Water at interface with proteins

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Abstract. Water is essential for the activity of proteins. However, the effect of the properties of water on the behavior of proteins is only partially understood. Recently, several experiments have investigated the relation between the dynamics of the hydration water and the dynamics of protein. These works have generated a large amount of data whose interpretation is debated. New experiments measure the dynamics of water at low temperature on the surface of proteins, finding a qualitative change (crossover) that might be related to the slowing down and stop of the protein’s activity (protein glass transition), possibly relevant for the safe preservation of organic material at low temperature. To better understand the experimental data several scenarios have been discussed. Here, we review these experiments and discuss their interpretations in relation with the anomalous properties of water. We summarize the results for the thermodynamics and dynamics of supercooled water at an interface. We consider also the effect of water on protein stability, making a step in the direction of understanding, by means of Monte Carlo simulations and theoretical calculations, how the interplay of water cooperativity and hydrogen bonds interfacial strengthening affects the protein cold denaturation.

Keywords: Water. Hydrated proteins. Confined Water. Biological interfaces.
1. Introduction

Water is ubiquitous in biological systems. It is a major component of cells and participates in the majority of the biological processes. It is usually considered essential for life, but it is still under debate why [1]. One possible reason is that water has many properties that are unusual with respect to other liquids [2].

The anomalous behavior of water is evident in the liquid phase. For example, fluctuations of volume and fluctuations of entropy have a minimum for liquid water, while in usual liquids they decrease when the temperature $T$ is decreased. Volume fluctuations can be observed by measuring the compressibility $K_T$, defined as how much the volume changes when the pressure $P$ is changed at constant $T$, and entropy fluctuations are proportional to the specific heat $C_P$ at constant $P$. For water at ambient pressure $K_T$ has a minimum at $46^\circ C$ and $C_P$ at minimum at $35^\circ C$.

The anomalies of water become more evident when $T$ is decreased toward and below $0^\circ C$. For example, water has a maximum in density at $4^\circ C$. Normal liquids, such as argon, reduce their density when the temperature decreases and reach their maximum density when they solidify in a crystal. Water, instead, below $4^\circ C$ expands. Therefore, liquid water at $0^\circ C$ has a density smaller than water at $4^\circ C$. By solidifying into ice, water expands even further, becoming “lighter”. For this reason ice cubes float in a glass of water. This property has a dramatic consequence in processes such as the cryopreservation of biological cells, because the large amount of water in each cell expands when forms ice and breaks the cell.

Another property of water that is relevant in these conditions is that water can stay in its liquid state even at $T < 0^\circ C$, i.e. it can be supercooled below its melting temperature. Bulk water can be supercooled to $-41^\circ C$ at atmospheric pressure, but in different conditions water can remain liquid even at lower temperatures. For example, it can be supercooled down to $-47^\circ C$ when confined in vegetable fibers, or down to $-92^\circ C$ when compressed at 2 kbars. An anti-freezing effect can be achieved also by dissolving in water polymers or proteins, with several practical applications. Understanding the details of this phenomena and how to regulate it could be very relevant for cryopreservation, food storage and refrigeration [3, 4].

Also in its solid state, water is peculiar. Water is a polymorph with many different crystal phases, more than fifteen, some of which are stable only at pressure $P > 100$ GPa. But water can easily form a solid that is not a crystal. If quenched rapidly below $-123^\circ C$ at ambient pressure, liquid water freezes in a metastable amorphous state, which is an arrested liquid configuration [2]. At low pressure water forms a low-density amorphous (LDA) state [3], while at high pressure it forms a high-density amorphous (HDA) state [6], separated by a volume discontinuity of $\approx 27\%$, comparable to that between crystalline ice I and ice VI. A smaller discontinuity has been observed more recently [7, 8], but its interpretation is under debate.

The discontinuities between amorphous states at different densities and the fact that quantities such as $K_T$ or $C_P$ largely increase in the supercooled state, show that...
water has a complex behavior at low $T$. This observation and the fact that water remains in its liquid state at very low $T$ when in contact with organic or inorganic interfaces suggest that water could play a main role in phenomena such as the so-called protein glass transition, or the protein cold denaturation at low temperature.

1.1. Experiments

To explore how the dynamics of proteins and water are related, S-H. Chen et al. in 2006 studied by high–resolution quasi–elastic neutron scattering (QUENS) the structure and dynamics of water molecules in the hydration layer surrounding lysozyme proteins at temperatures around 220 K (−53.15°C) [9]. Below this temperature the protein is in a solid–like “glassy state”, with no conformational flexibility and no biological functions. As the temperature is increased, the protein displays a harmonic atomic motion that, in hydrated proteins, suddenly becomes anharmonic and liquid-like at about 220 K. The change in the protein dynamics is believed to be triggered by the coupling with the hydration water through the hydrogen bonds because the hydration water displays a dynamic transition at a similar $T$ [10, 11]. Chen interpreted the hydration water dynamic transition as a change in a main structural relaxation and recently extended this interpretation to hydrated proteins at high-pressure.

This explanation has been questioned by Swenson et al. [12]. By using dielectric measurements on myoglobin in water-glycerol mixtures, they found a dynamic crossover at about 200 K and they interpreted it as an evidence that local (secondary) protein motions are controlled (slaved) by the local fluctuations in the hydration shell, as proposed by Fenimore et al. in 2004 [13] based on Mössbauer and neutron-scattering experiments.

On the other hand, Pawlus et al. in 2008, based on measurement of conductivity on hydrated lysozyme, found no crossover around 220 K. They also ascribe the apparent crossover observed with QENS to a secondary relaxation and to a lack of resolution on main structural relaxation of QENS [14].

