FRONTIERS IN WATER BIOPHYSICS

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BOOK OF ABSTRACTS
and
Conference Programme

May 23-26 2010
Trieste, Italy

"Frontiers in Water Biophysics"
May 23-26, Trieste, Italy

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Welcome

We warmly welcome all the participants and contributors to the Conference on “Frontiers in Water Biophysics”. A few words are always necessary before any activity that had an incubation period over one year: the machine-shop has been implemented by so many people and independent events that it would be quite impossible to list. All previous meetings and colleagues which dealt with water properties, water in food, pharmaceutical matter and biophysical properties of living systems and biomolecules, have contributed to some extent in developing the ideas merged in the present event. Some special thanks are deserved to the members of the Scientific Committee for their past, present and future advice.

The challenge and the difficulties of putting down the organization of such a composite meeting were always clear; it has been only after the enthusiastic response received from many colleagues and friends that our dream took the final shape of the expected “melting pot of a science broth”.

The lightening words of our mission were “Fostering mixing, learning and empathy between science cultures was and remains the main purpose of any project". As in every scientific endeavour, these concepts have supported us in pursuing ahead with the strongest consideration of achieving a fantastic goal.

We sincerely hope to see new contacts and collaborations, new ideas and new work, soon generated from the intense programme set for “Frontiers in Water Biophysics”.

Paola Pittia and Attilio Cesàro
on the behalf of the Organizing Committee

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Frontiers in Water Biophysics

Conference Programme

PLENARY LECTURES

MECHANOSENSITIVE CHANNELS – THEIR ROLE IN MODULATING WATER MOVEMENT IN BACTERIAL CELLS

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Mechanosensitive channels are gated by increases in membrane tension. In bacterial cells the channels the increase in tension is brought about by increased turgor across the cytoplasmic membrane. Turgor is generated by the accumulation of solutes in the cytoplasm that attracts water across the semi-permeable lipid bilayer. An increase in membrane tension occurs when either the solute gradient across the membrane is suddenly increased, leading to water flow into the cell, or when transient changes occur in the integrity of the cell wall (peptidoglycan and lipolysaccharide) that lowers the resistance to the transmembrane turgor. In both cases the resulting water flow poses a serious threat to cell integrity and can cause cell lysis. The problem is overcome by the activation of the mechanosensitive channels on a ms timescale, leading to the rapid transient loss of solutes and the concomitant reduction in the solute gradient. The talk will describe the structure of the channels and what is known of the gating mechanism and physiological functions of the multiple mechanosensitive channels found in bacterial cells.

WATER, WATER EVERY WHERE: PATHOPHYSIOLOGY OF MITOCHONDRIAL VOLUME REGULATION

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Regulation of mitochondrial volume is a key issue in cellular pathophysiology. Due to the high membrane permeability to water, mitochondrial volume changes closely match ion and solute transport across the inner membrane. In my presentation I will cover mitochondrial volume homeostasis that depends on (i) monovalent cation transport across the inner membrane, a regulated process that couples electrophoretic K^+ influx on K^+ channels to K^+ extrusion through the K^+-H^+ exchanger; and (ii) the permeability transition, a Ca^{2+}-dependent, regulated increase of inner membrane permeability that may be instrumental in triggering cell death. Specific emphasis will be placed on molecular advances on the nature of the transport protein(s) involved, and on diseases that depend on mitochondrial volume dysregulation.

WATER AND PHYSICAL CHEMISTRY CONCEPTS APPLIED TO THE STABILITY OF FOODS AND THEIR FUNCTIONALITIES

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Water and temperature are among the most important parameters controlling the physical properties of biological systems. Low moisture biopolymer-based systems are commonly encountered in food (e.g. cereal products) or non-food applications (e.g. packaging films). Obviously, understanding the physical basis of their quality or performance over time or as a function of their composition (water, or other added solutes) is of primary importance. Among the key parameters of their performance, one could mention their structural and chemical stability (e.g. respectively mechanical properties changes or oxidative reactions) which are generally more critical than their microbiological stability ensured by their low water activity. A polymer science approach using physical chemistry concepts based on physical state, phase transitions and molecular mobility can be applied to investigate these aspects.

Based on various examples, the role of water will be considered through different aspects. On the one hand, the effect of water on texture of cereal based products will be presented. The relations existing between the observed changes and physical state are investigated. The relations existing between the product process and its resulting physical state will be shown. The different levels of mobility remaining in the systems will be investigated by various techniques. A particular interest will be focused on two types of phenomenon associated to glass instability: i) secondary relaxations which are associated with localized motions of the amorphous material and ii) physical aging which is a structural relaxation corresponding to an thermodynamic evolution (enthalpy and volume changes) of the material towards a lower energy state.

On the other hand, a similar approach will be applied to consider the role of water on encapsulation matrices with a view to understanding the parameters controlling various functionalities or performances such as structure of the matrices, protection or release of encapsulated substances.

CONTROL OF ICE RECRYSALLIZATION IN FROZEN FOODS THROUGH THE USE OF NOVEL RECRYSTALLIZATION INHIBITION AGENTS

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Ice recrystallization in frozen foods refers to an increase in size and reduction in number of ice crystals at constant ice phase volume after initial freezing of the material. There are several operating mechanisms resulting in recrystallization, many of which are enhanced by temperature fluctuations during storage and distribution. Recrystallization generally results in a loss of quality in frozen foods due to structural changes associated with ice crystal growth. Polysaccharide stabilizers have been used for many years to help slow down the processes of recrystallization, owing primarily to enhanced viscosity in the freeze-concentrated unfrozen phase. Recently, two ingredients have also been introduced to the food industry to control ice recrystalization: ice structuring ("anti-freeze") proteins from natural plant or fish sources or through biotechnology; and propylene glycol monostearate (PGMS). We have examined the effectiveness of a water soluble extract of cold-acclimated winter wheat grass (AWWE) and of PGMS in frozen sucrose solutions and in ice cream, where ice recrystallization rapidly results in loss of textural quality. Ice crystal growth was significantly reduced with the addition of more than 0.05% total protein from AWWE in 23% sucrose solutions frozen under static conditions and temperature cycled. Ice recrystallization was reduced with increased ISP concentration until reaching a plateau after adding 0.13% total protein from AWWE. Reduction of ice crystal growth was as high as 74%, compared to the control. ISP's significantly reduced ice crystal growth in ice cream stored for a month under temperature fluctuations. PGMS (0.3%) dramatically reduced ice crystal sizes in ice cream and in sucrose solutions frozen in a scraped-surface freezer before and after temperature fluctuations. Our research suggests that PGMS directly interacts with ice crystals and interferes with normal surface propagation, similar to the mechanism of ISPs.

THERMODYNAMIC APPROACH TO OSMO-DEHYDRATION

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A thermodynamic approach is proposed to describe the osmo-dehydration treatment of apple pulp. Desorption isotherms were directly drawn from the traces of Knudsen thermogravimetry (KTG) runs.
It was found that these desorption trends of non treated and the partially osmo-dehydrated apple pulp reflect the heat-drying and the osmo-dehydration treatment, respectively, no matter the dehydration extent achieved before the KTG run. This supports the assumption that the desorption isotherms determined via KTG practically describe actual equilibrium states.
In an osmo-dehydration process the removal of water is concomitant with the migration of sugars from the syrup toward the apple pulp. As a result, the dry matter of the sample increases during the treatment, the water activity of the cytoplasm decreases and that of the hypertonic syrup increases.
Since the apple pulp may practically be referred to as a two aqueous phase system, namely, intra- and extra-cellular, the equilibrium condition imposes aW to be the same in either phase. This basic statement supports the view that the desorption trend of the non-treated apple pulp and that of the hypertonic sugar syrup are actually related to the intra- and extra-cellular aqueous phase, respectively.
The desorption trend of any partially osmo-dehydrated apple pulp remains between them and reflects the bi-phasic nature of the system.
The approach also allows a quantitative description of properties and phenomena related to the dehydration, namely, extent of the cell shrinkage, proportion between intra- and extra cellular mass, water diffusion coefficient through the cell membrane, expected sensorial differences related to the kind of dehydration process, heat-drying or osmo-dehydration performed with different sugar syrups.

