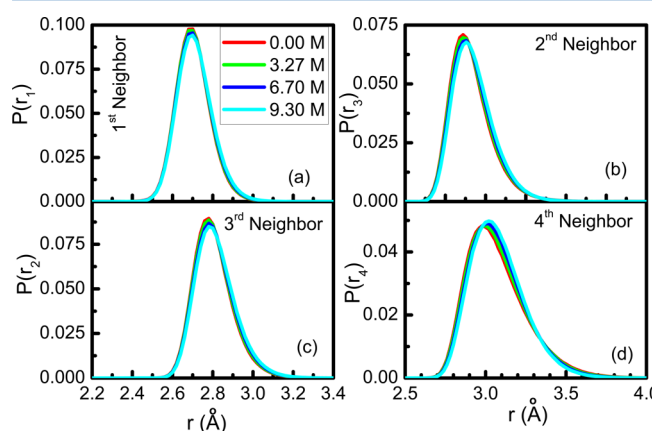


Figure 6. Same as in Figure 4 except that now in choosing the first n neighbors, sites of urea and water both are considered.

n th neighbor for $n = 1-4$. In this calculation, apart from water, we considered urea also as a neighbor if it is (distance-wise) within the first four neighbors of a reference water molecule. It is now clear from the plots in Figure 6 that irrespective of the urea concentration, the distributions $P(r_n)$ are almost the same for $n = 1-4$ (compare this figure with Figure 4).

To calculate average distance $\langle r_n \rangle$ of the n th neighbor and its fluctuation for $n = 1-6$, now we have considered first n nearest sites (of a reference water molecule) irrespective of whether it is from urea or water. The calculated quantities are shown in Figure 7. In this calculation, we have considered sites from both urea and water as neighbors. It is interesting to observe (see Figure 7a) that the average distance $\langle r_n \rangle$ of the n th neighboring site does not change at all up to $n = 4$ with the increase in urea concentration (compare these plots with the same in Figure 3a). If we calculate the fluctuations of these distances in the same way as above, then it is seen (see Figure 7b) that with the increase in urea concentration, fluctuations also do not change appreciably up to fourth nearest neighbor (compare this result with the same in Figure 3b). These results thus illustrate that one or more urea sites nicely substitute water molecules in the solvation shell of water in such a way that the radial arrangements of the remaining water neighbors in the first solvation shell remain the same as that in pure water. It is well-

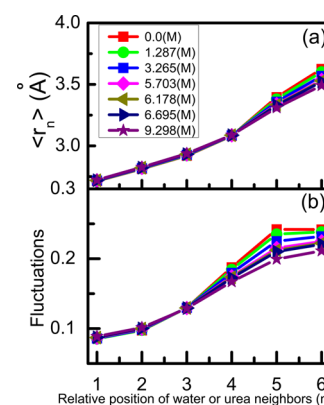


Figure 7. Same as in Figure 3 except that now sites of urea and water both are considered in choosing the neighbors of a reference water molecule.

known that hydrogen-bond formation is a driving force behind the tetrahedral network structure of water, and it is expected that if tetrahedral network is distorted or broken, then it will affect the hydrogen bonding geometry also. Thus, it will be useful to check the distribution of hydrogen-bonding angle, θ_{HB} as a function of urea concentration. In this calculation also, while selecting the first n neighbors of a reference water molecule, we have considered both urea and water as a neighbor. After designating the first four neighbors (whether it is water or urea sites), we calculate the angle θ_{HB} by considering only n th (where $n \leq 4$) neighboring water molecules (not urea). $P(\theta_{HB})$ distributions calculated in this way are shown in Figure 8. As the figure suggests, no distortion in the $P(\theta_{HB})$