More recently nuclear magnetic resonance (NMR) experiments on water hydrating elastin and collagen, performed by Vogel, showed no crossover at 220 K but a crossover at about 200 K. This data have been interpreted as consistent with thermally activated tetrahedral jump motion of hydration water [15].

These and other experiments, therefore, show that it is difficult to achieve a clear understanding of the protein-hydration water coupling solely based on the experimental data. A possible way to gain further insight is offered by the numerical results of detailed computer simulations.

1.2. Numerical results

In 2006 Kumar et al. [16], by simulating lysozyme or DNA surrounded by water represented with the TIP5P model, found a dynamic transition of the macromolecules at about the same temperature of a dynamic crossover in the diffusivity of hydration water,
in the range 242 K–250 K. They also show numerical indications that this crossover coincides with the maximum of the isobaric specific heat of the whole system and the maximum fluctuations in tetrahedral order of the hydration water.

This observation was confirmed in 2008 by Lagi et al. \cite{17}. They found a strong crossover in the water translational (\(\alpha\)) relaxation time and in the inverse of its self-diffusion constant at about 223 K, by simulating hydrated lysozyme with water represented by the TIP4P-Ew model. They corroborate that the crossover corresponds to the maximum structural change, and they found a low activation energy, showing consistency with the neutron scattering and the NMR experiments.

Nevertheless, in 2009 this view was disputed by Vogel \cite{18}. By simulating elastin or collagen hydrated by SPC-water he found only a weak crossover in the hydration water correlation time at about 200 K and a high activation energy associated to a secondary relaxation, consistent with activation energies determined in dielectric spectroscopy and NMR studies.

It is evident from these and other numerical studies that the comparison of experiments with simulation is useful, but is not enough to elucidate the mechanisms that regulate the coupling of the water dynamics with the biomolecules dynamics. In particular, the difficulties in finding clear answers to the open questions rise for the fact that experiments and numerical results are both affected by errors and uncertainties. Therefore, it is natural to look for a theory that could be able to find exact relations and make predictions to test in further experiments.

In the next section we describe a model for a hydration water monolayer that allows to develop a theory for these phenomena and to predict properties that could be verified in experiments. The model, moreover, allows for efficient simulations whose results complement the theoretical analysis. In section 3 we review results of this tractable model, including new data for the density and energy distributions at different pressures and very low temperature (subsection 3.3) and for the hydration percolation (subsection 3.4). In section 4 we discuss new results about protein stability and confined water in the context of food processing and cells in living organism. In section 5 we give our conclusive remarks and we discuss possible extensions of this model.

2. A tractable model for a hydration water monolayer

We first consider the case of water nanoconfined between two hydrophobic surfaces. To fix the idea, let’s consider the case with a distance \(\delta = 0.7\) nm between the two surfaces. Because the confining surfaces are hydrophobic, the water molecules will not form hydrogen bonds (HB) with the surfaces. Experiments shows that in bulk each water molecule is surrounded by four nearest neighbor molecules at a distance of about 3Å with a structure that resembles that of a tetrahedron at low \(TV\) and \(P\) \cite{19}. Hence, one would expect that each water molecule between the two hydrophobic plates will adjust in a way to form a distorted network of HBs with each molecules surrounded by other four. This has been indeed found by Kumar et al. \cite{20} by simulating TIP5P-water
To define a tractable model, we coarse-grain the structure described above, dividing the slab of space occupied by water into cells with volume $v = \delta r^2$, with a square section of size $r \geq r_0$, where $r_0 = 2.9\text{Å}$ is the closest approach (van der Waals) distance between water molecules (Fig. 1). If the density of water is $\rho$, and the number of water molecules in $N$, the water volume is $V = N/\rho$. If the system is uniform and we divide the system in $N$ cells, each cell has on average one water molecule. More in general if the system is not uniform, some cells can be empty or each cell can occupy a different volume $v_i$ and the distance $r_{ij}$ between two molecules in the cells $j$ and $j$ is the distance between their centers. For example, if $i$ and $j$ are the indices of nearest neighbor cells, the distance between the molecules in these cells is $r_{ij} = (\sqrt{v_i/\delta} + \sqrt{v_j/\delta})/2$.

We describe the isotropic (e.g., van der Waals) attractive and repulsive interactions between the molecules by a standard a Lennard-Jones interaction

$$U = \sum_{ij} \epsilon \left[ \left( \frac{r_0}{r_{ij}} \right)^{12} - \left( \frac{r_0}{r_{ij}} \right)^{6} \right] \quad (1)$$

where $\epsilon = 5.8 \text{ kJ/mol}$ is the attractive energy and the sum is over all the possible pairs of molecules $i$ and $j$. Some modifications of this interaction, such as the introduction of a maximum cut-off distance or a hard-core distance, have been adopted in previous analysis of this model to simplify the numerical simulations. For the theoretical analysis, instead, this interaction term has been replaced by a more tractable discrete interaction.

To take into account the energy and entropy variation when water molecules form HBs, we introduce for each water molecule $i$ four bonding indices $\sigma_{ij}$, one for each of the possible HBs with the four nearest neighbor water molecules $j$. The index can assume $q$ different states, $\sigma_{ij} = 1, \ldots, q$, where $q = 6$ is a parameter whose value we will discuss in the following. Therefore, each water molecule has $q^4 = 6^4 = 1296$ possible states, that can be interpreted as possible rotational configurations. The total number of configurations for the system is $q^{4N}$, that for $N = 10^5$ (the maximum number that we have considered in our analysis) is an astronomical number (about $3 \times 10^{31260}$). When a molecule forms a HB the number of its accessible configurations decreases, and the energy of the system is reduced.