STRUCTURAL SIGNATURE OF PLASTICIZATION AND ANTIPLASTICIZATION IN AMORPHOUS CARBOHYDRATES

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The last few decades have witnessed a considerable impact of the study of phase transitions in carbohydrate-based systems on the prediction and optimization of product stability and shelf life in [1,2] and pharmaceutics [3]. Such studies have in particular been aimed at the analysis of the glass transition and the role of water and other low-molecular weight compounds as plasticizers [1]. The picture which emerges from recent studies on the dynamic properties of binary glasses containing a low-molecular weight compound is more complex, however. It turns out that, in the glassy state, antiplasticization of the carbohydrate matrix impacts the local mobility and thereby the properties of the matrices in encapsulation and biostabilization [4 - 6].

In the lecture, I will provide an overview of developments in the molecular physics of carbohydrate matrices, including our own work on the elucidation of the carbohydrate nanostructure using the Positron Annihilation Lifetime Spectroscopy (PALS) facilities at the University of Bristol and Molecular Dynamics (MD) simulations. In particular, I will discuss the roles of water, glycerol and low-molecular weight carbohydrates in the plasticization and antiplasticization of amorphous matrices consisting of amorphous carbohydrate polymers [7-9]. In addition, I will report on our recent experiments combining PALS and Fourier-transform infrared (FTIR) spectroscopy as probes for molecular organization and hydrogen bonding respectively [10].

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SIMULATION AND NEUTRON DIFFRACTION STUDIES OF THE INTERACTIONS OF SMALL BIOMOLECULES WITH PROTEINS

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Neutron diffraction experiments on aqueous solutions of complex molecular solutes are notoriously difficult to interpret due to the complexity of the data obtained. Recently we have developed methods involving the combination of neutron diffraction with isotopic substitution (NDIS) experiments and molecular dynamics (MD) simulations that have permitted the interpretation of these complex experiments, and have applied them to problems not previously studied by neutron diffraction, such as the solution conformations of complex biomolecules and the interactions of large molecular ions. With these methods, it has been possible to identify components of the intramolecular conformations for several polyols. When applied to intermolecular interactions, this method has allowed the structure of aqueous solutions of electrolytes to be determined. As examples, MD simulations and NDIS experiments have found extensive aggregation in aqueous solutions of guanidinium sulfate and guanidinium carbonate, but not in guanidinium thiocyanate, as was confirmed by separate small angle neutron scattering (SANS) experiments. These findings have helped explain the underlying mechanism for the Hofmeister ordering of these species, and has explained the ability of sulfate to reverse the denaturant power of guanidinium. Using the comparison with such experiments to validate the results of MD simulations, studies of the interactions of several species with the functional groups of model peptides have been computed and used to characterize several interesting systems where small organic molecules interact with polypeptides.

WATER AT INTERFACE WITH PROTEINS

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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 [1]. Here, we review these experiments and discuss their interpretations [2,3] in relation with the anomalous properties [4] of bulk water [5] and confined water [6]. We summarize the results for bulk water and investigate the thermodynamic and dynamic properties of supercooled water at an interface [7]. We will 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 [8].

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WATER OF CRYSTALLIZATION AFFECTING THE FUNCTIONALITY OF PHARMACEUTICALS

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Introduction

Hydrate is the most commonly identified solvate within small organic drug molecules. It has been estimated that at least every third drug compound can form a hydrate [1]. Hydrate formation - or dehydration of a given hydrate - may affect dramatically the performance of the final medicinal product. Bioavailability of given compound is affected through difference in solubility and dissolution rate between anhydrate and hydrate. In the manufacturing environment, processability of these solid forms can also be affected through different habits. Hydrate can also be part of the overall IP protection strategy for a given compound. Therefore, it is of crucial importance to identify possible hydrate form(s) in the early phases of the drug development process.

Water is always present in the manufacturing of pharmaceuticals – aqueous process solutions are often needed. Later process phases may involve the use of heat. If not thoroughly understood, this may result in unstable product due to variation in hydrate content or uncontrolled hydrate formation/dehydration. Overall picture is further complicated when the conditions in GI track are taken into consideration. Bioavailability of the final drug product can be dramatically affected by the *in situ* processes involving hydrate formation in the biological environment.

These facts do underpin the need for thorough understanding of both the structural – crystallographic – aspects of hydrates and further, the mechanisms of hydrate formation and dehydration. Manufacturing of safe pharmaceuticals requires control over these phenomena.

Structural variety within pharmaceutically relevant hydrates

Various classification models have been proposed for drug molecules forming hydrates. A practical approach based on how water molecules are located in relationship to each other is commonly accepted [2]. We can identify three main classes of hydrates: i) isolated site hydrates – water molecules have main interaction with drug molecule, not with each other, ii) channel hydrates – crystal water molecules form hydrogen bonds with each other and by this means chains along a given crystal axis, and iii) ion associated hydrates containing ion coordinated water.

These packing differences can affect the processing characteristics and overall functionality of the final dosage form. Isolated site hydrates have

typically higher dehydration temperature in comparison with channel hydrates. Another structural challenge is the group of ion associated hydrates - pharmaceutical salts incorporating water molecule into crystal lattice. These three-component systems have a tendency to form series of hydrates with increasing molar ratio of water. Processing characteristics and biological performance can be affected through variation in pH.

Insight into water of crystallization during processing and performance testing of pharmaceuticals

It is important to understand the difference between the thermodynamic and kinetic aspects between a hydrate and corresponding anhydrous forms. Mechanistic understanding is needed to understand the overall performance of the final dosage form. In case of pharmaceuticals, it is not enough to study the active compound alone. The nature of water–solid interactions can be dramatically affected by excipient selection and therefore the effect of excipients in the final formulation has to be studied as well. The processing method of the final drug product also affects the anhydrate/hydrate system. Heat and mass transfer related phenomena, as well as degree of mechanical stress, are affected by unit-operation and processing equipment selection. Real time analytical methods are therefore needed to truly understand the anhydrate/ hydrate stability in the final dosage form.

Different vibrational spectroscopic approaches can provide an insight into the state of water during pharmaceutical processing [3]. The recent development of analytical tools together with increased computational power has led to the use of effective data analysis solutions. Fast analysis of multivariate spectroscopic information is a key tool for effective process analysis and by this means, increased level of product understanding. We can investigate the role of excipients, processing, and biological medium on the above-mentioned phase transitions. Using spectroscopic tools we can probe the molecular level interactions during processing and performance testing, and further, identify noninvasively the state of water from the moving material.