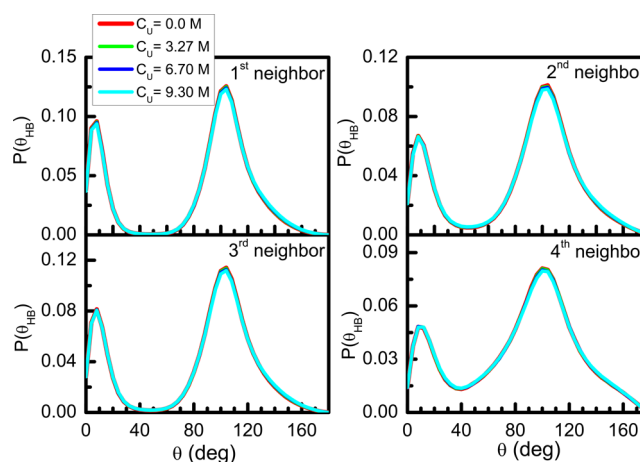


Figure 8. Same as in Figure 2 except that now we choose four nearest neighbors irrespective of whether it is urea or water and then calculate the required hydrogen bonding angle between the reference water molecule and that *water* neighbor, which is within the first four (distance-wise) neighbors.

distribution for any of the first four neighbors is observed as the concentration of urea is increased up to 9.3 M. The basic difference between this result and the same shown in Figure 2 originates from the fact that now we are considering a urea site also to be a neighboring site; thus discarding those water molecules that are not really within the first four nearest neighbors of a given water molecule (but they are within the first four *water* neighbors). In other words, the distortion shown in Figure 2 in the case of a third or fourth neighbor at

high urea concentration is originating from the water molecules residing outside the first solvation shell.

It is now worth examining how the average value ($\langle q_4 \rangle$) of tetrahedral orders parameter q_4 and its distribution change with the change in urea concentration. We have shown in Figure 9a,

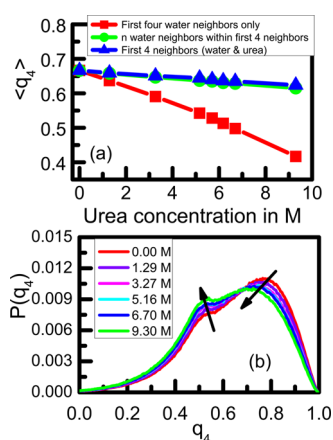


Figure 9. (a) Average tetrahedral order parameter ($\langle q_4 \rangle$) as a function of molar concentration of urea. Red line with squares represents results obtained when we choose the first four water molecules as the four nearest neighbors. Now, first we select four nearest neighbors irrespective of whether it is urea or water and then calculate q_4 by considering only those water molecules that are within the first four (distance-wise) chosen neighbors. Green line with circles represents results obtained by considering n ($n \leq 4$) water neighbors that are within the first four nearest neighbors. We have used proper normalization for the cases where the number of nearest neighbors is less than four (see Models and Methods section). The calculated result by considering both urea and water if they are within the first four neighbors is shown by the blue line with triangles. (b) Distributions $P(q_4)$ of tetrahedral order parameter q_4 corresponding to the green line in Figure 9a. Different colors correspond to different urea concentration as shown in the legend.

the average values of the tetrahedral order parameter ($\langle q_4 \rangle$) calculated in three different ways: (i) by considering all four nearest neighbors as “water” (without considering urea as a neighbor) as shown by red line with symbols; (ii) by considering first four nearest neighbors, where these neighbors can be both urea and water (blue line with symbol); and (iii) by taking only n (with $n \leq 4$) “water” molecules (not urea) that are within the first (distance-wise) four neighbors. In this case, if $n < 4$, we have used proper normalization (see Models and Methods section) for q_4 calculation. It is interesting to observe that if q_4 is calculated by the second method (i.e., by considering both urea and water while selecting first four neighbors and also considering all these four neighbors in the angle calculation), the average value of q_4 does not change (blue line with symbols) much with the increase in urea concentration. When we consider all the four neighbors as water (first method), there is a drastic decrease in the average q_4 values as a function of concentration of urea, C_U . This deviation occurs because of the probability that the third or fourth water molecule is outside the first solvation shell (or beyond the first four neighbors) has increased with increasing urea concentration (see Figure 5), and thus when we consider that all the four neighbors are water, basically we are including the outside (the solvation shell) water molecule, which contributes to the deviation. It is interesting to check whether the water molecules residing within the first solvation shell

(where the first solvation shell is assumed to be formed by four nearest neighbors irrespective of whether these molecules/sites are from urea or water), restore the tetrahedral arrangement even in highly concentrated urea solution. For that, we have calculated the average q_4 by considering only those water molecules residing within the first solvation shell (third method described above) and in this case also, we do not observe (see green line with symbols in Figure 9a) considerable change in average values with increasing concentration. This line is almost coincident with the blue line as calculated by considering four nearest water and urea neighbors. This confirms that the water neighbors within the first solvation shell are tetrahedrally arranged, and only those who are beyond the first four neighbors contribute to the deviation shown in Figure 1a.