To estimate the decrease in the number of accessible configurations, we observe that a HB is broken if it deviates from a linear bond more than $\pm 30^\circ$. Therefore, only $1/6$ of the whole continuous range of orientations $[0, 360^\circ]$ in the OH—O plane are associated to a bonded state. Hence, $5/6$ of the possible configurations are not—bonded. By allowing $q = 6$ possible states for each bonding index $\sigma_{ij}$, we can count correctly the entropy loss associated to the formation of a HB if only one out of $q$ states corresponds to a HB. This is achieved by allowing the formation of the HB between molecules $i$ and $j$ only if $\delta_{\sigma_{ij},\sigma_{ji}} = 1$, where by definition $\delta_{a,b} = 1$ if $a = b$ and $\delta_{a,b} = 0$ otherwise.

Next, we take into account that a HB is broken if the OH—O distance is too large.
To simplify the analysis, we introduce a condition on the O–O distance $r$, allowing the formation of HB only if $r \leq r^{\text{max}}$, with $r^{\text{max}} = r_0\sqrt{2} = 4.10\text{Å}$. Therefore, considering that the length of the OH covalent bond is $r_{\text{OH}} = 0.96\text{Å}$, the maximum bond length is $r^{\text{max}} - r_{\text{OH}} = 3.14\text{Å}$, consistent with other choices in literature \[36, 37]. In particular, we impose this condition by introducing a discrete variable $n_i$ for each cell $i$, with $n_i = 1$ if $r_i \leq r_0\sqrt{2}$, otherwise $n_i = 0$. A cell with $n_i = 1$ is liquid-like because its (dimensionless) density $\rho_i \equiv (\delta r^2_0)/(\delta r^2_i) \geq 1/2$, while a cell with $n_i = 0$ is gas-like because has $\rho_i < 1/2$. Hence, we define the number of HBs as

$$N_{\text{HB}} \equiv \sum_{\langle i,j \rangle} n_i n_j \delta_{\sigma_i,\sigma_j}(2)$$

where the symbol $\sum_{\langle i,j \rangle}$ denotes that the sum is performed over nearest neighbor cells $i$ and $j$.

From the experiments we know the formation of HBs leads to an open network of molecules with, on average, four neighbors instead of twelve as in argon-like fluids. The resulting volume per molecule with HBs is larger than the volume per molecule with no HBs. This is observable as the anomalous density decrease described in the introduction, that is a consequence of the formation of a macroscopic number of HBs. This effect is incorporated in the model by considering the total water volume to be given by

$$V \equiv V_0 + N_{\text{HB}} v_{\text{HB}},$$

where $V_0$ is the water volume in absence of HBs, and $v_{\text{HB}}$ is the increase of volume per HB. To estimate the parameter $v_{\text{HB}}$, we consider as reference values the increase between the density $\rho_{\text{IH}} = 0.92 \text{g/cm}^3$ of the ice Ih at atmospheric pressure and ice VI, with density $\rho_{\text{VI}} = 1.31 \text{g/cm}^3$, or ice ice VIII, with density $\rho_{\text{VIII}} = 1.46 \text{g/cm}^3$. Ice Ih is characterized by hexagonal rings of HBs with an almost perfect tetrahedral structure, while ice VI and ice VIII have a structure consisting of two interpenetrating tetrahedral networks of HBs. The relative increase of density in these cases is equal to 0.42 and 0.59, respectively. Hence, in the model we set the relative HB increase of volume per molecule equal to the average between the previous reference values, i.e. $v_{\text{HB}}/\delta r^2_0 = 0.5$.

Note that this increase of volume per molecule is due to a decrease of first neighbors and does not imply an increase of distance between molecules. Our model, being coarse-grained, does not include all the details about the structure, but maintains the increase of volume per molecule with no effect on the distance $r$ between molecules. In particular, the HB volume increase does not affect the calculation of $U_0(r)$ of Eq. (1).

The formation of a HB leads to an energy gain, represented in the model by an interaction term

$$\mathcal{H}_{\text{HB}} \equiv -J N_{\text{HB}},$$

where $J$ is the characteristic energy of the covalent (directional) component of the HB. This term only accounts for the two-body component of the HB interaction. However, in water many-body effects are relevant and, in particular, the three-body term \[39, 40\].
This can be observed from the \( T \)-dependence of the O–O–O angle distribution. This distribution becomes sharper around the tetrahedral angle when \( T \) decreases \[41\]. Hence, we include in the model the many-body (cooperative) effect due to HBs \[42, 43, 44\], which minimizes the energy when the HBs of nearby molecules optimize the tetrahedral orientation. This is accomplished by further adding to the Hamiltonian in Eqs. \((1)\) and \((4)\) the term
\[
\mathcal{H}_{\text{coop}} = -J_{\sigma} \sum_i n_i \sum_{(k,\ell)_i} \delta_{\sigma_{ik},\sigma_{\ell i}},
\]
where \( J_{\sigma} \) is the characteristic energy of the cooperative component of the H bond, and the sum over \((k,\ell)_i\) is performed over all the six different pairs of the four bonding indices of molecule \(i\).