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WATER AS ACTIVATING AGENT IN SWELLING CONTROLLED DRUG DELIVERY SYSTEMS

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Swellable matrices are reliable controlled release dosage forms for oral route. They are produced by tabletting a drug/hydrophilic polymer mixture.
The key element for drug release control is the formation of a gel layer on the external surface of the matrix. The thickness of this layer depends on the relative position of swelling front (the sharp moving boundary where the polymer glassy/rubbery transition takes place) and the erosion fronts (the interface between the gel and the surrounding dissolution medium).
A third front, located between the previous two that separates the solid drug from the completely dissolved drug, can be identified depending on drug loading and solubility.
An analogous, although lees sharp, interface can be evidenced for the complete polymer hydration into the gel layer.
All these fronts are associated with a specific water concentration, which can be measured or calculated from various types of experiments.
In fact the drug/water and the polymer/water interactions dominates the entire phenomena of gel layer formation and drug transport.
The latter, as well as the drug release kinetics are mainly affected by drug diffusion and matrix erosion, however, it can be demonstrated that drug dissolution and polymer relaxation play an important role as well.
All the four phenomena, i.e. diffusion, erosion, dissolution and relaxation, combine to determine the drug concentration gradient that represents the driving force for drug release control. This combination generates a complex scenario where the water plays the central role.
In the present paper, all aspects relevant to drug/water and polymer/water interaction will be will be discussed in relation with drug concentration gradient in the gel layer and eventually with release kinetics.

ORAL PRESENTATIONS

DIFFUSION OF SIGNAL MOLECULES IN MICROBIAL COLONIES: SIZE EFFECTS IN QUORUM SENSING

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Introduction

The system of self-regulation of bacterial populations known as Quorum Sensing (QS) has been shown to control the expression of different phenotypes with one common feature: cell-to-cell bacterial communication is achieved through the detection of the concentration of diffusible signal molecules [1]. In the case of gram-negative bacteria, the majority of signal molecules belongs to the class of N-acyl homoserine lactones, which can freely diffuse both in aqueous solution and in bio-films, semi-solid permeable matrices [2]. The current paradigm of quorum sensing, based on a concerted action of the bacterial population, implies a concept of minimum cell density to be reached by a growing bacterial population prior to inducing the previously silent phenotype. By using equations that simply describe the physical diffusion of the signal auto-inducer molecules, produced at a constant rate by each bacterial cell, one can calculate the gradient profiles that would occur either around a single cell or at the centre of volumes of increasing size and increasing cell densities [3]. The behaviour of water-based matrices at 25 ^{o}C and viscous bio-films at colder temperatures can be compared by taking into account their different diffusion coefficients.

Experimental

Rhizobium leguminosarum is a bacterium species that does not display autoinduction, that is the AHL production rate stays constant also after quorum activation [4]. We performed experiments to assess quorum sensing activation of *R.leguminosarum.* colonies of different cell densities in agar biofilms of different sizes. Quorum activation is visualized through a reporter bacterial strain, consisting in an *Agrobacterium tumefaciens* engineered to produce β-galactosidase enzymes which cleave the X-gal substrate into galactose and 5-bromo-4-chloro-3-hydroxyindole [5]. The latter is then oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product. Cells after quorum activation are then easily spotted through the deep blue colour surrounding them.

Results

Gradient-mixing dynamics between as little as two cells lying at a short distance is sufficient for reaching the known concentration thresholds for QS. A straight line in which the highest concentrations occur is created, providing an additional signal information potentially useful for chemo-tactic responses. In terms of whole population signalling, the concentration perceived by a cell in the centre is critically dependent not only on the cell density but also on the size of the bio-film itself. This size-dependence is confirmed experimentally by following the time-dependent quorum activation in agar bio-films for Rhizobium bacteria. Bigger biofilms are activated earlier than smaller ones sharing the same cell density.

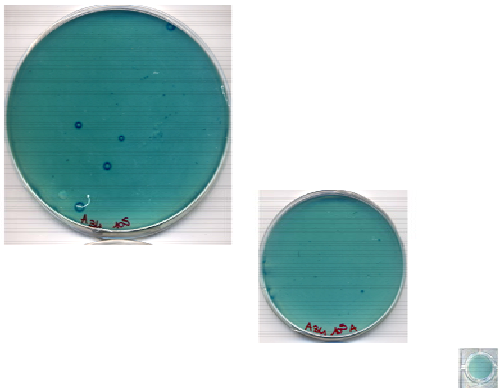

Figure 1. Agar plates of three different diameters (petri dish 150 mm Ø , petri dish 82 mm Ø, and microtiter 3.7 mm Ø) containing *Rhizobium leguminosarum* strain A34 cultures at 10^5 cells/mm and the reporter *A.tumefaciens* yielding blue color in response to the signal concentrations produced by the *Rhizobium leguminosarum* cells after a 17 h incubation.

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AQUAPORIN CHANNELS, WHEN WATER TAKES THE HIGHWAY TO CROSS THE CELLULAR MEMBRANE

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Water represents the major component of all living organisms and can be regarded as the "*solvent of life*", a prerogative underlying the dogma "*without water there is no life*". To make the cell to adapt to the surrounding environment and carry out its biological functions, driven by osmotic forces, water has to move into and out of the cell interior. For over a century, scientists studying the movement of fluid into and out of the cell struggled with a difficult biophysical question: how does water pass through the cell? Movement of water across the lipid bilayer was indicated almost as soon as the lipid bilayer was recognized as being the plasma membrane of cells, which was back in the 1920s. However, although simple diffusion of water across the lipid bilayer occurs through all biological membranes, its low velocity and finite extent soon became apparent, suggesting the existence of additional pathways for water moving through the membrane. In spite of the enormous amount of work carried out in this area, the precise and complete answer only came relatively recently, with the discovery of the aquaporins, membrane-channel proteins making the membrane 10- to 100-fold more permeable to water than membranes lacking such channels. The water conductance featured by the aquaporins is astonishing: each single aquaporin pore can conduct several billions of water molecules per second, a striking amount of water when considering that the plasma membrane surrounding the cell may possess tens to hundreds of thousands of AQP pores.

Aquaporins (AQPs) are widely found throughout nature from microorganisms to the plant and animal kingdoms. The biological importance of the aquaporin family of membrane channels was recently acknowledged by the 2003 Nobel Prize for Chemistry awarded to the discovering scientist Peter Agre [1]. Based on their selectivity in facilitating the movement of molecules across the membrane, aquaporins are grouped in *orthodox aquaporins* and *aquaglyceroporins*, the first ones being permeable only to water whereas the second ones being able to conduct glycerol and other small solutes in addition to water.

So far, thirteen different AQPs (AQP0-AQP12) have been described in mammals [1,2] where they are involved in a exceedingly high number of important functions, including (*i*) water (re)absorption in kidney, gallbladder, ear, eye as well as in the gastrointestinal and reproductive tracts, (*ii*) secretion of the gastric, intestinal and pancreatic juices, bile, saliva, sweat as

well as in pulmonary, cerebrospinal, ocular and seminal fluids, (*iii*) maintenance of brain water balance, (*iv*) skin hydration, (*v*) fat and carbohydrate metabolism, (*vi*) oxidative stress, (*vii*) apoptosis, (*viii*) cell migration, (*ix*) cell proliferation and (*x*) neural signal transduction [3]. Though appearing exceedingly rare, there exist loss-of-function mutations in human AQPs. Mutations in the gene coding for AQP2, an aquaporin expressed in the renal collecting duct, produce severe forms of non-X-linked nephrogenic diabetes insipidus (NDI) by a recessive mechanism. The few subjects that lack functional AQP1, are phenotypically normal but manifest defective urinary-concentrating function when deprived of water. Mutations of the lens aquaporin, AQP0, cause congenital cataracts. Disease-related aquaporin polymorphisms are being to be found. The recent discovery of AQP4 autoantibodies as a marker of the neuromyelitis optica (NMO) form of multiple sclerosis has allowed precise diagnosis of this disease. Valuable information on the patho-physiological relevance of AQPs is also coming from the study of AQP knockout mice.

