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Impact of urea on water structure: a clue to its properties as a denaturant?

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Abstract

A new investigation of the structure of urea–water solutions at a mole ratio of 1 urea to 4 water molecules is described. Neutron diffraction is used in conjunction with isotope labelling on the water and urea hydrogen atoms and on the nitrogen atom of urea. The diffraction data are analysed using the empirical potential structure refinement procedure to yield a set of site–site radial distribution functions and spatial density functions that are consistent with the diffraction data. The results are discussed in relation to recent and past X-ray and neutron diffraction experiments and theoretical studies of this system. It is found that urea incorporates readily into water, forming pronounced hydrogen bonds with water at both the amine and carbonyl headgroups. In addition the urea also hydrogen bonds to itself, forming chains or clusters consisting of up to approximately 60 urea molecules in a cluster. There, is however, little or no evidence of urea segregating itself from water, in marked contrast to a recent study of the methanol–water system. This behaviour is discussed in the context of the great propensity of urea to effect protein denaturation.

Keywords: Neutron diffraction; Isotope substitution; Protein denaturation; Hydration

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1. Introduction—some history of the water–urea system

Frank and Franks gave one of the earliest discussions of the effect of urea on water structure in 1968 [1]. The motivation for that study was the (at the time) relatively recent observations that many hydrocarbon gases, but not methane, were more soluble in urea–water solutions than in pure water alone, while the evolution of heat on dissolution was reduced. Fully admitting the limitations of their basic assumptions, Frank and Franks modelled water as a two phase system consisting of a bulky, ice-like or clathrate-like, component and a dense, normal liquid component which each dissolve the hydrocarbon gas in different ways. It was supposed that urea entered only the denser phase because urea as a planar molecule would not easily fit into the tetrahedral arrangement of water molecules in the bulky phase. Urea was

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assumed to form an ideal solution in the dense phase, and hence effectively dilute the hydrocarbon solute in that phase, so altering the equilibrium of hydrocarbon between the dense and bulky phases. The model gave qualitative agreement with the observed solubility changes of the different hydrocarbon gases in the presence of different urea concentrations.

The basic tenet of that model, that water can be regarded as consisting of two phases in equilibrium has always been controversial and has received much attention right up until the present. Indeed Kauzmann, to whom this paper is dedicated, and Eisenberg [2] already discussed this matter at length in their book in 1969 and came to the conclusion that so-called continuum models of water structure were more likely to give the correct thermodynamic properties of water. Later Henn and Kauzmann [3] derived a plausible equation of state for water within the continuum framework. More recent theories, [4,5] which have also been quite successful at predicting water properties, imply that water, under conditions where it is a stable liquid, is in a continuum state, and computer simulations also appear to come to the same conclusion [6,7]. Experimental evidence in favour of the continuum model for ambient water has come from several small angle X-ray and neutron scattering experiments (see for example Xie et al. [8] and references therein), where there is no sign of the significant density correlations (above what is known from the compressibility) that would be needed if liquid water were a mixture of two phases.

A more recent and quantitative theoretical treatment of saturated hydrocarbons dissolved in the water–urea system by Muller [9] takes a different approach compared to the Frank and Franks model. Perhaps the most difficult problem raised by Muller is that if urea is simply a ‘structure breaker’ as claimed by Frank and Franks, then how does the presence of urea raise the solubility of hydrocarbons, whereas increasing the temperature, which presumably also breaks structure, lowers the solubility? Interestingly Muller's model also starts with the hypothesis that there are two populations of water molecules in equilibrium, but this time only at the molecular level, namely those in the hydration shell (of the hydrophobic entity, and therefore of a ‘bulky’ nature) and those outside of it. As in Frank and Franks these two populations are in equilibrium with each other, but the hydrogen bonds in the hydration shell have greater enthalpy *and* greater entropy than in the bulk, implying that the hydration shell bonds are enthalpically stronger, but also more of them are broken, than in the bulk liquid outside the hydration shell. Unlike Frank and Franks model, however, the urea-like cosolvent can enter the apolar hydration sphere and when it does so it simply reduces the number of water–water hydrogen bonds in the solute hydration sphere, without modifying the properties of those bonds. Good quantitative agreement between measured and calculated thermodynamic quantities was found for a wide range of solutes from what is fundamentally a straightforward model. So far there has been no experimental confirmation of the molecular scale assumptions behind this work, although two simulation studies give significant support to the Muller concept [10,11] the first having been executed well *before* Muller's work was published, but not referenced by him.

Diffraction studies of urea in water have often been justified in relation to its ability to affect the conformation of biological macromolecules in solution. One X-ray study attempted to determine the extent of urea–urea pairing in the solution [12], but was inconclusive on account of the degree of overlap of different interatomic distances in the X-ray diffraction pattern, although there was some evidence for longer range urea–urea correlations at high concentrations. Later Turner et al. [13] attempted neutron diffraction work, using isotope labelling on both the water hydrogen atoms and on the nitrogen atoms of the urea molecule. They concluded that urea could fit into the water network without causing significant perturbation of the water structure. However, this interpretation was made in the days when interpreting diffraction data was based solely on the extracted radial distribution functions, and for this reason it is likely that much of the detail that might be occurring in this system was overlooked. They did, however, conclude that urea apparently could fit into the water network quite easily whether it is in the bulk or in the hydration sphere of an apolar species, lending additional support to the Muller model.

Apart from these two reports there appear to be few if any other recent experimental studies of the atomic scale structure of aqueous urea solutions, and certainly no attempt to see if the two phase ideas of Frank and Franks or the simpler substitution ideas of Muller have any experimental basis at the molecular level. On the other hand, computer simulation has been applied to this system fairly extensively and some recent reviews of the earlier theoretical and simulation work include Hernández-Cobos et al. [14], Tsai et al. [15], and Sokolić et al. [16]. Virtually all the recent simulations [14,15,17–21] highlight the importance of understanding the way urea at high concentration, namely at concentrations greater than approximately 3.5 M, causes protein denaturation, although the conclusions obtained thus far are not in overall agreement. However, the simulation work of Sharp et al. [20], which looks at the distribution of water–water H-bond angles in the first hydration shell of a series of solutes, including urea at a concentration of 1:7, seems to lend support to the Muller model, without referencing it directly, namely that urea fits into the water structure without modifying the local H-bond structure appreciably. However it also concludes that there is the additional factor that urea may bond directly to the peptide groups, so destabilising the protein. Clearly, therefore, the problem of protein denaturation by aqueous urea is potentially much more complex than simply a problem of altered hydrophobic interactions in the presence of urea. It is not obvious that any of the quoted studies, including the present one, can really tackle the problem of protein denaturation by urea at the molecular level directly. Perhaps the direct computer simulation study of Tirado-Rives et al. [22] of this particular problem is the closest we have come so far to achieving that goal.

One feature to emerge in many recent simulations of various solutes in water is the phenomenon of solute aggregation in the simulation. Such molecular segregation was observed *experimentally* in a recent neutron diffraction investigation of concentrated methanol in water (molar ratio 7:3), where it was seen as a decrease in the near-neighbour methyl–hydrogen to methyl–hydrogen distance compared to pure methanol [23]. Because the water existed in clusters of up to ~ 20 molecules within the simulated methanol fluid phase, the water structure itself did not appear greatly different from that in the bulk phase, there being a significant second shell peak at 4.5 Å in the water–oxygen to water–oxygen radial distribution function, $g_{O_WO_W}(r)$, despite the high concentration of methanol. The observation of a bulk-like $g_{O_WO_W}(r)$ at high solute concentrations is one indicator of possible solute–solute clustering: if the water molecules were distributed randomly amongst the solute molecules at high concentration the solute molecules would prevent the longer range water network implied by the 4.5 Å peak from forming.