As discussed by Stokely et al. in Ref. \[33\], Eqs. \((4)\) and \((5)\) have two free parameters: \( J \) and \( J_{\sigma} \), respectively. Experiments estimate the HB in ice Ih to be \( \approx 3.0 \text{ kJ/mol} \) stronger than in liquid water \[45\]. Attributing this increase to a cooperative interaction among HBs \[46\], we can estimate the value of \( J_{\sigma} \) in the cell model to be \( \approx 1.0 \text{ kJ/mol} \), because for each HB there would be \( 6/2 \) pairs of \( J_{\sigma} \)–interactions. The optimal HB energy, \( E_{\text{HB}} \), has been measured to be \( \approx 23.3 \text{ kJ/mol} \) \[47\]. By considering tetrahedral clusters of H-bonded molecules, with HB and van der Waals interactions up to the third nearest neighbor molecules, the value for the directional component of the HB is estimated by Stokely et al. \[33\] as \( J \approx 12.0 \text{ kJ/mol} \). Other experimental estimates suggest that breaking the directional component of the HB requires \( J \approx 6.3 \text{ kJ/mol} \) \[48\]. It is, therefore, a reasonable estimate to set \( J_{\sigma}/J = 1/10 \) and to consider \( J \) as the only free parameter of the model.

In the following we will briefly summarize some recent results for this model, to show that it reproduces in a qualitative way the properties of water. An appropriate choice of the free parameter \( J \) leads to results that, as for more detailed models, can be fairly rescaled on the known properties of a water monolayer. In this respect, this model is not better then detailed models, but, it has two features that detailed models have not. i) It is less computationally expensive, because it is coarse-grained. This allows to simulate very large number of water molecules (about a million) on simple desktop computers in a few hours. ii) More importantly and differently from the detailed models, this model is tractable for theoretical calculations. The trade-off for these advantages is that the model is coarse-grained and cannot give informations about some properties of water, such as the structure. Nevertheless, works is in progress to overcome this limitation.

3. Results for water between hydrophobic plates

We study the model described in the previous section by mean-field (MF) analysis and Monte Carlo (MC) simulations. The MF approach follows the Bethe-Peierls and the cavity method \[49\], by expressing the molar Gibbs free energy in terms of an exact partition function for a portion of the system, and taking into account the effect of all
the rest of the system as a mean field acting on the border of this portion, as described in Ref. \[2, 50\].

MC simulations are performed at constant \(N, P, T\), allowing the volume \(V_0\) in Eq. (3) to fluctuate as a stochastic variable. To minimize the boundary effects, we consider periodic boundary conditions in the directions parallel to the confining surfaces. To study the thermodynamic properties of the model we adopt an efficient cluster MC dynamics, defined in Ref. \[31\], or a continuous \(T\) algorithm, the histogram reweighting method, as in Ref. \[24\]. To study the dynamics of the HBs we adopt a standard Metropolis algorithm \[35\], while to study the diffusion properties we use the Kawasaki algorithm \[29\].

3.1. The gas and liquid phases for nanoconfined water: transport properties

For water nanoconfined between hydrophobic plates, the model display a gas–liquid first-order phase transition ending in a critical point \(C\), that qualitatively resembles the gas–liquid transition for bulk water (Fig. 2a). In their work, de los Santos et al. \[30, 51\] verify that the water diffusion constant \(D\) decreases when water changes from gas to liquid. The change in \(D\) is strong far from the critical point \(C\), and it disappears at \(C\). In the liquid phase, de los Santos et al. \[30\] observe that, as for bulk water, the nanoconfined system has a region in the \(P–T\) plane where \(D\) increases for increasing \(P\). This behavior is an anomaly of water, because in normal fluids \(D\) decreases for increasing \(P\) \[52, 53\]. This anomalous behavior is qualitatively rationalized as a consequence of the HB formation and it has been observed both in bulk and confined water \[54\].

At lower temperature, by decreasing \(T\) at constant \(P\) the model displays the line of temperatures of maximum density (TMD) \[35, 29\], as in the bulk case (Fig. 2a). The TMD line in the \(P–T\) plane has a positive slope at low \(P\) and negative slope at high \(P\).

The nanoconfinement does not allows the formation of crystal ice. Nevertheless, it is possible to calculate where in the \(P–T\) plane \(D(P,T)\) is constant and to show that the lines of constant-\(D\) qualitatively resemble the melting line of bulk water \[30, 51\].

3.2. Water monolayer compared to protein hydration water

At low \(T\) the diffusion constant largely decreases and, eventually, the monolayer becomes subdiffusive, i.e. the mean square displacement of water molecules never reach the diffusive regime \[30, 51\]. This property has been observed experimentally below 320 K by neutron scattering in a monolayer of water hydrating a myoglobin surface at low hydration level \((h = 0.35 \text{ g H}_2\text{O/g of protein})\), corresponding to a number of water molecules sufficient to cover the entire protein surface \[55\].

Franzese and de los Santos in 2009 \[29\] found that a water monolayer partially hydrating a hydrophobic surface, described by the model considered here, would display a very slow dynamics for the HBs at low \(P\) and \(T\). In these conditions the HB correlation function \(C(t)\), that quantifies how much the HBs are correlated in time, is almost not changing in time, showing that the water dynamics is completely frozen. Hence, water
Water at interface with proteins

is in its glassy state at low \( T \) and low \( P \), consistent with the observed freezing of the incoherent intermediate scattering function of water hydrating myoglobin at hydration level \( h = 0.34 \text{ g H}_2\text{O/g of protein} \) at about 180 K \cite{56}. Glassy water is observed also in bulk, but below 150 K.

This slowing down of the dynamics is well understood in our model where, at low \( T \) and low \( P \), the number of HBs largely increases when the \( T \) decreases. This progressive building up of the HB network traps the water molecules in a percolating network of HBs that leaves small areas of the hydrophobic surface completely dehydrated.