An extraordinarily high number of aquaporin water channel proteins have been described in plant cells where they are present both in the plasma and intracellular membranes [4]. Other than facilitating the transport of water some of them are found to transport small neutral solutes (urea, boric acid, silicic acid) or gases (ammonia, carbon dioxide). Plant AQPs play multiple integrated functions being central to water relations of roots, leaves, seeds, and flowers. Unexpectedly, they have also been linked to plant mineral nutrition and carbon and nitrogen fixation. A function in stomatal regulation is also being investigated.

Microorganisms also contain AQPs. Many homologues have been identified since the 1995's discovery of the first microbial aquaporin, the *Escherichia coli* AqpZ. The physiological meaning of AQPs in bacteria remains so far mostly elusive also in consideration that prokaryotes have high surface-to-volume ratios and water permeability should not be rate limiting in volume regulation. In yeasts, aquaporin overexpression improves the freeze-thaw resistance and maintenance of the viability.

AQPs trigger exciting biotechnological interest. Incorporation of AQPs into compatibile synthetic polymers is an innovative approach for making biomimetic membranes for medical, industrial, and municipal desalting applications. Controlled AQP expression for biotechnological improvement of plant tolerance to water stress and during water stress recovery and in conditions similar to those experienced by crops in the field is an appealing approach. Bacterial AQPs may prove to be useful targets for new antibiotic development. New perspectives are open for the successful development of freeze-resistant baker's yeast strains for use in frozen dough applications. The many roles exerted by animal AQPs as well as the identification of AQP-specific inhibitors could be exploited for clinical benefits in cancer, obesity, glaucoma, immune cell dysfunction, wound healing, edematous states and epilepsy. Translational research in this area could lead to the development of

new therapeutic strategies. There is no doubt that the movement of water across aquaporin channels can help us to improve our lives, our world.

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ELECTRON TRANSFER KINETICS IN FILMS OF PHOTOSYNTHETIC REACTION CENTERS AT DIFFERENT HYDRATION LEVELS

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The central role of hydration water in protein dynamics and function is intensively studied both theoretically and experimentally [1].

In bacterial photosynthetic reaction centers (RC) the recombination kinetics of the light-induced charge separated state $P^+Q_A^-$ between the primary electron donor (P^+) and quinone acceptor (Q_A^-) is a sensitive probe of the internal RC dynamics. Hindrance of the RC dynamics (at low temperature in water-glycerol systems [2] or at room temperature in dehydrated trehalose glasses [3]) prevents stabilization of $P^+Q_A^-$, resulting in strongly accelerated and distributed charge recombination kinetics.

We have studied these effects in room temperature films of RC purified from *Rhodobacter sphaeroides*, in the presence of the detergent N,N-dimethyldodecylamine N-oxide (LDAO) or octyl glucoside (OG), as a function of the residual water content under controlled relative humidity (RH). The water content was evaluated by FTIR spectroscopy from the area of the combination band of water around 5155 cm^{-1} [3]. Different adsorption isotherms were obtained at 297 K in the presence of the two detergents. Analysis in terms of the Hailwood and Horrobin equation [4] suggests a significant involvement of the protein detergent belt in water sorption. Decomposition of the water combination band into Gaussian components [5,6] provided information on the evolution of the hydrogen bond network at different hydration levels of the RC-detergent system.

Dehydration of the RC films strongly affects the kinetics of $P^+Q_A^-$ recombination, which upon decreasing the content of residual water become progressively faster and strongly distributed over a continuous spectrum of rate constant. At RH≅10% the average rate constant, <k>, and the width σ of the rate distribution measured at room temperature are comparable with those reported for the hydrated system at cryogenic temperature [2], showing that extensive dehydration dramatically inhibits the interconversion between conformational substates of the RC. In films equilibrated at RH values lower than 10% a second strongly non-exponential kinetic phase is resolved in the hundreds of μs time-scale (Figure 1). We propose that the appearance of this faster phase, previously undetected even at cryogenic temperatures in the water-glycerol system [2], is related to the removal of

tightly bound water molecules, which play an essential role in stabilizing the primary charge separated state under physiological conditions.

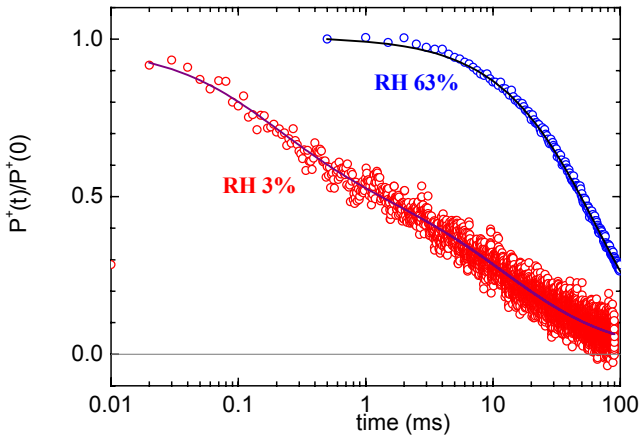

Figure 1. $P^+Q_A^-$ recombination kinetics measured in LDAO-RC films equilibrated at different levels of relative humidity. At RH=63% the continuous line represent a best fit to a single power law (<k>=15.3 s^{-1}, σ=6.6 s^{-1}). The kinetics at RH=3% is fitted to the sum of two power laws, yielding <k>= 71.1 s^{-1}, σ= 70.4 s^{-1} for the slow phase and <k>= 5100 s^{-1}, σ=5370 s^{-1} for the fast phase.

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SOYBEAN 15-LIPOXYGENASE: AN ENZYME WORKING AT THE WATER-MEMBRANE INTERFACE

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Introduction

Lipoxygenases (LOXs) are non-heme, non-sulfur iron containing enzymes that catalyze the dioxygenation of polyunsatured fatty acids containing one or more pentadiene systems to the corresponding hydroperoxy derivatives and play critical roles in plants and animals. The LOX activity is also of fundamental importance in the formation of all the volatile substances responsible for the aroma of fruit and vegetables. As yet, attempt to investigate the role of iron in this family of enzymes has not been successful. Aim of this study was to investigate the role of iron removal on the structural, kinetic and membrane binding properties of this type of enzymes. Our results clearly demonstrate that metal removal and substitution not only alter the catalytic activity of the enzyme, but also modulate the membrane binding ability of the protein. This role for iron in LOX is rather unprecedented. Overall, our findings support the hypothesis that iron removal induces a conformational change that leads to a more relaxed structure reducing the hydrophobic surface involved in membrane binding. These observations seem of particular relevance because LOX-1 is considered a paradigm of enzymes working at the water-membrane interface.

Experimental

Materials and enzymes: All chemicals were purchased from Sigma Chemical Co. LOX-1 was purified from soybean (*Glycine max* [L.] Merrill, Williams) seeds and miniLOX was produced by tryptic digestion of LOX-1 as described (1). Apo-miniLOX and metal derivatives preparation was obtained as already described (1). LOX activity was assayed spectrophotometrically as already reported in (2). Near UV-circular dichroism, and steady-state fluorescence measurements were done on a Jasco-710 spectropolarimeter. Steady-state fluorescence spectra were recorded using a Perkin Elmer LSB50 fluorimeter. Liposome preparation and FRET studies of Ca^{2+}-dependent membrane binding were done as reported in (1). Experimental data were analyzed by nonlinear regression through a hyperbolic binding isotherm, using the Sigma Plot 2000. Statistical analysis was performed by the non-parametric Mann-

Whitney U test, analyzing experimental data by means of the InStat 3 program.