Of the urea–water simulations cited here the issue of solute–solute aggregation is not widely discussed, but is referred to specifically in the simulations of Hernández-Cobos et al. [14], where the claim is that it is limited to dimers only, and Sokolić et al. [16,24], who say that solute aggregation occurs with all their model potential functions to a greater or lesser extent. Six of the cited simulations show the estimated $g_{O_WO_W}(r)$ explicitly [14–17,19,24]. In all six cases the estimated $g_{O_WO_W}(r)$ look remarkably bulk-like even at high concentration, implying perhaps that urea clustering is occurring to some extent in these simulations.

The above list of references to previous work on urea in water is certainly not complete and more studies could have been cited. It does, however, underline the ongoing considerable uncertainty that still surrounds the question of how urea incorporates into water structure, and the considerable importance that is attached to understanding this system at the molecular level.

Since the previous neutron study [13] much has changed in the way diffraction experiments are analysed. Fundamentally it is now possible to perform a computer simulation of the system being studied [25] closely analogous to the ones cited above alongside the diffraction experiment and use the diffraction data to help refine the configurations of molecules in the simulation. One manifestation of this method is called empirical potential structure refinement (EPSR) [26], which is particularly appropriate for molecular systems. For water [27] and tertiary butanol [28] this approach has led to much insight into local correlations, in particular the spatial density function [29–31], around solute and water molecules, which was not available by traditional methods of analysing measured radial distribution functions. Thus, many of the measures of structure available to the simulator become available to the experimentalist as well, albeit with the caveat that the result will still depend on the extent that the data are sensitive to the particular measure of structure chosen. In EPSR, there is a reference potential that is essentially the potential that would be used in a conventional simulation without diffraction data, so on that basis the conclusions drawn are no less valid than in any other simulation. The fact that the configurations of molecules are guided by the diffraction data *as well as* the reference potential can in our view only benefit the outcome.

In the rest of this paper we describe a new neutron diffraction experiment on the urea water system, using deuterium isotope labelling on the water and urea hydrogen atoms, and ^{15}N labelling on the urea nitrogens. The system has been studied at three concentrations and two temperatures, but for the present purpose we will concentrate on the study at 298 K and molar ratio 1 urea molecule:4 water molecules. The data are analysed via the EPSR technique that is used to establish the salient features of the structure of this solution.

2. Method

Samples of deuteriated and protiated urea were obtained from Aldrich Chemical Company as well as protiated urea with ^{15}N substitution. The chemicals were used as supplied by Aldrich without further purification. Three concentrations of urea were studied with molar ratios urea:water of 1:4, 1:8 and 1:16, respectively, but only the 1:4 data, corresponding to an atomic number density of 0.103 atoms/Å^3 will be discussed further here. At each concentration 5 solutions were prepared, namely (1) deuteriated urea in heavy water, D_2O; (2) protiated urea in H_2O; (3) a 50:50 mixture of (1) and (2); (4) protiated urea-^{nat}N in D_2O; and (5) protiated urea-^{15}N in D_2O. Diffraction data for each sample were measured on the small angle neutron diffractometer for amorphous and liquid samples at ISIS, UK, corrected for attenuation and multiple scattering, and normalised to the scattering from the incoherent scatterer vanadium, using the ATLAS suite of programs [32]. The result is a differential scattering cross-section per atom of sample for each sample.

The differential cross-sections for samples (1), (2) and (3) were analysed using the method of Soper and Luzar [33] to yield three composite partial structure factors, HH, XH and XX, where H represents a weighted sum of the water hydrogen atoms (H_W) and urea (H_U), and X represents a weighted sum of the non-substituted atoms, namely water oxygen (O_W), urea carbon (C) urea nitrogen (N) and urea oxygen (O). In addition, a first order difference was taken between the data of samples (4) and (5) to yield a weighted sum of the nitrogen—other correlations. Thus, there were four sets of composite structure factor that could be applied to the EPSR computer simulation. In terms of the individual site–site partial structure factors these composite structure factors are expressed as weighted averages of the relevant partial structure factors:

$$S(Q)=\frac{\sum_{\alpha} c_\alpha c_\beta b_\alpha b_\beta S_{\alpha\beta}(Q)}{\sum_{\alpha} c_\alpha c_\beta b_\alpha b_\beta} \tag{1}$$

where c_α, b_α are the atomic fraction and coherent neutron scattering length of species α, respectively, the partial structure factors, $S_{\alpha\beta}(Q)$ are defined in terms of the corresponding site–site radial distribution function between sites α and β, $g_{\alpha\beta}(r)$:

$$S_{\alpha\beta}(Q)=4\pi\rho\int_0^\infty r^2(g_{\alpha\beta}(r)-1)\frac{\sin Qr}{Qr}\mathrm{d}r \tag{2}$$

and ρ is the atomic number density of the sample (0.103 atoms/Å^3 in the present case). In Eq. (1) the sum is done only over those pairs of atom types relevant to each specific composite partial structure factor.

The specific weighting factors for each of the measured composite structure factors in the present experiment were:

$$S_{NX} = 0.074S_{NC} + 0.065S_{NO} + 0.175S_{NN} + 0.143S_{NH_U} + 0.259S_{NO_W} + 0.285S_{NH_W} \quad (3)$$

$$S_{HH} = 0.111S_{H_UH_U} + 0.444S_{H_UH_W} + 0.444S_{H_WH_W} \quad (4)$$

$$S_{XH} = 0.041S_{CH_U} + 0.036S_{OH_U} + 0.114S_{NH_U} + 0.143S_{O_WH_U} + 0.082S_{CH_W} + 0.071S_{OH_W} + 0.228S_{NH_W} + 0.285S_{O_WH_W} \quad (5)$$

and

$$S_{XX} = 0.015S_{CC} + 0.026S_{CO} + 0.084S_{CN} + 0.105S_{CO_W} + 0.011S_{OO} + 0.073S_{ON} + 0.092S_{OO_W} + 0.117S_{NN} + 0.293S_{NO_W} + 0.183S_{O_WO_W} \quad (6)$$

where C, O, N and H_U represent the carbon, oxygen, nitrogen and hydrogen atoms on the urea molecule, respectively, and O_W, H_W represent the oxygen and hydrogen atoms on the water molecule, respectively. In these equations all the partial structure factors $S_{\alpha\beta}$ depend implicitly on the wave vector change in the diffraction experiment, $Q = 4\pi \sin \theta/\lambda$, where 2θ is the scattering angle in the diffraction experiment and λ is the neutron wavelength.

Assumed molecular structure parameters for the urea molecule are given in Table 1 where they are seen to be very close to the values obtained in a combined microwave spectroscopy and electronic structure study of urea [34]. The tabulated values were obtained by comparing the computed radial distribution for each composite structure factor with the direct Fourier transform of the diffraction data and then comparing peak positions in the 1–3 Å region.

One aspect of the urea molecular structure that does not seem to be well established is the orientation of the–NH_2 groups in solution. X-ray and neutron crystallography demonstrate unambiguously that the NH_2 groups lie planar with the rest of the molecule [35]. Careful examination of the urea crystal structure shows a coordination number of eight H-bonds: one each from the four protons, with the carbonyl oxygen accepting four H-bonds, instead of the canonical maximum of two H-bonds per oxygen atom.

Table 1
Assumed geometry of the urea molecule

Atom pair	Average intramolecular distance or angle
C–O	1.22 Å
C–N	1.39 Å
N–H_U	1.01 Å
N–O	2.34 Å
N–N	2.31 Å
H_U–H_U	1.74 Å
C–H_U(1), C–H_U(2)	2.04 Å, 2.11 Å
∠NCN	112.4°
∠NCO	127.3°
∠HNH	118.9°
∠CNH(1), ∠CNH(2)	115.5°, 122.3°

Assumed intramolecular distances for the urea molecule used in the EPSR simulations (a) and (c), i.e. planar geometry. H_U(1) refers the hydrogen atom furthest from the central axis of the urea molecule, H_U(2) refers to the hydrogen atom closest to the central axis. For simulation (b) the amine hydrogens are allowed to rotate about the C–N axis, but the other molecular parameters are unchanged.