To get further insight, we have shown in Figure 9b the distributions ($P(q_4)$) of q_4 , calculated in the following way: First designate first four neighbors of a water molecule considering both water and urea as neighbors and then calculate the distribution $P(q_4)$ by considering only n (with $n \leq 4$) water neighbors that are within the first four neighbors (this situation corresponds to the green line in Figure 9a). The distributions at different urea concentrations are almost coincident (compare with Figure 1b) showing that urea does not appreciably break the water structure. However, a critical observation of the distributions reveals that there is a slight decrease in the tetrahedral peak appearing at high q_4 and a slight increase in the nontetrahedral peak at low q_4 values. Not only that, the decrease of the tetrahedral peak is also associated with a slight shift toward low q_4 value. A pertinent question then arises: is this minute change in the $P(q_4)$ distribution with increasing urea concentration a result of breakdown of the tetrahedral water structure?

To understand this, it is important to know the origin of the appearance of the nontetrahedral low q_4 peak, which appears even in $P(q_4)$ of bulk water. The angle involved in the q_4 calculation is formed by two neighboring water molecules extended at the central, reference water molecule. In a recent publication,⁵⁶ other water molecules high q_4 tetrahedral peak originates if both the neighbors are hydrogen-bonded to the central molecule and the low q_4 (nontetrahedral) peak originates if both the neighbors are *not* hydrogen-bonded to the central molecule and also if one of them is hydrogen-bonded but the other is not hydrogen-bonded (to the central water molecule). So, to get correct angular distribution, one requires knowledge about the hydrogen-bonding pattern of water.

In Figure 10a, we have shown the average number of hydrogen bonds, $\langle n_{HB} \rangle$, as a function of urea concentrations (C_U) for three different cases. The red line shows the $\langle n_{HB} \rangle$ formed by a central water molecule with other water molecules and it decreases with increasing C_U . The blue line depicts the change in $\langle n_{HB} \rangle$ formed by a central water molecule with a urea neighbor as a function of urea concentration, and clearly it is seen that $\langle n_{HB} \rangle$ is increasing as a function of C_U . It is interesting to observe that when we calculate $\langle n_{HB} \rangle$ formed by a central water molecule with both water and urea molecules, we find almost no change (see green line) in $\langle n_{HB} \rangle$ as a function of C_U . Thus, it clearly illustrates that some of the water–water hydrogen bonds of bulk water are nicely substituted by the water–urea hydrogen bonds in concentrated aqueous urea solution. A similar observation was made by Stumpe et al.⁷¹ To get further insight, we have also calculated percentages of n -hydrogen-bonded water molecules of two different types,

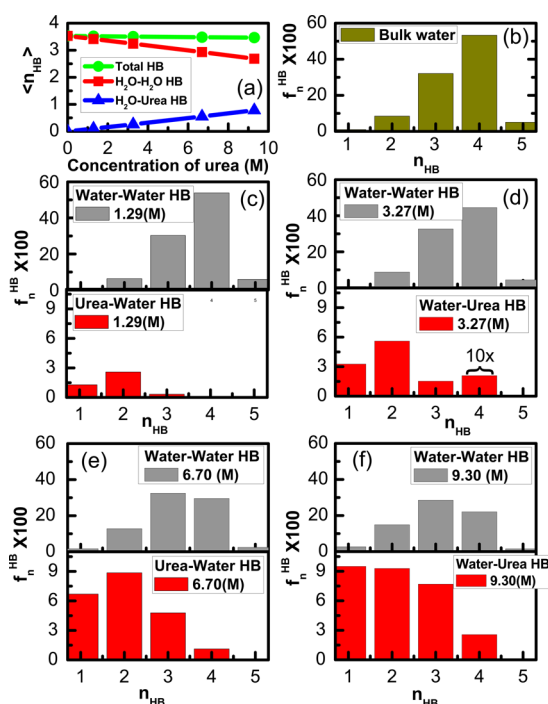


Figure 10. (a) Average number of hydrogen bonds $\langle n_{HB} \rangle$ as a function of concentration of urea. (b) Distribution of hydrogen bonds in bulk water. Plots c–f represent distributions of water–water (upper panel in each figure) and water–urea (lower panel in each figure) hydrogen bonds at different urea concentrations.