Results

Here, we took advantage of miniLOX, the trimmed form of soybean LOX-1, that shows larger accessibility of the active site to the solvent water, potentially representing a more suitable protein for iron extraction and for reconstitution/substitution with vicariant metals. Thus, we succeeded in isolating an apo-form of soybean mini-LOX that allowed to identify a novel role of metal ion in enzyme binding to membranes. In particular, our functional and structural analyses clearly show that the removal of iron yields to an inactive form of miniLOX, with a looser and more relaxed conformation. Therefore, our results suggest that beyond catalytic activity, the iron ion plays a crucial role in the structural stability of the enzyme. The structural role of metal ion within the active site can result in overall conformational changes of the apo-form, that could impair not only the catalytic activity, but also the ability to work at the water-membrane interface. The association of LOXs with membranes is a complex process, which seems to be driven mainly by hydrophobic interactions between water-exposed non-polar amino acids and membrane lipids. Instead, calcium ions support membrane binding, plausibly by forming salt-bridges between acidic surface amino acids and negatively charged constituents of membrane phospholipids. The here reported 2-fold increase of the membrane binding ability of the trimmed enzyme seems to fit with this picture. Indeed, despite the absence of the N-terminal region, that contains at least one binding site for Ca^{2+}, the larger hydrophobic regions of mini-LOX compared to LOX-1 seem to sustain a significant increase of membrane affinity. More interestingly, metal removal not only alters the catalytic activity of the enzyme, but also impairs its membrane binding ability. This role for iron in LOX is rather unprecedented. Overall, our findings support the hypothesis that iron removal induces a global conformational change starting from the iron coordinating residues, and then it leads to a more relaxed structure. Thus, the presence of iron in the active site with proper coordination geometry seems to stabilize a protein conformation that shows an increase of hydrophobic residues exposed to the solvent and then is more competent for the selective binding to the membrane surface. This mechanism underlies that the increase of water entropy due to hydrophobic effect can allow a more effective substrate recognition by the enzyme.

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WATER EFFECTS ON THE INTERACTION OF LIGHT WITH MATTER AND THEIR IMPLICANCES ON FOOD APPEARANCE

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Introduction

Visual appearance greatly contributes to establish consumer preferences and to define the acceptance or rejection of a food product. The studies on the chromatic aspects of visual appearance have prevailed over other appearance attributes. Color measurement has a great interest in the food industry since it represents a non destructive tool of analysis, it is adaptable to automatisation and control of product processing. However, there are several appearance characteristics of food materials which are related to the interaction of light with matter. These properties do not involve the chromatic components of appearance, but they can influence the color perception. Some studies have been performed in relation to the appearance of materials in the areas of Architecture. César Janello in the `60 has proposed the name “cesía” to designate the modes of visual appearance produced by the transformation of the quantity and/or spatial distribution of the luminance flux that reaches the eye after being absorbed or re-emitted by an object. Caivano (1) organized cesia charts that can be used in a similar way in which color charts are employed. It is, however, surprising that these aspects have only been partly investigated in the food research areas (2). Although the Kubelka-Munk theory for turbid media has been applied to food materials (3), and gloss measurements have also been performed in relation to food quality (4), current procedures for the measurement of some of the above-mentioned properties are farm from being standarised. Water is one of the food components that most affects those light transformations which give rise to appearance properties such as transparency/opacity, specular gloss/matte characteristics with different degrees of luminosity. Water may cause appearance changes indirectly, through its effects on phase and state transitions or directly, by filling pores and eliminating interfaces in the food material.

Methodology

In the present work, appearance changes (chromatic and those derived from spatial charateristics of light distribution) in vegetable and sugar food products were analyzed as related to their water content and/or to the processing conditions. A reflectance spectrophotometer, digital camera and an adequate software were employed in order to measure appearance

(chromatic and cesia) properties. A standarised cabin with different standard illuminats at several illumination and observation angles were also used. The samples were presented over black and white backgrounds. The temperatures of phase and state transitions, and the macromolecular characteristics of the samples, as related to water content, were obtained by differential scanning calorimetry, X rays and time-resolved ^{1}H-RMN.

Results and discussion

The physical characteristics food materials under several processing conditions were determined and phase/state diagrams were constructed. In most of the products, especially those dehydrated to low water contents, water and its effects on phase and state transitions played a significant role in the establishment of appearance characteristics. Transparency changes have been associated to the glass transition temperature in glassy candies, to starch gelatinization in corn grits and to the presence of bulk water in re-humidified dehydrated vegetables. Opacity and gloss loss have been correlated to sugar crystallization in candies.

Under certain conditions a strong correlation exists between appearance changes and supramolecular and macroscopic structural changes occurring due to Bajo ciertas condiciones, existe una correlación estrecha entre los cambios de apariencia que ocurren en los sistemas analizados en función de las transiciones estructurales y supramoleculares

Conclusions

Water and its influence on phase and state transitions in food materials determine the kinetics of appearance changes during food processing and/or storage. Besides chromaticity diagrams, the construction of cesia charts allow a better characterization of food appearance. For the adequate development of innovative food products the availability of data on appearance characteristics (both chromatic and those derived from the interaction of light with matter) of the materials under study are of fundamental importance Appearance parameters can be obtained by non-destructive and precise methods, and they can be very helpful tools for the control during processing of several food products if standarised procedures are developed for their determination.

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WATER IN FOOD: THE KEY TO PERCEPTION

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Introduction

Water is inherent to food products, irrespective of the product a hard, yielding of flowing character exhibits. The presence of water provides inevitably a source of system instability: dry crispy crusts become tough, gels may show syneresis, or liquids could exhibit phase separation, with all negative sensorial consequences for consumers. In this presentation the role of water in the (changing) perception of a complex product is discussed with an emphasis on the mechanisms behind the deterioration of sensory attributes. It is shown that control over the water phase in a broad range of products is a most important property to engineer or restore product functionality. These insights are derived from experimental studies providing insights at length scales varying from nanometers to macroscopic properties, combined with the development of models that describe the migration of water according to physical mechanisms.

Control over water vapour permeability to increase crispness retention

Bread crusts act as a barrier for water. After baking a driving force for water migration will develop from the moist crumb to the dry outer layer. In due course the water content of the crust will rise and once it reaches water contents higher than 13-15% crispness will gradually get lost. Next to transport of water through the solid matrix (lamellae) of breads the vast majority of transport occurs via the gas phase. Morphological properties of the crumb and crust are therefore very relevant for this process. Employing technologies that allowed us to vary the morphology of the crumb independently of that of the crust, enabled us to determine the importance of water vapour permeability for accumulation of water in a crust. The ability to engineer these morphological properties in for example breads, resulted in a retained crispness for already a factor of eight.

Water in gels as energy dissipater to control sensory properties

During oral processing of gelled foods between tongue and palate the spatial (protein) networks become deformed. The energy exerted on these networks can be either elastically stored, be used for fracture, or become dissipated. The relative balance between these contributions determines the structural break-down properties of the product. It has been shown that sensorial mouth feel attributes, like ‘spreadable’, ‘crumbly’, or ‘creamy’, are directly related to these properties. Water flow within the gel during applied deformation is a major determinant for the part of the energy available for

fracturing of the gel. We have shown that the impact of water flow during deformation can be accurately varied by small changes in viscosity of the serum enclosed in the gel, or by varying the connectivity or deformability of the spatial (protein) network. The consequence of the fracturing process has been mapped and it has been demonstrated that in this way a number of sensorial attributes of gelled products can be directly controlled.