At higher \( P \) the decreased number of HB allows to the small dry cavities to slowly equilibrate, due to large-scale rearrangements of the HBs and leading to partial dehydration of the surface. In this case water can slowly flow on the surface because the high pressure reduces the volume per molecules and disfavors the formation of HBs. Hence, the HB network builds up at lower \( T \) with respect to the low pressure condition \cite{29}. In this case the long time behavior of \( C(t) \) is well described by a stretched exponential function

\[
C(t) = C_0 \exp \left[ - (t/\tau)^\beta \right]
\]

where \( C_0 \), \( \tau \) and \( \beta \leq 1 \) are fitting constant. For \( \beta = 1 \) the function is exponential, and the more stretched is the function, the smaller is the exponent \( 0 < \beta \leq 1 \).

Franzese and de los Santos \cite{29} predicted that at low \( P \), by increasing \( P \), (i) the time needed for the HBs to decorrelate decreases, i.e. water can relax more rapidly, and (ii) the \( \beta \) exponent decreases going from \( \beta = 0.8 \) to \( \beta = 0.4 \) for a pressure approaching a characteristic value \( P_{C'} \). We will discuss further about \( P_{C'} \) in the following. By increasing the pressure even further, for \( P > P_{C'} \), the HB correlation function relaxes faster and the exponent becomes \( \beta = 1 \), i.e. \( C(t) \) becomes an exponential function.

The prediction about \( \beta \) is consistent with the experimental findings of Settles and Doster showing that, for water hydrating myoglobin at low hydration level, the incoherent intermediate scattering function at large \( Q \) vector, i.e. the dynamics of density fluctuations at short distance, are well described by stretched exponential functions with \( \beta \) varying between 0.4 and 0.3 at 320 K \cite{55}.

We observe that the theoretical lower limit for \( \beta \) is expected to be \( 1/3 \) \cite{57, 58} and that \( 1 - \beta \) is a measure of the heterogeneity in the system. Therefore, the prediction \cite{29} that the stretching parameter \( \beta \) approaches its smallest possible value when the pressure tends to \( P_{C'} \) implies that the water monolayer reaches its maximum in heterogeneity at \( P_{C'} \). In the following we will discuss further this point.

### 3.3. Thermodynamics at very low temperature

To understand the origin of the heterogeneity at \( P_{C'} \), we recall here that the properties of water are consistent with theories that propose different mechanisms and different phase behaviors at very low temperature (approximately 150 K \( \leq T \leq 200 \text{ K} \)). At these temperatures, supercooled bulk water forms ice, while confined water in appropriate
conditions can be kept liquid \[59\]. The different theories can be summarized in four possible scenarios for the $P - T$ phase diagram.

(i) In the stability limit (SL) scenario \[60\] it is hypothesized that the limits of stability of superheated-stretched liquid water changes its slope in the $P-T$ plane from positive at high $T$, to negative at low $T$ and negative $P$, giving as a consequence the anomalous increase at low $T$ of quantities such as $K_T$, $C_P$ and $\alpha_P$.

(ii) In the liquid–liquid critical point (LLCP) scenario \[61\] it is hypothesized the existence of a first–order phase transition line in the supercooled liquid region, with negative slope in the $P-T$ plane and terminating in a critical point $C'$. This phase transition separates two liquid phases, both metastable with respect to the crystal phases: one with low density, resembling the LDA disordered ice, and one with high density, resembling the HDA disordered ice. The low-density-liquid (LDL), high-density-liquid (HDL) critical point $C'$ has been predicted at positive $P$ \[61\] or negative $P$ \[62\], depending on the water model adopted in simulations. The anomalies of water are the consequence of approaching $C'$.

(iii) In the singularity–free (SF) scenario \[63\] it is hypothesized that the HBs have no cooperativity. In this case it is shown that the anomalous increase of $K_T$, $C_P$ and $\alpha_P$ is a consequence of the low-$T$ anticorrelation between volume and entropy, also responsible for negative slope in the $P-T$ plane of the line of temperatures of maximum density (TMD).

(iv) In the critical–point free (CPF) scenario \[64\] it is hypothesized that the LDL-HDL first–order phase transition line extends to $P < 0$, reaching the superheated limit of stability of liquid water and with no critical point. As a consequence the HDL has a superheated-stretched limit of stability similar to that predicted in scenario (i).

As discussed by Stokely et al. \[33\], it is still unclear which of the scenarios best describes water, because there is no definitive experimental test. In Ref. \[33\], the same tractable model presented in the previous section is analyzed by means of theoretical calculations and numerical simulations, showing that the four scenarios (i)–(iv) may be mapped in the space of the parameters $J$ and $J_\sigma$, representing the strength of the HB directional component and the strength of the HB cooperative component, respectively. The relation $J_\sigma/J = 1/10$ discussed at the end of the previous section, and based on estimates from experimental data, supports the prediction of a liquid–liquid critical point $C'$ at positive pressure for supercooled water (Fig.2b). The model allows to distinguish the LDL phase and the HDL phase by their different densities and characteristic energies. The presence of two maxima in the distributions of these quantities marks the coexistence of the two phases and corresponds to the occurrence of two minima in the free energy of the system (Fig.3).

At the critical point $C'$ the cooperativity of the HBs is maximum. Hence, cooperative rearrangements of the HB are necessary to allow the relaxation of the dynamics. These rearrangements occur on different length-scales, each associated with a different time-scale. As a consequence a single time-scale cannot be defined for the dynamics, resulting in a stretched decay of the correlation function, i.e. in heterogeneous
dynamics. It is, therefore, \( C' \) the origin of the heterogeneity described in the previous section and occurring at \( P_{C'} \) of the liquid–liquid critical point.