namely, (i) water–water and (ii) water–urea hydrogen bonds. In Figure 10b we have shown HB distribution of bulk water, in which only water–water hydrogen bonds are present. In Figure 10c–f we have shown both water–water (upper panel) and water–urea (lower panel) HB distributions for aqueous urea solutions of different urea concentrations. As the concentration of urea is increasing (see upper panels of Figure 10c–f), we find that there is a steady and substantial decrease in the percentage of *four* hydrogen-bonded ($n_{HB} = 4$) water molecules. Same is the case for *five* hydrogen-bonded ($n_{HB} = 5$) water molecules. On the other hand, the number of *two* hydrogen-bonded water molecules ($n_{HB} = 2$) increases steadily with the increasing urea concentration. It is interesting to observe that the percentage of *three* hydrogen-bonded ($n_{HB} = 3$) water molecules remains almost the same as C_U is increased. It may be due to the fact that when one of the four hydrogen-bonds (in the case of $n_{HB} = 4$) of a water molecule breaks due to the addition of urea, the water molecules with $n_{HB} = 3$ is generated, but this increase in number of $n_{HB} = 3$ water molecules is offset by the decrease in number of $n_{HB} = 3$ water molecules resulting from the conversion of *three* hydrogen-bonded ($n_{HB} = 3$) to *two* hydrogen-bonded ($n_{HB} = 2$) water molecules due to the presence of urea. Now, looking at the water–urea hydrogen-bond distributions (see lower panels of Figure 10c–f), we find that at the lowest urea concentration studied here, there is only *one* hydrogen-bonded water (forming HB with urea) and at higher urea concentrations, populations of *two* and *three* hydrogen-bonded (with urea) water molecules increases. In general, as the urea concentration is increased, the proportion of water–urea hydrogen-bonded molecules increases. This is consistent with the above observation regarding water–water hydrogen bonds. Thus, it shows how water–water hydrogen

bonds are nicely replaced by water–urea hydrogen bonds as the concentration of urea increases.

Now the change in the $P(q_4)$ distribution due to the addition of urea can be better understood if we analyze the distribution $P(\cos \theta)$ of the cosine of the angles formed by two of the first four neighbors at the reference, central water molecule. First, we choose four nearest neighbors considering both urea and water sites as neighbors. For calculating the angle formed by two neighbors extended at the central molecule, we restrict ourselves only to water neighbors (not urea neighbors). The $P(\cos \theta)$ distributions at different urea concentrations as a function of $\cos \theta$ as shown in Figure 11a show a large broad

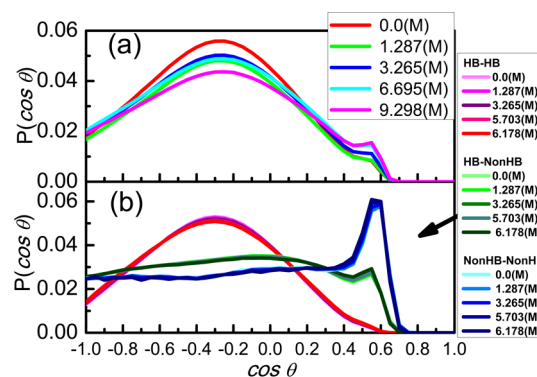


Fig. 11

Figure 11. (a) Distribution $P(\cos \theta)$ of cosine of the triplet angle $\cos \theta$ (for definition see Models and Methods section) at different urea concentrations (see the legend for different concentrations). (b) Distributions $P(\cos \theta)$ of the cosine of angle θ made by (i) two hydrogen-bonded (family of red curves), (ii) two non-hydrogen-bonded (family of blue curves), and (iii) one hydrogen-bonded and the other non-hydrogen-bonded (family of green curves) nearest neighbors at the central molecule.