Water segregation to reduce salti- or sweet-ness in flowing products
Depending on the microstructure of yielding or flowing (food) products the water volume exuded by the product during oral processing can be very different. Protein based spatial networks can be obtained that release about 35-40% of the total volume, whereas subtle changes during processing may lead to different structural arrangements that do not release water at all under those conditions. It has been shown that the perception of salt or sweeteners present in these products can be boosted significantly with increasing segregation of water, opening opportunities to produce products with less salt or sugar but with comparable perception of salti- or sweet-ness. This segregated water could also be used as carrier for components that facilitate lubrication properties of the oral cavity or to specifically trigger saliva-production. The generic principles to control the extent of water segregation during (oral) manipulation will be further explored in this presentation.

PHASE TRANSITIONS IN PROTEIN-STABILISED EMULSIONS

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Introduction

Water interactions with solutes and dispersed lipid droplets play a key role in determining the physicochemical properties of oil-in-water emulsions. Phase and state transitions occurring in food emulsions during thermal treatments (e.g. freezing and thawing) are important factors for the production of stable emulsion-based food products such as ice creams, desserts, beverages and sauces [1]. Ice formation during freezing may force the oil droplets into close proximity in the restricted volume of the freeze-concentrated phase. This event can disrupt the interfacial membranes which can result in emulsions destabilisation upon ice melting [2, 3]. In the present study, phase and state transitions of oil-in-water emulsions with different water phase formulations, lipid type and interfacial compositions were studied as crucial factors affecting emulsions stability during freezing and thawing.

Materials and Methods

Coarse emulsions were prepared by blending an aqueous solution of 0 to 40% w/w sucrose and emulsifiers (Whey Protein Isolates, WPI, or Sodium Caseinate, SC, 1 and 2% w/w) at pH 7 with 10% w/w lipid phase (sunflower oil, SO or hydrogenated palm kernel oil, HPKO) using a high speed blender. These pre-emulsions were added with 0.02% w/w sodium azide and 1 mg/Kg chloramphenicol and homogenised using a two stage valve homogeniser at 850 bars. Emulsions were cooled at 20°C then frozen at -10; - 20 and - 40°C with two different cooling rates obtained by using a blast freezer or a chest freezers. Phase/state behaviour of the continuous and dispersed phases was studied by differential scanning calorimerty (DSC) [2]. Emulsion stability was followed by DSC, gravitational separation and mean particle size analysis [3, 4, 5].

Results and Discussion

The absence of sucrose in emulsion formulations led to emulsion instability. Emulsions formed a self-supporting cryo-gel type structure or a partially coalesced bulk solid fat phase. The presence of sucrose greatly improved emulsion stability even at the lowest concentration. Both SC and WPI

showed good stabilisation properties but instability was detected when SC was used at 2% w/w in the presence of SO as carrier as a result of depletion flocculation. The cryoprotective effect of sucrose on the emulsion was confirmed from improved freeze/thawing stability.

Conclusions

The results of this study could provide guidelines for improving freeze-thaw stability of protein-stabilised food emulsions.

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WATER STRUCTURAL AND DYNAMICAL PROPERTIES CHANGES INDUCED BY HOMOLOGOUS DISACCHARIDES: A BIOPHYSICAL STUDY

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The innovative research field on glass-forming systems having bioprotective action has been boosted from a growing demand from many biotechnological industrial research laboratories and processing industries to improve the quality and safety of high added-value products, as well as to establish new approaches to improve existing pharmaceutical treatments. Recently homologous disaccharides, and trehalose to a greater extent, have received a growing attention, both from the academic and applicative point of view. The interest is due not only to their effectiveness as bioprotectant systems but also to their nature of glass-forming systems. Light and neutron scattering [1,2] allows to unravel some features which are responsible for the higher effectiveness of trehalose in comparison with sucrose and maltose. It emerges that disaccharides promote, with a different strength, a destructuring effect on the hydrogen bond network of pure water, pointing for trehalose a higher frustration capability on ice formation together with a greater slowing down effect on water dynamics. Furthermore, the experimental findings are consistent with the existence of a direct connection between the fragility degree of the ensuing glassy matrix and the bioprotective effectiveness.

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MOLECULAR DYNAMICS OF SOLUTE AND SOLVENT IN AQUEOUS SOLUTIONS OF SUGARS BY BROADBAND DEPOLARIZED LIGHT SCATTERING EXPERIMENTS

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Depolarized light scattering (DLS) spectra of aqueous solutions of carbohydrates (glucose and trehalose) were measured in a wide frequency range, going from 0.01 to 1000 cm^{-1}, combining the use of a double monochromator and a multipass Fabry-Perrot interferometer [1,2]. The observed spectral features relates to dynamical events which modulate the anisotropic polarizability of the system in temporal scales ranging from fractions of to hundreds of picoseconds (1 ps = 10^{-12} s) [1-3].

The spectral profiles obtained in a range of temperatures and concentrations, were analysed considering the susceptibility formalism and the presence of three distinct relaxation terms was ascertained [1-4]. The slow process has characteristic relaxation times of hundreds of picoseconds and strongly depends on temperature and concentration. This is interpreted in terms of the Stokes-Einstein-Debye rotational diffusion of the sugar molecule and is discussed in comparison with the findings of other related techniques.

The spectral susceptibilities also evidence the existence of two distinct relaxation processes at picosecond timescales due to the solvent dynamics [4]. From a physical point of view these dynamical events involve molecular rearrangements connected to the rapid hydrogen bond restructuring within the system [3,4]. The fast process (fractions of picoseconds) is attributed to the dynamics of bulk water molecules scarcely affected by the sugar, while the slow one (few picoseconds) to local rearrangements of water molecules strongly influenced by the solute (hydration water). Experimental data of trehalose solutions were quantitatively interpreted considering that the dynamics of proximal water molecules is 5-6-fold slowed down compared to the bulk and that each sugar molecule basically restricts the motions of 16-18 water molecules, these latter being the solvent molecules directly hydrogen bonded with the solute [4]. These findings represent an experimental counterpart of recent theoretical results and shed some light about the influence of carbohydrates on the fast restructuring dynamics of water molecules.

The studied sugars besides representing an important class of compounds due to the role they play in biological and pharmaceutical processes, may serve as a model to gain important insights on basic solvation properties of more complex systems in aqueous media. In this respect the same experimental approach was lately applied to other biorelevant compounds, such as model peptides and proteins. These results will be also considered.

Overall, broadband DLS experiments were proven useful to improve our knowledge on dynamical properties of complex hydrogen bonding systems.

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MOLECULAR DYNAMICS SIMULATION AND RAMAN SCATTERING STUDY OF LYSOZYME IN DISACCHARIDE AQUEOUS SOLUTIONS

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Most of proteins are sensitive to moderate changes in temperature, pressure, hydration level, ionic strenght or pH. Therefore, various additives are used in the pharmaceutical, cosmetics or food industries to prevent their partial or even complete denaturation, which might occur during industrial conservation processes such as lyophilisation (freeze-drying) as a result of low temperatures and dehydration stresses. Disaccharides ($C_{12}H_{22}O_{11}$) like trehalose and sucrose have been increasingly studied in the last two decades because of their high efficiency in preserving biological molecules. These sugars are synthesized in large amounts by organisms that can withstand severe conditions of high/low temperatures and/or almost complete dehydration during long periods of time and then resume their normal activity. In spite of numerous experimental and numerical studies, the molecular mechanisms responsible for the enhanced efficiency of trehalose, and more generally, for the protection of proteins by sugars are still not fully understood. Many hypotheses have been proposed (formation of a glass, replacement of the protein hydration shell, etc.), but none of them is fully satisfying, since it generally covers only a limited range of temperature and/or hydration level.