Quantum calculations show that in the free molecule the hydrogen atoms are out of the plane of the N_2–C=O backbone [34]. In fact, when the electronic structure calculation is done with urea constrained to the (crystalline) planar geometry, a vibrational frequency calculation obtains two imaginary frequency normal modes, showing that the isolated planar urea geometry is a saddle point, not a stable ground state minimum.

Another interesting feature of the electronic structure of urea is the electrostatic potential (ESP), which is correlated with the probability for H-bonding. The ESP for urea is shown in Fig. 1. Unlike most other organic molecules containing oxygen atoms, the urea oxygen atom is surrounded by a hemispherically symmetric ESP. This indicates that H-bonding can occur for all angles

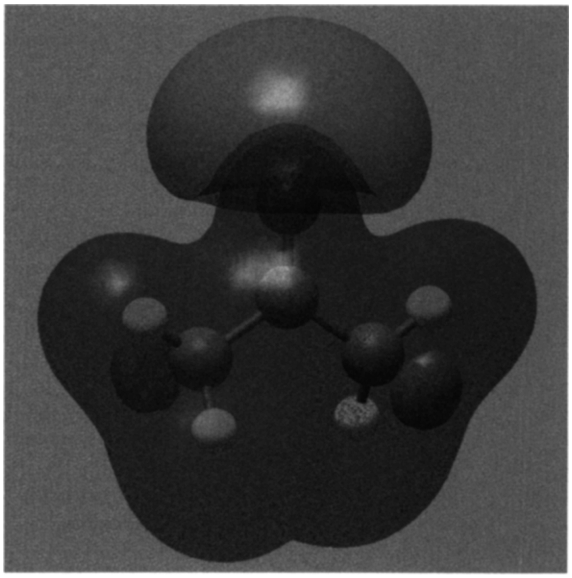

Fig. 1. Estimated ESPs for the (gas-phase) urea molecule, [36] using MP2/6-311++G(d,p) optimization, no symmetry constraints. The envelope shows the contour at the 0.04 $e^-/Å^3$ density level.

surrounding the urea O, and also is consistent with the H-bonding observed in the crystal structure.

Ishida et al. have investigated aqueous urea at low concentrations [36] using an extended reference interaction site self-consistent field (XRISM-SCF) model [37]. This model solves the quantum electronic structure of the urea in a classical electric field provided by a model water potential. Their results showed aqueous urea at infinite dilution to have nonplanar NH_2 groups, and substantial H-bonding with water. Integration of the relevant radial distribution functions indicates that there is an average of 5.6 water molecules hydrogen-bonded to urea.

Therefore we ran two simulations, one with all of the atoms confined to the plane of the molecule, and another where the hydrogen atoms could rotate about each of the respective C–N axes, to see if there was any sensitivity in the neutron data to this rotation. In addition, a further simulation was run using identical conditions as the first two, but using the reference potential on its own without empirical potential refinement to establish the influence of the diffraction data on the calculated distribution functions.

The simulations were performed in a cubic box of dimension 28.9539 Å, containing 125 urea molecules and 500 water molecules, giving an atomic number density of 0.103 atoms/$Å^3$. This 1 urea:4 water ratio corresponds to an ~8.55 M solution, which is frequently used for denaturation of proteins. Table 2 lists the Lennard–Jones parameters and assumed effective charge for each atom type [38]. Lorentz–Berthelot mixing rules were used for dissimilar atom pairs [39]. Note, however, that whilst these values are the same as used in the previous simulation study [22], the assumed molecular geometries for urea and water are guided by the data. Thus, for example, the assumed average O_WH_W bond distance in water is 0.976 Å and the assumed average H_WH_W distance is 1.55 Å, in accordance with their experimental values, rather than the values used in the original SPC/E potential [40]. In addition, a Gaussian distribution is used to distribute the actual bond distances for any given molecule about their average values, to simulate the observed zero-point disorder in the molecules. For these reasons a simulation of the system using these potential values on their own would give somewhat different results compared to those where the original molecular geometries are used. In EPSR the standard minimum image convention is applied, but no correction is made for long-range forces because the diffraction data

Table 2
Reference potential parameters for the urea–water system

Atom	ε (kJ/mol)	σ (Å)	Charge (e)
C	0.439	3.750	+0.1420
O	0.878	2.960	−0.3900
N	0.711	3.250	−0.5420
H_U	0.000	0.000	+0.3330
O_W	0.650	3.166	−0.8476
H_W	0.000	0.000	+0.4238

Lennard–Jones parameters and effective charges used to generate the reference potential in the EPSR simulation. The parameters for urea are taken from the OPLS model. [38] The parameters for water are those of the SPC/E model [40]. For atoms of different type the standard Lorentz–Berthelot mixing rules were applied.

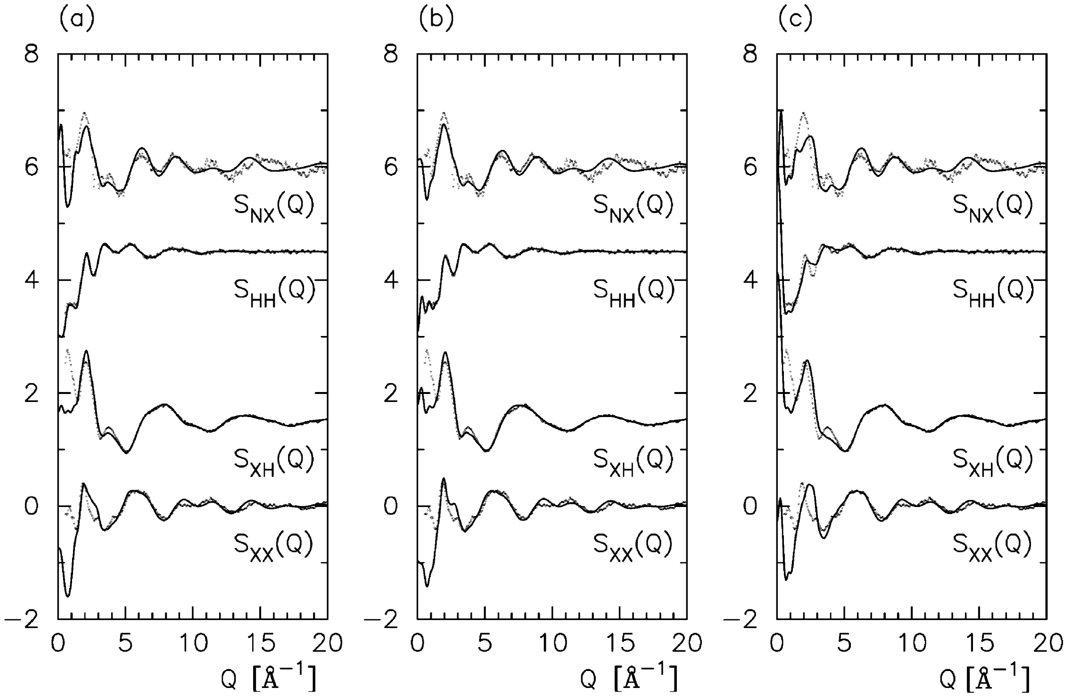

Fig. 2. Diffraction data (circles) for each of the functions listed in (3)–(6) for a 1:4 molar ratio urea–water solution. The EPSR simulations of these data (solid lines) are shown in (a) for the case where the NH_2 groups are held close to the plane of the urea molecule, (b) where these groups are allowed to rotate out of this plane, and (c) the same as (a) but with the reference potential on its own and no refinement of the empirical potential.

do not provide useful information beyond approximately 10–15 Å. Instead all potentials are truncated smoothly at half the box dimension.

Once the EPSR simulation is equilibrated, a number of other structural quantities can be calculated from the simulated molecular ensembles, including the site–site radial distribution functions, the solvent–solvent, solute–solute and solute–solvent spatial density functions, and other correlation functions as described below.