### 3.4. Hydration percolation

It possible to study the cooperative regions, and their length-scales, by using a geometrical approach based on the concept of correlated percolation \([65, 31]\). We define a cluster of correlated water molecules as described in the following steps.

- The first step consists in including in the cluster one of the bonding indices \( \sigma_{i,j} \) of a randomly selected water molecule \( i \) in the hydration shell.

- The second step is to add to the cluster another bonding index of the same molecule \( i \) with probability \( p_{\text{same}} \equiv \min\{1, 1 - \exp[-J_{\sigma}/(k_B T)]\} \) (where \( k_B \) is the Boltzmann constant), or the facing bonding index \( \sigma_{j,i} \) of the nearest neighbor molecule \( j \) with probability \( p_{\text{facing}} \equiv \min\{1, 1 - \exp[-J'//(k_B T)]\} \) where \( J' \equiv J - P v_{HB} \). In this expression, \( J' \) is the \( P \)-dependent effective coupling between two facing indices as results from the enthalpy \( U + \mathcal{H}_{HB} + \mathcal{H}_{coop} + PV \) of the system Eq.s (1)-(5). The quantity \( J' \) can be positive or negative depending on \( P \). If \( J' > 0 \), an index can be added, with probability \( p_{\text{facing}} \), to the cluster only if it is in the same state as the other indices already in the cluster. Instead, if \( J' < 0 \), the index can be added only if it is in a different state with respect to the index to whom it will be connected.

- The third step is to randomly select a bonding indices on the border of the cluster and pick at random one of the indices on the same molecule, or the facing index on a bonded molecule, that is not already in the cluster, and to include it in the cluster with probability \( p_{\text{same}} \) or \( p_{\text{facing}} \), respectively. This step is repeated until all the possible directions of growth for the cluster have been considered.

The resulting cluster statistically represents the region of correlated HBs, as can be shown \([66]\), and its characteristic linear size statistically corresponds to the correlation length of the water molecules. Therefore, in the vicinity of the liquid-liquid critical point, where the correlation length increases, it is possible to observe that the size of the clusters of correlated water molecules increases. At the liquid-liquid critical point the correlation length diverges and a cluster of correlated water molecules spans (percolate) the whole monolayer. General results on correlated percolation theory allow to find mathematical relations between thermodynamics quantities and percolation quantities. In particular, it can been shown that the mean size of the clusters defined above diverges with the same power–law exponent as the compressibility of water and that the distribution of number \( n(s) \) of finite cluster of size \( s \) per water molecule is exponential far from the liquid-liquid critical point \( C' \), while follows a power law with exponent \( \tau \) near \( C' \). From general considerations it is possible to show that \( \tau = 1 + d/D_F \) where \( d = 2 \) is the effective dimensionality of the monolayer and \( D_F \) is the fractal dimension of the clusters \([67]\). Preliminary estimate of \( \tau \approx 2 \) suggests that the clusters of correlated water molecules are compact with \( D_F \approx 2 \) (Fig.4) \([68]\). Since the compressibility is proportional to
the density fluctuations, the clusters allows for a geometrical analysis of the diverging density fluctuations near the liquid-liquid critical point [32].

3.5. Hydrogen bonds dynamics on Hydrated Protein

The density fluctuations are observable also far from the liquid-liquid critical point $C'$. In particular, they can be observed along a line in the $P-T$ phase diagram that emanates from $C'$ into the one-phase region and marks the maxima of the correlation length. This line has been named after Widom [69, 2, 70] and can be characterized in the study of hydrated protein. For example, Kumar et al. [28] used the tractable model described above to investigate the case of a percolating monolayer of water molecules adsorbed on a protein surface, with hydration level about $h \approx 0.4 \text{ g H}_2\text{O/g dry protein}$. Under these conditions the protein is immobile and inhibits the ice crystallization because it forces the water molecules out of the positions corresponding to a crystal configurations. The authors studied the HB dynamics, regardless if the HBs are formed within the water molecules or with the surface.

They first locate the Widom line, by Monte Carlo simulations, and observed that it corresponds to the locus where there is the largest change in the number of HBs. At $P$ and $T$ above the Widom line, water has a few HBs, while at $P$ and $T$ below the Widom line, it has a well-developed network of HBs. This change of structure is reflected by a maxima in constant-$P$ the specific heat along the Widom line. The simulations also reveal the presence of a dynamic crossover in the HB correlation function $C(t)$ when the Widom line is crossed at constant $P$. Using mean field theory and making the hypothesis that the dynamics is dominated by the rearrangements of the HBs, the authors calculate the activation energy for the relaxation of the system and show that it gives the same relaxation time calculated by Monte Carlo simulations [35]. The proposed mechanism consists in breaking a HB that does not fit into the tetrahedral arrangement and reorient the molecule to optimize locally the tetrahedral configuration. This mechanism has been confirmed also by simulations of the hydration shells of elastin-like and collagen-like peptides [18]. In particular, Kumar et al. [35] predict (i) how this barrier is affected by the variation of $P$, (ii) how the crossover $T$ is affected by the variation of $P$, and (iii) that for any $P$ the correlation time at the crossover $T$ is the same (isochronic crossover). Experiments with hydrated lysozyme, spanning a range of pressures going from ambient pressure up to 1600 bar, performed by the group of S.-H. Chen at MIT, have confirmed these three predictions [71, 34].