Molecular dynamics (MD) simulations and Raman scattering experiments of hen egg white lysozyme in presence of three homologous dissacharides - namely trehalose, sucrose and maltose - have been performed to shed light on the influence of these sugars on some structural, vibrational and dynamical properties of proteins[1,2,3]. The sugars are found preferentially excluded from the surface of lysozyme in line with the preferential hydration hypothesis[4]. Furthermore, an important strengthening of the hydrogen bond (HB) network of water is induced by the presence of sugars and appears as a blue shift in the low frequency range [0-350] cm^{-1} of the vibrational density of states (VDOS) of water[3]. This might partly explain the protection of sugars against the thermal denaturation of lysozyme observed experimentally by Raman scattering[5]. A privileged interaction of trehalose with water is observed only up to a concentration of 50 weight %[1,2], in contrast to binary sugar/water solutions where trehalose was systematically

the most hydrated sugar[6]. This result is interpreted as a competition between sugars and lysozyme to bind to water molecules. Finally, a significant slowing down of protein dynamics is found and stems from the percolation of the HB network of sugars[1].

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UNIVERSAL RELAXATION BEHAVIOUR OF WATER IN BIOLOGICAL MATERIALS AND ITS IMPLICATION FOR BULK WATER

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General introduction

This presentation consists of two parts. In the first part we discuss the universal behaviour of water in biological materials and in the second part we discuss possible implications of these findings for bulk water.

Universal behaviour of water confined in biological materials

From dielectric relaxation studies of a wide range of water containing biological materials, such as proteins, lipid membranes, food materials etc., and even aqueous solutions it is clear that the low temperature (≤ 180 K) relaxation behaviour of the interfacial water is very similar in all these systems provided that the water content is high enough to allow the formation of smaller water clusters (see e.g. Ref. 1). The reason for this universality is that the interfacial water has reached its glass transition temperature and consequently the observed low temperature relaxation is not the viscosity related structural (α) relaxation, but rather a local secondary (β) relaxation, determined by hydrogen bond relaxations. An apparent crossover in the temperature dependence of the most pronounced relaxation process occurs close to the glass transition of the interfacial water (or in some cases at the glass transition of the whole system) when the local β-relaxation decouples from the cooperative α-relaxation. Below, it is argued that a similar crossover behaviour is expected for supercooled bulk water when the build-up of an ice-like tetrahedral network structure is completed.

The glass transition and relaxation behaviour of bulk water

Due to the widespread importance of water and the difficulty to study it in the so-called no man's land between 150 K and 235 K, deeply supercooled (or glassy) bulk water is currently heavily debated. It speculates about its properties from extrapolations of experimental data on bulk water above 235 K and below 150 K, computer simulations, as well as experiments on confined water for which the finite size effects may prevent crystallisation in the no man's land. In this presentation the latter approach is used and the dynamical properties of supercooled bulk water are tried to be understood from experimental results on interfacial water and comparisons with bulk water. We propose [2] that the commonly accepted glass transition

temperature of bulk water at about 136 K is not a true glass transition, but rather due to the freezing in of a local secondary β-relaxation, as shown in Fig. 1. The true glass transition should instead be located close to the critical temperature $T_s \approx 228$ K when the build-up of an ice-like tetrahedral network structure is completed, as also illustrated in Fig. 1. The proposed interpretation is the simplest one that is able to explain many of the peculiar properties of supercooled water. For instance, it explains (a) the weakness of the calorimetric feature at 136 K, (b) why bulk water crystallizes immediately slightly above 228 K, (c) the unusual density behaviour below 277 K, and (d) the similarities to ice at low temperatures. Furthermore, the model is closest to the findings for confined water and aqueous solutions, and no speculative liquid-liquid transition or fragile-to-strong transition is needed to explain the properties of supercooled and glassy water.

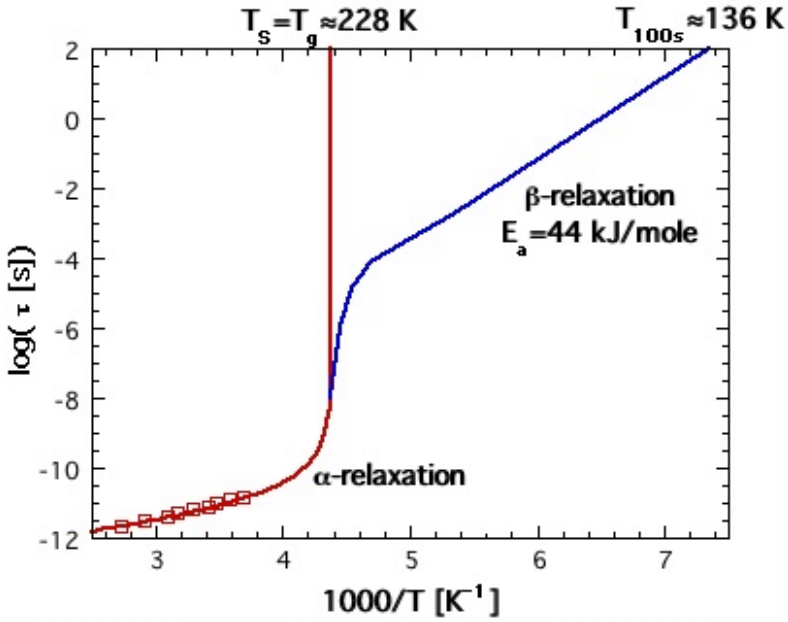

Fig. 1. A possible relaxation scenario for supercooled water, with the dielectric relaxations times measured by Rønne *et al*. [3] included. The relaxation time of the viscosity related α-relaxation goes rapidly to infinity around the critical temperature of 228 K. Below this temperature the slowest relaxation process is the more local β-relaxation, which behaves as the β-relaxation in confined water and reaches a relaxation time of 100 s at 136 K. Thus, below 228 K water is macroscopically a solid, although relaxation and diffusion processes can still take place, as in ice.

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POLARIZED HYDRATION SHELLS AROUND PROTEINS AND THE ELECTROSTATICS OF THE WATER/PROTEIN INTERFACE

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Many properties related to biological activity of enzymes are driven by electrostatics. These include redox activity, substrate binding, optical and spectral properties, etc. All these properties are traditionally understood within the linear response paradigm suggesting that the water shell is polarized in proportion to the electric field of the solute. The hydration shell of a protein shows a quite different behavior. We found from numerical simulations of a number of redox [1,2] and photosynthetic [3] proteins that their hydration shells are spontaneously polarized by the extended protein/water interface to carry a gigantic dipole moment with the average magnitude of several hundred debyes (Fig. 1).

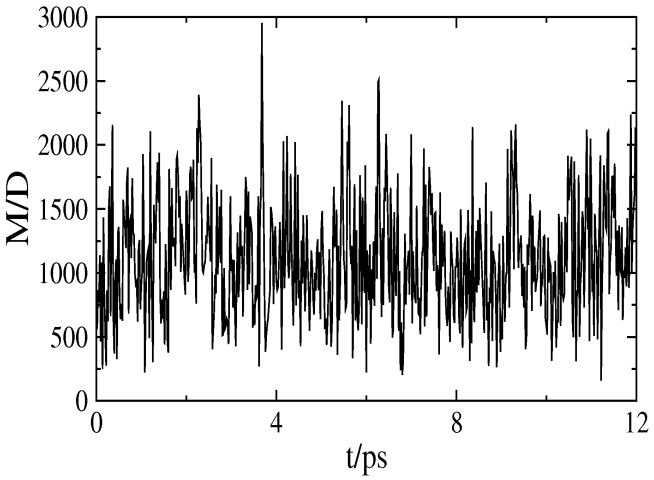

Figure 1. Trajectory of the dipole moment of the first solvation shell of plastocyanin from MD simulations.