3. Results

3.1. Fits to the diffraction data

Fig. 2 shows the comparison of calculated structure factors with the diffraction data (a) using structure refinement, but keeping the urea hydrogen atoms close to the plane of the urea molecule; (b) using structure refinement, allowing the urea hydrogen atoms to rotate (about each of their respective C–N axes) out of the plane of the urea molecule; and (c) the same as simulation (a) but using the reference potential on its own with no empirical potential refinement. Fig. 3 shows the same comparisons in *r* space. As can clearly be seen from the figures, the empirical potential gives a marked improvement in fit compared to running the simulation with the reference potential on its own. The average RMS deviation between diffraction data and fit for (a) was 0.029 compared to (b) where it was 0.026 and (c) where it was 0.037. This indicates that allowing the urea hydrogens to rotate out of the plane makes only a slight improvement in the quality of fit. The present data for the XX, XH and HH structure factors is much improved over the previous neutron data [13] due to the more advanced instrumentation and analysis techniques now available. Note that the XX function was not available in the previous neutron

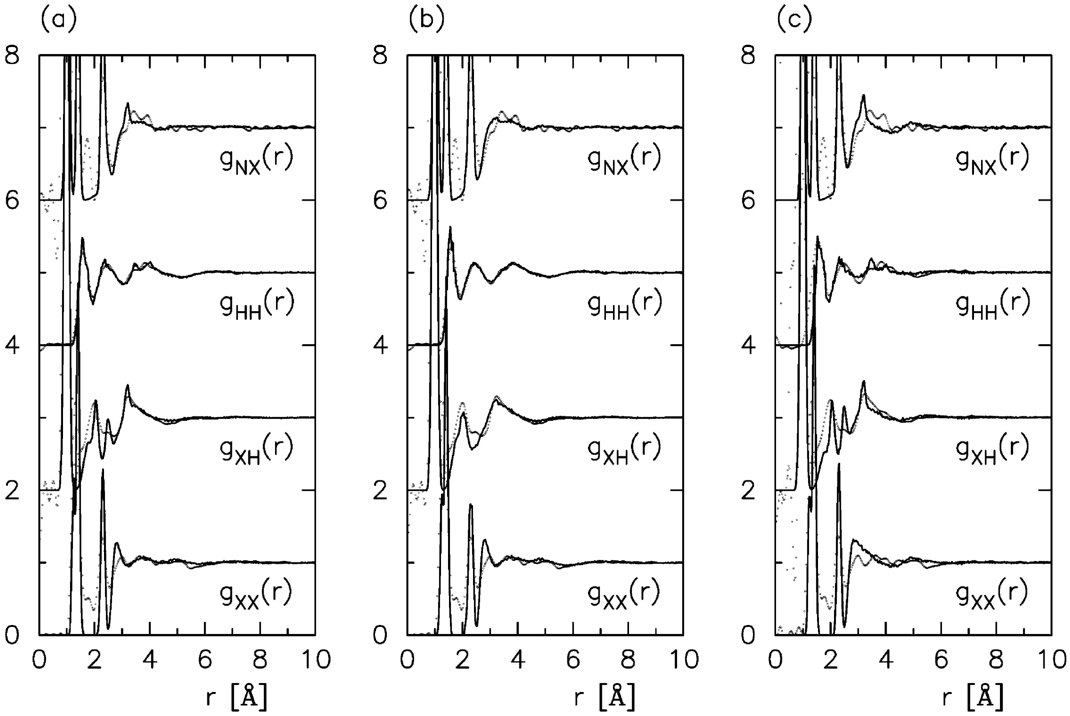

Fig. 3. EPSR simulations the same as in Fig. 2, but this time showing the results in *r* space.

experiment. The radial distribution function from the XH structure factor of the present data shows a much more pronounced peak at ~1.85 Å compared to previously [13], which is almost certainly a consequence of the much better counting statistics and instrument resolution that are now available. On the other hand, the nitrogen first order difference data, although showing good overall agreement with the previous result, do not appear quite as reliable as before—some tiny Bragg oscillations are discernible in the *Q* range 2–4/Å, and these have subsequently been traced to the neutron beam not being sufficiently well collimated near the edges of the sample containment. It is not believed these small peaks have an undue effect on the final outcome of the structure refinement. In any case they only affect one out of the four sets of data.

The distribution of rotation angles for the NH_2 headgroups about the C–N axis was calculated for simulations (a) and (b). It was found that for simulations (b) the distribution of rotation angles was almost random, indicating there was nothing in the diffraction data nor in the assumed intermolecular potentials that restricts the urea hydrogen atoms to lie near the plane of the urea molecular backbone. Given that the fit for this case is only marginally better than (a) there is no clear verdict from these data on the degree to which the hydrogen atoms lie out of the molecular plane, except to say that equally there is no strong evidence that these *must* be confined to that plane. (Bear in mind that due to the Gaussian distribution of atom positions that has been used to simulated zero-point disorder, even for simulation (a) the NH_2 groups did not lie precisely in the plane of the urea molecule. Rotation angles of up to ±50° were found in that case.)

3.2. Water radial distribution functions

Fig. 4 shows the water–water site–site radial distribution functions derived from the EPSR simulation for the 1:4 urea:water solution and com-

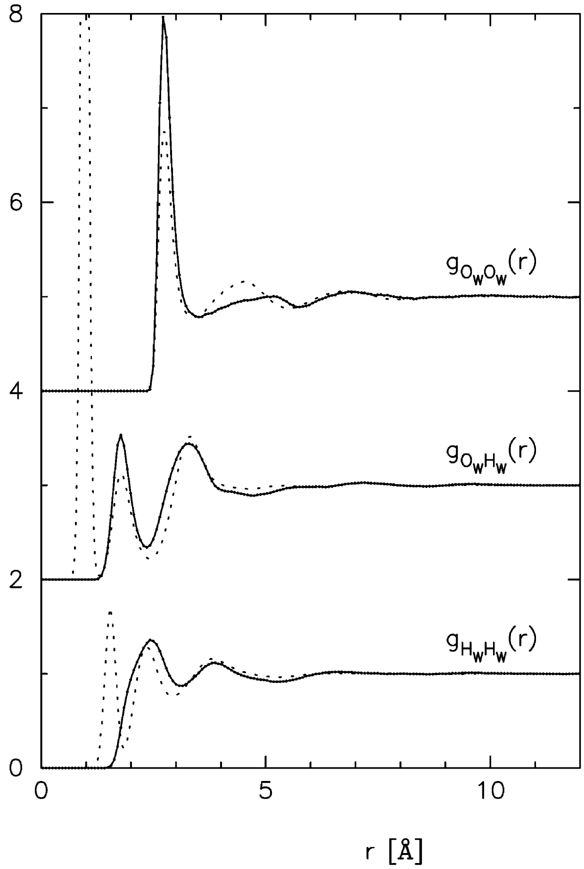

Fig. 4. Water–water site–site radial distribution functions (O_WO_W, O_WH_W, and H_WH_W; lines) for 1:4 urea–water solutions, compared to the same functions in pure water (dashed lines). Significant differences are observed particular in the second peak in the O_WO_W function.

pares them to the equivalent functions for pure water. It is noted that all three functions are markedly affected by the presence of urea at this concentration, particularly the O_WO_W function, since the 4.5 Å peak in this function for pure water has largely disappeared in urea water at this concentration. It is therefore evident that urea *does* have a marked affect on local water structure in solution, and, in the light of the discussion in the introduction, the severely depleted 4.5 Å peak at such a high concentration probably means that urea does not aggregate appreciably, even at this high concentration.