More recent analysis of lysozyme proteins at a lower hydration level ($h = 0.3 \text{ g H}_2\text{O/g dry protein}$) reveals another surprising results [72]. At this very low hydration, dielectric spectroscopy, probing the proton relaxation, displays that at ambient $P$ not only there is at about 250 K the dynamic crossover described above, but also another crossover at about 180 K. The study of the tractable model presented here associates this lower-$T$ crossover to the saturation of the cooperative ordering of the HB network. Specifically, the HBs rearrange to maximize the number of tetrahedral orientations
among the bonds. This ordering is marked by a new specific heat maxima that can be calculated in the model at about 180 K. Therefore, summarizing, the model predicts a broad specific heat maximum at about 250 K, due to the saturation of a macroscopic HB network, and a sharper specific heat maximum at about 180 K, due to the saturation of the tetrahedral ordering of the HBs. By increasing the pressure of the hydrated protein, Mazza et al. predict that these two maxima merge and then diverge at the liquid-liquid critical point $C'$.

4. Discussion: Implications in food science

The presence of a liquid-liquid critical point $C'$ at low $T$ in confined water could be an undesirable property for the storage of frozen food and, more in general, biological cells. This is because in the vicinity of a critical point between two liquid phases, both metastable with respect to the crystal phase, large density fluctuations occur. These enhanced fluctuations would drastically change the pathway for the formation of a crystal nucleus, because the crystal would form from the dense fluid, instead that from the low density fluid. As a consequence, there would be a strong reduction of the crystal nucleation free-energy barrier and, hence, an increase by many orders of magnitude of the crystallization rate, as theoretically predicted by ten Wolde and Frenkel.

Under this conditions the enhanced formation of ice could destroy the cells as a consequence of the increase of volume of ice with respect to the liquid water. Hence, the best way to preserve the cells would be to freeze them at a $T$ that is far away from the liquid-liquid critical temperature. Nevertheless, further analysis show that the situation is even more complex. Indeed, simulations of a model with a metastable liquid-liquid critical point display enhanced crystallization rate not only in the vicinity of the critical point, but in the vicinity of the whole region of liquid-liquid coexistence and possibly also in the one-phase region above the critical point along the Widom line. Work is in progress to elucidate these implications.

In the evaluation of these effects is extremely important to properly include the interaction with the confining surfaces. We are presently studying how to incorporate these effects in our tractable model. A first step in this direction is to include a description of the hydrophobic effect. Frank and Evans and Silverstein et al. proposed that supercooled water forms highly structured “ice-like” regions in the hydration shell of nonpolar solutes. Stillinger proposed that HBs in the hydration shell are not significantly perturbed near small hydrophobic solutes, while the HB network is strongly affected by hydrophobic particles with size above a characteristic value. Chandler estimated this value of the order of 1 nm on the basis of free energy calculations. Muller explained the vibrational and NMR spectroscopy results by suggesting enthalpic strengthening of the hydration HBs with a simultaneous entropy increase in the hydration shell. We are presently including the enthalpic strengthening in our model, and properly accounting for the entropy increase for the study of water in confined by hydrophobic nanoparticles. Our preliminary results...
show a surprising change of thermodynamic fluctuations at low $T$, whose implication is a large decrease of compressibility also at very low nanoparticles concentration. This finding suggests that adding hydrophobic particles at low concentration in organic solutions would decrease the density fluctuations and the formation of ice.

Further confirmation of the validity of our assumption about the hydrophobic effect comes from another study that we are performing to establish if our tractable model is able to describe the stability of proteins with respect to changes of temperature and pressure. In this study we consider how the hydrophobic interaction with water of a coarse-grained protein induces hot denaturation, folding, cold denaturation and pressure denaturation. Our preliminary results [82] display a region of stable folded configurations that, in the $P-T$ phase diagram, has the same qualitative features of the experimental stability diagram of myoglobin [83] (Fig.5).

5. Conclusions

We have introduced a tractable model for a water monolayer hydrating surfaces of proteins and, more generally, confined water. The model includes HB cooperativity and elucidates how the many-body component of the HB is important to understand the low-$T$ behavior of water. In particular, parameters estimated from the experiments suggest the occurrence of a critical point at $T \simeq 180$ K and $P \simeq 0.13$ GPa at the end of a first order coexistence line between two liquids with different densities [23, 24, 33].

The model shows that the liquid-liquid critical point affects the low-$T$ dehydration of a hydrophobic surface [29]. The cooperativity of water induces dynamic heterogeneities that reach their maximum when the cluster of correlated HBs percolate [31, 32, 68].

This heterogeneous dynamic behavior is revealed by a strongly non-exponential relaxation of the HB dynamics and by a subdiffusive translational motion of the water molecules in the hydration shell [29], as observed in hydrated proteins at low $T$ [55, 56].

The model predicts that, at low protein hydration, shell water should be characterized by two structural transitions. One associated to the macroscopic formation of HBs [35] [28], occurring at about 250 K for low-hydrated lysozyme, and another associated to the tetrahedral reordering of the HBs, at about 180 K [72]. These two structural changes are at the origin of two dynamic crossovers: the one at higher $T$ has been observed by QENS experiments on hydrated lysozyme [71, 34], and both have been measured by dielectric spectroscopy on hydrated lysozyme [72]. The pressure behavior of these crossover is consistent with the presence of the liquid-liquid critical point $C'$ at high $P$ and low $T$ [34, 73].

The large increase of density fluctuations in the vicinity of the critical point $C'$ is expected to enhance the water crystallization process. As a consequence of the expansion of ice, this process could destroy biological structures in a crowded environment, as for example in food stored at low temperature [3, 4]. It is, therefore, relevant to understand if this process is affected by confinement or if it could be controlled. Our
preliminary results [81] show that hydrophobic confinement has a strong effect of the thermodynamics of water, suppressing the density fluctuations associated to the critical point $C'$. This result suggests that by dissolving hydrophobic nanoparticles at low concentration it could be possible to control the water compressibility and the formation of large crystals.