peak near $\cos \theta = -(1/3)$, corresponding to the tetrahedral peak and a small kink at around $\cos \theta = 0.5$ corresponding to nontetrahedral peak of the q_4 distribution. As the concentration of urea increases, the intensity of the tetrahedral peak decreases and that of the nontetrahedral peak increases. If we now classify the two neighbors that form the angle in terms of whether they are hydrogen-bonded to the central, reference molecule, three different combinations are possible: (1) both the neighbors are hydrogen-bonded to the central molecule (let us call it HB–HB) (2) one neighbor is hydrogen-bonded to the central molecule and the other is not (HB–nonHB) and (3) both the water neighbors are *not* hydrogen-bonded (nonHB–nonHB) to the central molecule. If we look at the $P(\cos \theta)$ of these three combinations (see Figure 11b), we find that the tetrahedral peak near $\cos \theta = -(1/3)$ originates from HB–HB combination (see the group of reddish colored curves); whereas the nontetrahedral peak at around $\cos \theta = 0.5$ arises because of the angles formed at the central molecule by HB–nonHB or nonHB–nonHB combinations of neighbors (see groups of bluish and greenish graphs). It is interesting to observe that the intensities of these normalized distributions do not change with urea concentration. But as we have seen in Figure 10, the relative proportion of these H-bonded neighbors changes as a function of urea concentration, the HB–HB, HB–nonHB, and the nonHB–nonHB combinations also change leading to an intensity change in the $P(\cos \theta)$ distribution shown in Figure 11a. Thus, in essence, the slight change in the

distribution $P(q_4)$ as a function of urea concentration is a result of change in the proportions on n -hydrogen-bonded molecules, not due to the breaking of local tetrahedral structure of water.

The effect of urea concentration on the dynamics of the urea–water system has also been studied. The mean square displacements for urea and water (oxygen) are shown in Figure 12. The translational diffusion coefficient of the water

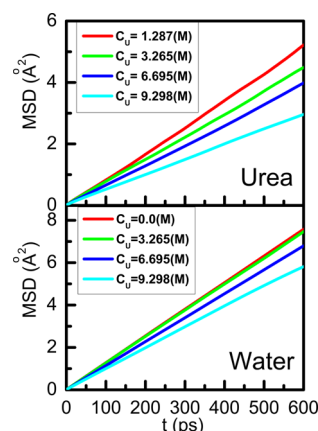


Figure 12. Mean squared displacements (MSDs) of (a) urea and (b) water molecules as a function of time at different urea concentrations (see the legends for different concentrations).

molecules is related to the slope of the mean squared displacement (MSD) in the long time limit. Thus, from Figure 12 it is evident that presence of urea modifies the translational diffusivity of both urea (see Figure 12a) and water (see Figure 12b). As the concentration increases, the slope of the MSD decreases, indicating that diffusivity decreases as a function of urea concentration. A similar trend has been observed in many earlier studies.^{45,87} The orientational dynamics in terms of the dipole moment vector of the water molecules has also been studied. Although not shown here, we have found negligible change in the dipole moment time correlation functions as a function of urea concentration. This observation is consistent with that obtained from the experimental study of Rezus et al.³⁷

3. SUMMARY AND CONCLUSIONS

Aiming at elucidating the effect of urea on the local structure of water, we have performed molecular dynamics simulations of aqueous urea solutions of different concentrations ranging from 1.29 M to 9.30 M. As already mentioned, previous investigations have contradictory conclusions about the effect of urea on water structure. In a recent investigation, Stumpe et al.⁷¹ have inferred through their analysis of various local structural parameters that urea does not break the tetrahedral structure of water. However, their analysis of energetic quantities suggests a structure-making ability of urea. On the contrary, the results presented by Idrissi et al.⁷⁵ using various orientational and radial order parameters to quantify local structural arrangements of water show that the tetrahedral structure of water is broken in the presence of urea at high concentrations. The order parameters calculated in this work are based on the nearest neighbor approach, in which angular or radial arrangement of the first four neighbors with respect to the central reference water molecule is measured. Thus, this approach heavily relies on the correct choice of the four nearest neighbors. In bulk water, all the four nearest neighbors of a reference water molecule are always water, whereas in a