3.3. Urea–urea, urea–water and water–water hydrogen bonding

The radial distribution functions associated with urea–urea and urea–water hydrogen bonding are shown in Fig. 5. The NO_W and NH_W radial distribution functions are also shown for comparison. A pronounced first peak is evident for all the oxygen–hydrogen radial distribution functions, indicating clear hydrogen bonding between solute

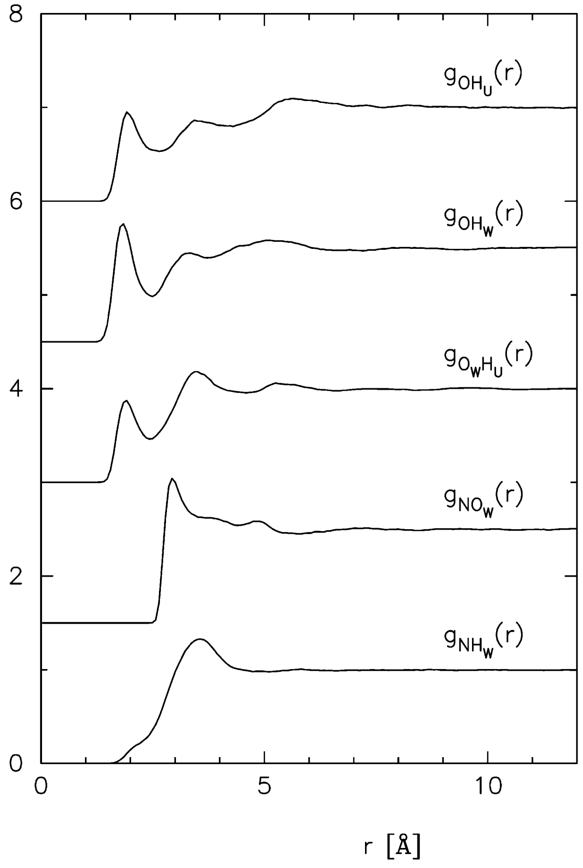

Fig. 5. Some of the site–site radial distribution functions associated with hydrogen bonding between urea molecules and between water and urea at a 1:4 urea–water ratio. Also shown are the relevant nitrogen–water radial distribution functions. In contrast to the oxygen hydrogen functions, the nitrogen–water functions show no sign of direct water–nitrogen bonding, despite the negative charge on the nitrogen in the reference potential. This can be seen by the lack of a characteristic nitrogen to hydrogen peak at a distance shorter than the corresponding nitrogen oxygen peak.

Table 3
Coordination numbers associated with hydrogen bonding

Atom pair	Relevant rdf	$r_{\min}$ (Å)	Coordination number, $C_{\alpha\beta}$ (atoms)
H_W about O_W	O_WH_W	2.35	1.37
H_U about O_W	O_WH_U	2.45	0.59
O_W about H_W	O_WH_W	2.35	0.69
O about H_W	OH_W	2.50	0.21
H_W about O	OH_W	2.50	1.70
H_U about O	OH_U	2.62	0.79
O_W about H_U	O_WH_U	2.45	0.59
O about H_U	OH_U	2.62	0.20

This table lists the coordination numbers of different pairs of oxygen and hydrogen atoms for 1:4 urea:water solutions. These are estimated from the formula $C_{\alpha\beta} = 4\pi\rho_\beta\int_0^{r_{\min}} r^2 g_{\alpha\beta}(r)\mathrm{d}r$, where $C_{\alpha\beta}$ is the coordination number of β atoms about α and $r_{\min}$ is the radius of the first minimum of the relevant radial distribution function (rdf).

molecules, between solvent molecules, and between solvent and solute. In contrast to this there is no evidence for hydrogen bonding to the (negatively charged) urea nitrogen, even though it has a more negative charge than the urea oxygen in the simulation.

Table 3 lists the relevant coordination numbers of both urea and water hydrogen atoms about both urea and water oxygen atoms, likewise for oxygen about hydrogen, and the limits of integration as derived from the radial distribution functions of Figs. 4 and 5. We will associate these coordination numbers as being representative of the number of hydrogen bonds between each respective atom pair. This is obviously an oversimplified definition, but since the precise definition of the hydrogen bond is unknown in a simulation of this nature the coordination numbers should at least be *proportional* to the degree of hydrogen bonding in each case. From the table we see that around the water oxygen, there are 0.59 bonds to urea hydrogen atoms and 1.37 H-bonds to other water hydrogen atoms. The ratio of these two numbers is close to the stoichiometric ratio of urea and water hydrogen atoms in the mixture (4:8), indicating that there is no preference for water or urea to hydrogen-bond to a water oxygen. On the other hand, around the water hydrogen atom there are on average 0.22 bonds to urea oxygen atoms and 0.69 bonds to other water oxygen atoms. This ratio is slightly larger than the stoichiometric ratio of urea oxygen atoms to water oxygen atoms (1:4) in this case. This may not be significant given the likely uncertainties on these numbers, but if it were true it would indicate a slight tendency for water hydrogen atoms to prefer bonding to urea, rather than to other water molecules. From the urea point of view, the number of water hydrogen atoms about the urea oxygen atom is 1.70, while the number of urea hydrogen atoms about the urea oxygen atom is 0.79, giving a total urea oxygen H-bond number of 2.49. This is well in excess of the 2 or less that might be expected on the basis of the charge on this atom in the reference potential, but note again that the ratio of these numbers is closely in proportion to the stoichiometric numbers. For urea hydrogen atoms the total oxygen number is 0.79 atoms per hydrogen atom, so the total urea hydrogen to other oxygen atom H-bond number is approximately 3.16. Overall therefore the urea molecule has a high degree of hydrogen-bonding, approximately 5.7 bonds per urea molecule at the present molar ratio of 1:4, compared to the crystalline number of 8 H-bonds. (As proven by the crystal structure, the urea oxygen atom can accept as many as four H-bonds, so one might expect the urea hydrogen bond number to change with decreasing concentration as more water becomes available to form bonds. We will discuss the effect of concentration on this number in a separate paper.) Thus, both urea and water have substantial H-bonding in the solution and the fact that the hydrogen bond numbers at each site are on average close to stoichiometry at the concentration of the experiment suggests that there is no marked preference for water or urea to bond either to themselves or to each other. In other words urea and water appear to readily substitute for each other in solution, while maintaining a high degree of H-bonding in each case. However, this is achieved only by substantial disruption to the water network, as shown by the substantial decrease in the 2nd coordination peak at ~4.5 Å in $g_{\mathrm{OwOu}}(r)$ (Fig. 4).

3.4. Spatial density functions and the degree of chain formation

Fig. 6 shows the spatial density functions of (a) water around water and (b) urea around water for

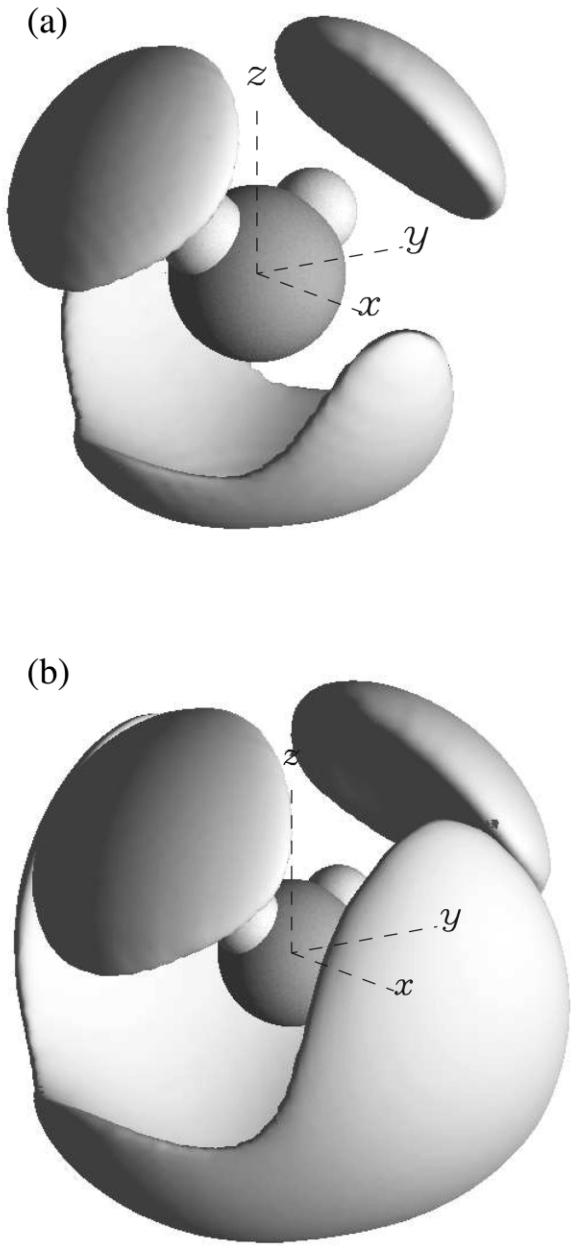

Fig. 6. Spatial density function of water about water (a), and urea about water (b). The contour level for this isosurface plot has been set to include 15% of the molecules in the distance range 0–5 Å for both water and urea. Distances are measured from the oxygen atom on the water molecule and from the carbon atom on the urea molecule. We note the great similarity between water about water spatial distribution function, and water about urea spatial distribution function.