These predictions are based on a modelization of the hydrophobic interaction that is able to reproduce a stability diagram for a coarse-grained protein. In particular, the model shows that the protein cold denaturation and the pressure denaturation can be explained as a consequence of the strengthening of interfacial water-water HBs at the hydrophobic interface and the proper account of the entropy change due to the presence of the interface [82].

Work is in progress to include other features in the model and to use it to make other predictions in different contexts. For example, we are extending the model in such a way to describe the ice formation and analyze how the crystallization process is affected by interfaces. We are also developing the generalization in three dimensions to allow the study of many layers of hydration water and to extend the investigation to the bulk case. In this way our research about protein stability will be developed also in bulk water. All together these generalizations will allow us to explore situations of possible interest in food science and biology, as for example the effect on water structure and dynamics of preservative agents such as trehalose, or antifreeze proteins, or cryoprotectants such as glycerol or dimethyl sulphoxide.

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Figure 1. Schematic representation of a water monolayer. Top view of water molecules with an oxygen atom (red) and two hydrogen atoms (blue) distributed over a surface, between two hydrophobic plates (not represented). Possible hydrogen bonds are represented by gray sticks. The total surface area is divided in equal-size square cells (dashed lines). In the tractable model adopted here, the coordinates of each molecule inside a cell are coarse-grained. A configuration of water molecules is represented by the occupancy state of the cell (local density) and the states of the four bonding indices of each molecule, accounting for the hydrogen bonds formed with water molecules in the nearest neighbor cells.

Figure 2. Density at constant pressure for a water monolayer nanoconfined between hydrophobic slabs of infinite section and separated by a distance $\delta = 0.7$ nm. Results are from Monte Carlo simulations of a system with $N = 15625$ water molecules. The pressure $P$ is calculated in GPa, the temperature $T$ is rescaled with respect to the liquid-gas critical point temperature $T_C$ of the water model, the density $\rho = N/V$ is rescaled by the volume $v_0 = r_0\delta \simeq 0.059$ nm$^3$, where $r_0 = 2.9$ Å is the van der Waals distance. (a) Isobars at high $T$, around the liquid-gas critical point $C$, marked by a full large circle. At $P < P_C \simeq 0.18$ GPa a discontinuity in the isobars denotes the coexistence of the gas (at $\rho v_0 < 0.5$) with the liquid (at $\rho v_0 > 0.5$). In the circled region for $0.02 \leq P \leq 0.06$ GPa the isobars reach the temperature of maximum density (TMD) at $\rho v_0 \simeq 0.65$ and $T/T_C \simeq 0.35$. (b) At much lower $T$, the isobars display another discontinuity in density that is evident at $P = 0.18$ GPa, and smoothly disappears, within the calculation error, for $P < 0.13$ GPa. This discontinuity denotes the coexistence of a liquid at higher density with a liquid at lower density. Where the discontinuity disappears, the system displays a liquid-liquid critical point $C'$ (large open circle, with $P_{C'} \simeq 0.13$ GPa and $T_{C'} \simeq 180$ K. In both panels errors are of the order of the size of symbols.
Water at interface with proteins

Figure 3. The histogram of rescaled density $\rho v_0$ and rescaled energy $[U + \mathcal{H}_{\text{HB}} + \mathcal{H}_{\text{coop}}]/\epsilon$ above [panels (a)-(b)] and below [panels (c)-(d)] the liquid-liquid critical point $C'$. In the one-phase region, density histogram (a) and energy histogram (b) display one single maximum. At the liquid-liquid phase separation, density histogram (c) and energy histogram (d) display two maxima separated by a minimum, corresponding to two coexisting phases with different densities and energies. Calculations are from Monte Carlo simulations of a monolayer with $N = 15625$ water molecules at $T = 175.1$ K and $P = 0.12$ GPa (a)-(b), and $T = 173.8$ K and $P = 0.13$ GPa (c)-(d). The liquid-liquid critical point $C'$ is estimated at $T_{C'} \approx 180$ K and $P_{C'} \approx 0.13$ GPa.

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Figure 4. Histograms of number $n(s)$ of clusters of size $s$ near the liquid-liquid critical point $C'$ for a monolayer with $N = 25600$ water molecules. For $T < T_{C'}$ (a) and $T > T_{C'}$ (c) the distribution of $n(s)$ is exponential, while for $T \simeq T_{C'}$ (b) it becomes approximately a power law, whose leading term is $n(s) \simeq s^\tau$ with an estimate $\tau \simeq 2$. Lines are fits of the histogram with exponential functions in (a) and (c) and with $n(s) \simeq s^\tau$ in (b).

Figure 5. Folding and unfolding of a coarse-grained protein suspended in water at different temperatures $T$ and high pressures $P$. Typical configurations in a system in two dimensions are represented for the sake of schematic description of the process. The protein is represented as a fully hydrophobic chain (in white), surrounded by water molecules (turquoise background). (a) At high pressure and high $T$, the protein is unfolded and the surrounding water has only a few hydrogen bonds (HBs) formed, represented as colored sticks. Different colors of the HBs correspond to different relative orientations of the HBs. (b) At the same pressure but lower $T$, the protein start to fold in a molten globule state. (c) At lower $T$ the protein folds, while the surrounded water has a large number of HBs. (d) At much lower $T$ we observe cold denaturation of the protein when the number of water HBs is largely reduced due to the combined action of $T$ and $P$. (e) At higher $P$ the denaturation effect is observed at higher $T$, and we observe the protein only in open configurations.

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