concentrated aqueous urea solution, a nearest neighbor position can be occupied either by water or by (the site of) a urea molecule (see Figure 5). In a concentrated aqueous urea solution the probability of one or more water neighbors being replaced by (the site/s of) one or more urea molecules is more. In the present investigation, we have shown that if we choose four neighbors as the first four *water* molecules [without considering urea as a neighbor], the average tetrahedral order parameter and its distribution (see Figure 1) change significantly from the bulk water results; indicating breakdown of the tetrahedral water structure. Results with the same conclusions can be drawn from the measurements of average and fluctuations of distances of all the four neighboring water molecules (see Figures 3–4). It is interesting to observe the distributions of the hydrogen-bonding angle formed by one of the four nearest *water* neighbors with the reference (central) water molecule. The distribution of this angle does not change from its bulk water value for the first or second *water* neighbor, whereas the same for the third or fourth *water* neighbor changes significantly with increasing urea concentration. It may prompt us to conclude that the tetrahedral structure of water is broken. However, in a concentrated urea solution there are around 26% and 37% probabilities that the third and fourth nearest neighboring positions respectively can be occupied by urea and not by water (see Figure 5). Therefore, it is very important to consider urea molecules (apart from water molecules) as a neighbor of a reference water molecule. If we do not consider the fact that urea can also be a neighbor and take all the four neighbors as water, then the third or fourth *water* neighbor may not be actually the third or fourth nearest neighbor. These water molecules in concentrated aqueous urea solution are in the second solvation shell. The deviation in the calculated quantities from their bulk water values in concentrated aqueous urea solution is thus resulting from these outside (the first solvation shell) water molecules. When we consider both urea and water molecules in choosing nearest neighbors and calculate all the order parameters representing different orientational and radial orders, we find almost negligible changes in these order parameters as a function of urea concentration. These results thus suggest that the usual tetrahedral structure of water is maintained; only some of the water molecules are substituted by (site/s of) urea molecules. A careful observation of distributions of tetrahedral order parameters (see Figure 9 and 11) indicates that there is a small change in the distribution as a function of urea concentration and therefore further observation is required. At this point it is important to remember that liquid water at a finite temperature is not perfectly tetrahedral and it is a broken hydrogen-bonded network consisting of water molecules with one, two, three, four, five, and more hydrogen bonds. The proportion of these different types of hydrogen-bonded water molecules changes with various external parameters such as temperature, pressure, etc. Recently, it was shown⁵⁶ that if the proportions of these different types of hydrogen-bonded water molecules change (due to change in temperature in that case), the tetrahedral peak in the distribution of q_4 diminishes and the nontetrahedral peak increases. In the present investigation also, we find that there is a considerable change in the proportions of n -hydrogen-bonded molecules with the increase in urea concentration and we have shown that the slight change in $P(q_4)$ is due to this change in the hydrogen bonding pattern of water in the presence of urea. The present investigation thus asserts that radial, tetrahedral, and other orientational structures

of water remain largely unaffected by the presence of urea even at high concentrations. The present result thus supports the conclusion made by Grubmüller and co-workers⁷¹ and others^{18,19,37,40,45,47,62–67} that the water structure is not changed significantly because of urea and oppose the idea that urea acts as a structure-breaker as demonstrated by Idrissi et al.⁷⁵ and others.^{35,38–44,57–60} The present results ascertain that the water structure is not broken with the addition of urea. However, the interpretation of protein denaturation by urea requires further analyses of the thermodynamic link between solvent structure in terms of solute–solvent and solvent–solvent radial distribution functions and protein stability,^{20,88} and this can be easily studied through the Kirkwood–Buff approach.^{89,90} Denaturant urea can have a considerable effect on the manifestation of hydrophobicity at the nanoscale.⁹¹ Further investigation in this direction is in progress. We have also found that translational diffusivities of water and urea change considerably but orientational dynamics remains almost unchanged upon the addition of urea. Modification of water dynamics^{37,92} may have a definite role in the protein denaturation process. In fact, there are contradicting views^{37,63} in this matter, and therefore, further investigations are required to resolve the issue. Another important area of very recent research deals with the role of urea on nucleic acid conformations and stability.^{93,94} Further studies are required in this direction.

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Notes

The authors declare no competing financial interest.

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