the 1:4 urea:water solution. Apart from the change in centre to centre distance between the two graphs, one is immediately struck by the great similarity between these functions. Given the much larger size and different geometry of the urea molecule it is remarkable these two functions should look so similar. These plots therefore also suggest that urea molecules and water molecules readily substitute for each other in the solution. Fig. 7 shows the spatial density functions obtained from EPSR for (a) urea about urea and (b) water about urea. Here again there are many similarities, for example the broad band over the top of the central molecule and the two lobes along the positive and negative *y*-axis.

The ‘head to tail’ configuration of the urea molecules seen Fig. 7 is reminiscent of the stacked linear ordering along the crystalline *c*-axis. This suggests that it might be worth checking the degree to which urea molecules are clustered or have formed chains in the liquid. To this end an analysis of the simulated molecular configurations was performed in which urea molecules hydrogen bonded to another urea molecule (according to the specification given in Table 3, namely the hydrogen atom on one molecule is no more than 2.62 Å from the oxygen atom of another urea molecule) were classified as belonging to the same cluster. The distribution of cluster sizes was averaged over approximately 2400 molecular configurations, with the result shown in Fig. 8. No analysis has been done to ascertain the degree of branching in these clusters, but it is clear from this figure that a high degree of linking between urea molecules can occur at this concentration. The variance on these numbers is as large as the numbers themselves, indicating that the clustering is not static, but fluctuates rapidly as the simulation proceeds, as was seen in the simulation of Sokolić et al. [16]. We note that approximately 11% of the urea molecules occur singly, but the remainder are in clusters or chains of size ranging from 2 up to ~60 urea molecules in the cluster. This clustering is achieved without the urea segregating from the water—if the latter happened we would not have seen the near-stoichiometric ratios in the hydrogen bond numbers for water vs. urea hydrogen bonding. Presumably therefore the 4 amine hydrogen

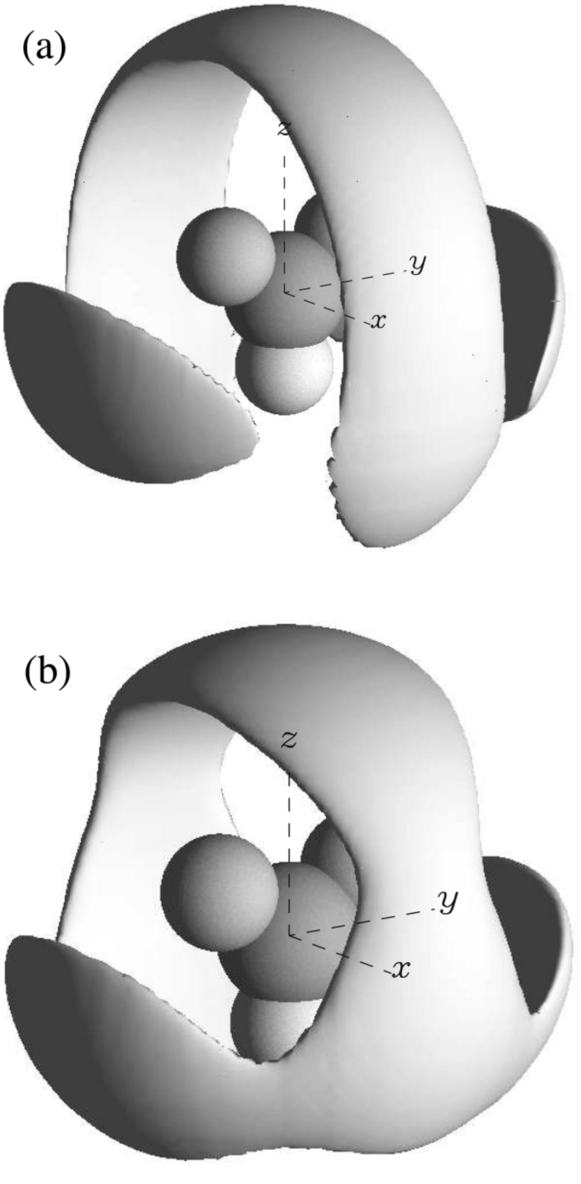

Fig. 7. Same as Fig. 6, but now for urea about urea (a) and water about urea (b).

atoms leave plenty of scope for water–urea hydrogen bonding, even in the presence of a significant chains or clusters of urea molecules.

Finally, in Fig. 9 we compare the second neighbour shell of water about water in 1:4 urea solutions with the same function in pure water, at a lower contour level that contains 40% of the molecules within the 0–5 Å distance range. Fig. 9 clearly shows that the local water structure in concentrated aqueous urea solutions is substantially disrupted compared to pure water.

4. Discussion

The results described in the previous section appear to give a consistent view of the way urea enters aqueous solution, although they do not appear particularly consistent with many of the previous simulations of this system. Overall, we see a situation where urea substitutes for water in the water hydrogen bond network. The molecular volume of urea is nearly three times that of water. However, water at ambient conditions is far from close-packed, so that the molecular volume of urea actually displaces approximately two water molecules, not three. Consider water dimers and trimers that have an ice-like configuration. The two water molecules of such a dimer will have a combined total of eight possible H-bonds, with six of the H-bonds extending to waters outside the dimer. Similarly, there would be a possible 12 H-bonds for an ice-like trimer, with 8 of them to molecules outside the trimer. In contrast, a crystalline urea molecule can make 8 H-bonds, occupying a volume between 2 and 3 water molecules. Quite simply, urea can dissolve in water for a broad range of concentrations, keeping a similar number of H-bonds (relative to water), while providing a similar range of geometries for the collection of H-bonds.

Studying the water–water radial distribution functions in some of the previous simulations of urea in water [14–17,19,24], one gets the impression in these simulations that water structure is not much affected by urea even at high concentrations. In the sense that the present results indicate that urea can readily replace water as a hydrogen-bonding neighbour, then they agree with those conclusions. On the other hand, in terms of the 3-dimensional network of water molecules all the present evidence points to a substantial disruption induced by the presence of urea. Probably it is too simplistic to think of this as a simple compression of the water structure because the topology of the

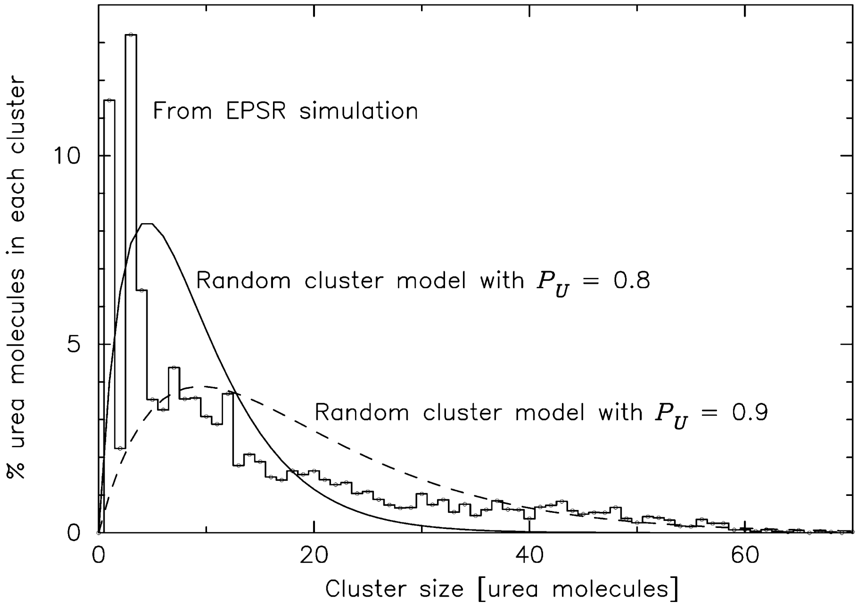

Fig. 8. Distribution of urea molecules amongst hydrogen-bonded urea clusters. Two urea molecules are considered to belong to the same cluster when a hydrogen atom on one urea molecule is within 2.6 Å of the oxygen atom of another urea molecule.

second shell changes to some extent, but certainly the spatial density functions of Fig. 9 show significant reduction in the second shell distances compared to pure water, just as happens when water is pressurised [27].

The finding that the high probability of urea–urea hydrogen bonding leads to significant chains or clusters of urea molecules forming in solution also seems to be in accord, at least qualitatively, with the simulation results of Hernández-Cobos et al. [14]. Although the authors did not perform a formal cluster analysis as here, they reported that 'no other polymerisation than dimers can be observed', with the dimers being both 'cyclic', (i.e. 1 hydrogen atom on each urea molecule bonding to the oxygen of the other), or 'head to tail' dimers, with 1 or 2 hydrogen atoms on one molecule bonding to the oxygen on the other. The network they refer to is then formed from these urea dimers. Clearly in the present EPSR simulation there is a preference for small H-bonded clusters or chains to form, but in addition a significant fraction of the urea molecules are involved in much longer chains or clusters. In fact Fig. 8 shows that only approximately 2% of the urea molecules are involved in isolated dimers, while the percentage of molecules in trimers and larger clusters is much higher. It is possible that stoichiometry, combined with optimum occupancy of H-bond sites, may be the principal explanation for urea oligomers observed here at high concentration. The discussion about H-bond numbers presented in the previous section indicates that one expects a urea molecule at the 1:4 urea:water concentration to have ~5.7 H-bonds, ~3.2 from N–H donors, and the carbonyl oxygen accepting ~2.5 H-bonds. It is highly unlikely that the stoichiometry of four water molecules per urea can permit so many H-bonds to urea without urea clustering.

It is interesting to speculate whether these clusters are larger or smaller than what might be expected on a purely random basis. To illustrate the meaning of this statement Table 3 shows that on average the probability of a single urea hydrogen atom being bonded to a urea oxygen atom is 0.2 on average. Since there are 4 such hydrogen atoms on each urea molecule, the probability of

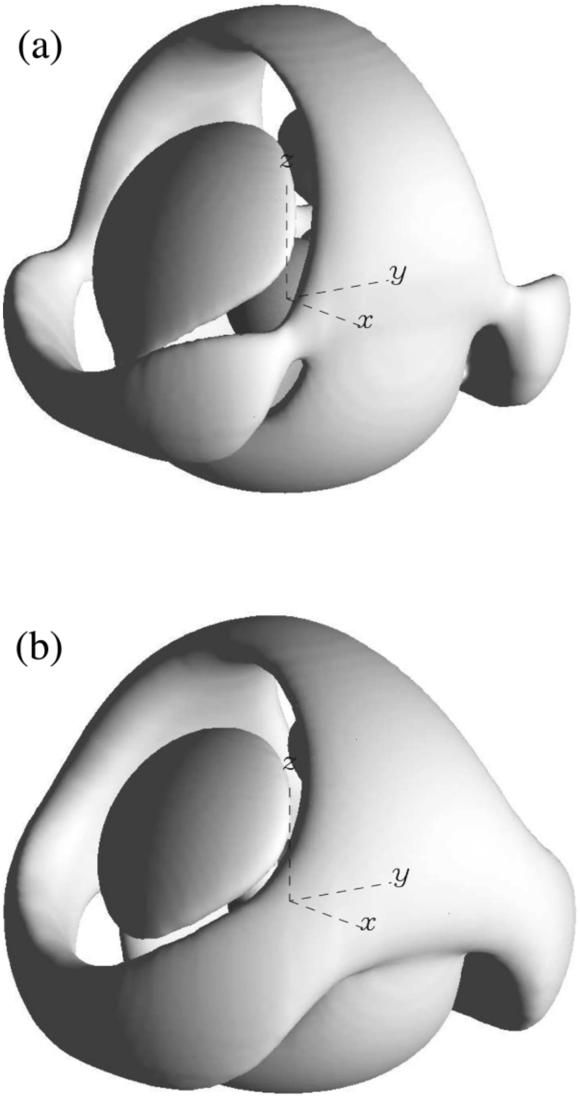

Fig. 9. Comparison of the spatial density function of water about water in urea–water 1:4 (a), with water about water in pure water (b). The contour level has been set so that the isodensity surface encloses 40% of the molecules in the distance range 0–5 Å. We note a substantial distortion of the second shell in urea–water compared to pure water.

any urea molecule being H-bonded to another is $p_U = 4 \times 0.2 = 0.8$. If we assume this value is independent of how many other urea molecules are bonded to the first urea molecule, or *where* they are bonded, then the likelihood of a cluster or chain containing N urea molecules is $p_U^{(N-1)}$. Thus the number of urea molecules involved in the cluster of size N is $Np_U^{(N-1)}$. Expressing this as a fraction, $F_U(N)$, of the total number of urea molecules in the solution we obtain

$$F_U(N) = \frac{Np_U^{(N-1)}}{\sum_{N=1} Np_U^{(N-1)}} \tag{7}$$

This function is shown for $p_U = 0.8$ alongside the data of Fig. 8, as well as for $p_U = 0.9$. The obvious lack of agreement between Eq. (7) and the simulation signals immediately that such a simple model is not appropriate in this case. The difference however indicates that once a chain or cluster has started to form in the simulation, it is self-propagating, i.e. once more than approximately 4 urea molecules have formed into a cluster or chain, this combined entity somehow raises significantly the probability of further molecules being added to the chain or cluster. This emphasis on the formation of longer chains then automatically depletes the population of smaller clusters relative to the random bonding model. Visual inspection of the present simulation box, Fig. 10, certainly reveals both 'ring' and 'head to tail' conformations but these are usually incorporated into longer chains, which then may bond back onto the same chain after several bonds involving additional intervening molecules. Hence the propensity for clusters to form from more than 2 urea molecules.

Further inspection of Fig. 10 reveals what appear to be some cavities in the urea network that would be filled with water (not shown here). It is not clear whether this was a feature of this particular configuration or occurred throughout the simulation. It is distinct from the type of segregation that was observed in methanol–water solutions [24], since in urea–water the water is incorporated by hydrogen bonding directly into the clusters and chains, as well as into the region between them.

To investigate further the degree to which water and urea segregate in this system a triplet angle distribution function was calculated for all the water molecules in 1:4 urea–water, and compared with the same function for pure water. Triplets of water molecules in the simulation box are identi-

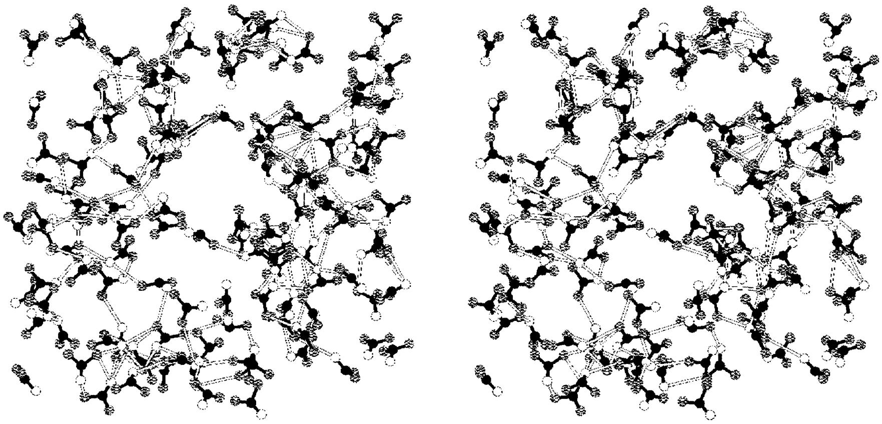

Fig. 10. Stereo plot of one EPSR simulation box of urea–water 1:4 showing only the urea molecules. Carbon atoms are shaded black, urea oxygen atoms are white and urea nitrogen atoms are grey. Black lines show the CN and CO intramolecular bonds, while the white bonds represent nitrogen–oxygen distances less than 3.6 Å between different molecules. These coincide closely with the hydrogen bonds between urea molecules. For clarity the urea hydrogen atoms and water molecules are not shown.

fied when at least two pairs of molecules within the triplet are nearest neighbours, i.e. the separation of their oxygen atoms is $\leqslant 3.3$ Å. The angle between the two pairs of neighbours is used to generate a histogram of such angles. Fig. 11 shows the result of this calculation and compares it to the result of the same calculation for pure water. In a further investigation, a constraint was placed on the water molecules that they must *not* lie within the first coordination shell of any urea carbon ($r > 5.1$ Å), urea oxygen ($r > 3.6$ Å) or urea nitrogen ($r > 4$ Å). This however yielded only a handful of water molecules available out of 500 in the box, and none of them satisfied the condition to calculate the triplet distribution, indicating that virtually all the water in this system is in the first shell of one or more of the urea atoms. We therefore conclude from this and from Fig. 11 that water and urea do not segregate appreciably in this system, and that the water structure is indeed radically modified compared to bulk water.

The present results may also give us at least some speculative insight into the solubility of alkanes in urea water, and into the denaturing abilities of urea, closely analogous to what previous work has indicated [15,21,41]. We have seen that urea readily intercepts the water–water hydrogen bond in solution and appears to substitute for

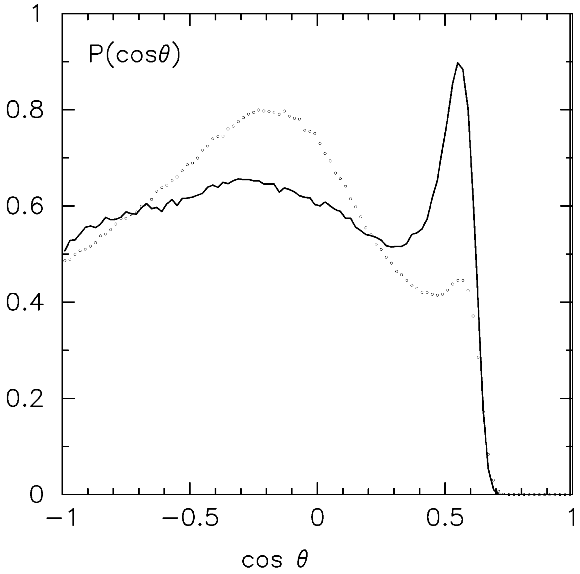

Fig. 11. Triplet angle distribution function $P(\cos\theta)$ for water molecules in 1:4 molar ratio urea–water (line) compared to the same function for pure water (circles). Note the sharp diminution of the broad feature near $\cos\theta = -0.2$, which in pure water is indicative of the tetrahedral H-bond network being present at short distances. Note also a slight shift to larger angles. At the same time there is marked growth in this function around $\cos\theta = 0.5$, corresponding to near neighbour interstitial molecules, and indicating a highly distorted local water structure in the 1:4 molar ratio urea solution.

it easily. When a small alkane molecule like methane dissolves in water, the water needs to form a shell around it consisting of 16–20 molecules, which it can do fairly easily with only slightly distorted hydrogen bonds and little change in the overall number of H-bonds per water molecule. On the other hand, with urea present, the relatively large size of the urea molecule would make it awkward to fit around the small methane molecule. Thus, to get methane into aqueous urea at high concentrations of urea requires significant breaking of the water–urea bonds so that a shell consisting purely of water molecules can be created for the methane. This is likely to be energetically unfavourable, leading to lower methane solubility compared to pure water. On the other hand, for larger alkanes the shell becomes elongated along the alkane chain, and so in that case the larger urea molecule *can* cooperate with the water to form a shell without breaking the water–urea hydrogen bonding, and so increase the solubility of the alkane.

A similar kind of argument could be used to explain the protein denaturing that takes place in urea solutions. Being a larger molecule which nonetheless readily hydrogen bonds to water, urea is able to assist hydration of the hydrophobic residues of the protein molecule around which water on its own would have greater difficulty forming a solvation shell. The increase in the solubility of the residue, together with any direct interaction the urea molecule might have with hydrophilic residues, would lower the threshold for protein denaturation. This would only work at high urea concentrations since the mechanism requires the urea to be readily available for hydrophobic hydration.

Obviously these qualitative ideas need to be explored more quantitatively, but they do seem to support the earlier simulation results of Kuharski and Rossky [10] and the basic assumptions of Muller [9] that in concentrated urea water systems, both the urea and the water will be incorporated into the solvation shell of hydrophobic entities, due to the strong propensity for urea and water to form hydrogen bonds with each other. If the geometry of that shell prefers only water hydrogen bonding then the solubility of that entity will be reduced, whereas if the solvation shell can accommodate urea as well, then the solubility is enhanced. On the other hand, the observations derived from Figs. 10 and 11 that there is little segregation taking place in this system, is in agreement with the analysis of Tsai et al. [15], and argues against the Frank and Franks hypothesis [1] that water somehow segregates into two forms, with urea going only into the denser form. Interestingly, however, we do see a break up of the tetrahedral network of pure water as a result of the addition of urea.

5. Conclusion

A new neutron diffraction experiment looking at the urea–water system at a 1:4 molar ratio has been described here. Using EPSR a molecular model of the system was set up to give structure factors closely similar to the diffraction measurements. Overall the agreement is good, with some fairly minor discrepancies that are interpreted as arising from unknown errors in the corrections that have to be made for attenuation, multiple scattering, and inelastic recoil of the atomic nuclei. The model derived from this process has been used to investigate a number of structural quantities of the solution, in particular the change in water structure induced by the urea compared to pure water, the degree of possible hydrogen bonding in the solution, and the extent to which transient chains and clusters of urea molecules occur in the solution.

Compared to previous simulations of this system, and in contrast to recent data on the methanol-water system at an even higher concentration [23], urea mixes in well with the water molecules, but in doing so has a drastic effect on the water–water second neighbour shell. On average the degree of possible hydrogen bonding between urea and water compared to that between urea and urea is in close proportion to the relative numbers of each molecule in solution, suggesting that urea simply replaces water on a more-or-less random basis as a hydrogen bonding neighbour. This, however, does not stop the formation of transient hydrogen-bonded urea chains and clusters within the solution, due to the large number of sites available on the urea molecule for hydrogen bonding. In contrast to some recent simulations of urea in water [24] there is little evidence for urea molecules segregating themselves from the water in this system.

The same experiment produced data on urea in water at different concentrations and at different temperatures, and it is hoped to describe these data, and explore further some of the issues raised in this paper, in a later publication. It is clear that mixtures of urea and water provide a most fascinating and important system to study, and with the application of modern diffraction methods, combined with computer simulation and other approaches, some of the uncertainties surrounding these solutions will start to disappear.

Since this article was typeset it came to our notice that the correct theoretical description for the cluster size distribution in this case might be a power law rather than the exponential distribution show in Fig. 8 and Eq. (7). See for example A. Oleinikova, I. Brovchenko, A. Geiger and B. Guillot, J. Chem. Phys., vol. 117, p. 3296 (2002). We will review this power law dependence when we make the comparison of the present urea water data with those at two lower urea: water concentrations, 1:8 and 1:16 in a future publication.

Acknowledgments

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