Thermal fluctuations of this polarized water shell are reflected in the non-Gaussian statistics of the electrostatic potential measured within the protein or at the protein/water interface. The linear-response relation between the average electrostatic potential and its variance breaks down, with the breadth of the electrostatic noise far exceeding the expectations of the linear

response theories. The dynamics of these non-Gaussian electrostatic fluctuations are dominated by a slow (1 ns) component which freezes in at the temperature of dynamical transition of proteins. We therefore observe that the temperature dependence of the variance of the electrostatic potential matches that of atomic displacements typically observed by neutron scattering.

The dynamics of the hydration shells turns out to be significant for the overall performance of energy chains in respiration and photosynthesis. Fast reactions in bacterial photosynthesis show a severe breakdown of ergodicity on the time scale of primary charge separation, with the consequent inapplicability of the standard canonical prescription to calculate the activation barrier [3]. Common to all photosynthetic reactions studied is a significant excess of the charge-transfer reorganization energy from the width of the energy gap fluctuations over that from the Stokes shift of the transition. This property of the hydrated proteins allows the reaction center to significantly reduce the reaction free energy of near-activationless electron hops and thus raise the overall energetic efficiency of the biological charge-transfer chain. It is suggested that the experimentally observed break in the Arrhenius slope of the primary recombination rate, occurring near the temperature of the dynamical transition in proteins, can be traced back to a significant drop of the solvent reorganization energy close to that temperature.

Finally, it is suggested that the appearance of the polarized water clusters around proteins can be detected by means of THz dielectric spectroscopy [4]. The application of standard dielectric theories to protein solutions results in the decay of dielectric absorption with increasing protein concentration. In contrast, experiment shows a maximum of the absorption coefficient at the protein's volume fraction of about 1%. The appearance of "elastic ferroelectric cluster" around proteins [5], extending 10-15 $\mathring{A}$ into the bulk, allows one to explain the experimental observations [4].

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SUPERCOOLED WATER DYNAMICS IN HYDRATED PROTEINS AND BIOPOLYMERS: THE ROLE OF SECONDARY β-RELAXATION

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Introduction

Dynamic properties of hydrated proteins change in vicinity of some temperature. The change of dynamics has been likened to glass transition. However, a complete understanding of the conformation fluctuations of hydrated proteins and their relation to the dynamics of the solvent is still not available, possibly due to the protein molecules being more complex than ordinary glass-formers. For this reason, we turn our attention to the experimental findings of the dynamics of mixtures of water with simpler glass-formers (small molecules and polymers). Two major relaxation processes are observed in these aqueous mixtures. One is the structural α-relaxation of the hydrophilic glass-former hydrogen bonded to the water, which is responsible for glass transition. The other one is the local secondary β-relaxation of water in the mixture. Remarkably, these two relaxation processes in aqueous mixtures have analogues in hydrated proteins with the same properties. The conformation fluctuations of the protein and the relaxation of the solvent in hydrated proteins respectively behave like the α-relaxation and the β-relaxation of water in aqueous mixtures. At low temperatures, the Arrhenius activation energy of the solvent relaxation time in a hydrated protein is almost the same as that of the β-relaxation in aqueous mixtures in the glassy state. The solvent relaxation time changes its temperature dependence when crossing the 'glass' transition temperature of the hydrated protein defined as the temperature below which the conformation relaxation time exceeds some very long time. This behaviour is exactly analogous to the change found in the β-relaxation of water in aqueous mixtures at the glass transition temperature of the mixture. Furthermore, the same was found in the faster component of binary mixtures of two van der Waals glass-formers, where there is the absence of hydrogen bonding. The experimental data of these mixtures are amenable to theoretical explanation. Since the properties of hydrated proteins, aqueous mixtures, and the mixtures of van der Waals liquids are similar, we transfer the theoretical understanding gained in the study of the last system to the other two systems [1]. In this presentation, we review all the aforementioned experimental facts together with new experimental data of two systems: (1)

hydrated bovine serum albumin (BSA) and (2) water/glycogen at various levels of hydration. The new data confirm the general behaviour.

Experimental

Bovine Serum Albumin (BSA) was purchased from Sigma and it was used without further purification. The appropriate amount of distilled and deionized water obtained from an ultrapure water product (Millipore, MILLI-Q Lab.) was added to the BSA to form 20 and 40 %wt solution. Dielectric measurements were performed in the frequency range between 2mHz and 1.8 GHz at temperatures between 80K and 270K. Glycogen (Polyglumyt@, ACRAF) was mixed with water to form solutions with concentration from 23 to 77%wt. Mixtures were studied by dielectric spectroscopy in the frequency range from 10 mHz to 10 MHz and from 350 K down to 100 K.

Results and Discussion

Dielectric spectra for BSA/water and glycogen/water mixtures showed two major relaxation processes originating from water in the hydration shell and the hydrated glycogen or BSA. All these relaxation times of water in the hydration shell have Arrhenius T-dependence from 100 K and up to about 200 K. Above 200 K, the relaxation times of water in the hydration shell of BSA change to a stronger non-Arrhenius T-dependence. The relaxation times of the hydrated BSA, due to cooperative motion of water physically coupled to the BSA, show a Vogel-Fulcher T dependence and reach the range of 10^2–10^3 s at 200 K, suggesting that it is the structural α-relaxation and that there occurs the glass transition of the hydrated BSA, as shown by Kawai et al. [3] by heat capacity and enthalpy relaxation rate measurements.
An outstanding feature of the dielectric data is the change of the temperature dependence of the relaxation time of water in the hydration shell of BSA when crossing the glass transition temperature. This property, also found in aqueous mixtures, mixtures of van der Waals glass-formers, and even in a variety of neat glass-formers, indicates that the local structural dynamics of protein and water in the hydration shell are coupled, and the glass transition of solvated protein is governed or 'slaved' by the β-relaxation of the solvent. Consequently, the glass transition temperature of the solvated protein depends on the solvent as observed by others [4]. We expect the theoretical advance made will stimulate interest from researchers in food science, life science, and pharmaceutical science.

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WATER AND TREHALOSE: HOW MUCH DO THEY INTERACT WITH EACH OTHER?

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Introduction

The observation made by early naturalists that some organisms could tolerate extreme environmental conditions, and "enjoy the advantage of real resurrection after death" [1] stimulated research that still continues to this day. Cryptobiosis, the ability of an organism to tolerate adverse environments, such as dehydration and low temperatures, still represents an unsolved and fascinating problem. It has been shown that many sugars play an important role as bioprotectant agents, and among the best performers is the disaccharide trehalose. The current hypothesis links the efficiency of its protective role to strong modifications of the tetrahedral arrangement of water molecules in the sugar hydration shell, with trehalose forming many hydrogen bonds with the solvent. Here we show, by means of state-of-the-art neutron diffraction experiments combined with EPSR simulations, that trehalose solvation induces very minor modifications of the water structure. Moreover the number of water molecules hydrogen bonded to the sugar is surprisingly small.

Experiment and data analysis

Neutron diffraction experiments with isotopic H/D substitution [2] have been performed on trehalose aqueous solutions, at two concentrations and standard T,P conditions. We have used the SANDALS diffractometer [3] installed at the ISIS pulsed source [4]. Data have been analyzed applying the EPSR Monte Carlo code [5, 6, 7] in order to refine data in Q space and obtain accurate radial distribution functions, relative to the relevant molecular sites. We have identified four distinct sites on the trehalose molecule, which are in principle good candidates for HB with water: these are labeled according to Fig. 1 (circled sites). We will show and comment radial distibution functions relative to hydration of these sites, along with the water-water ones.

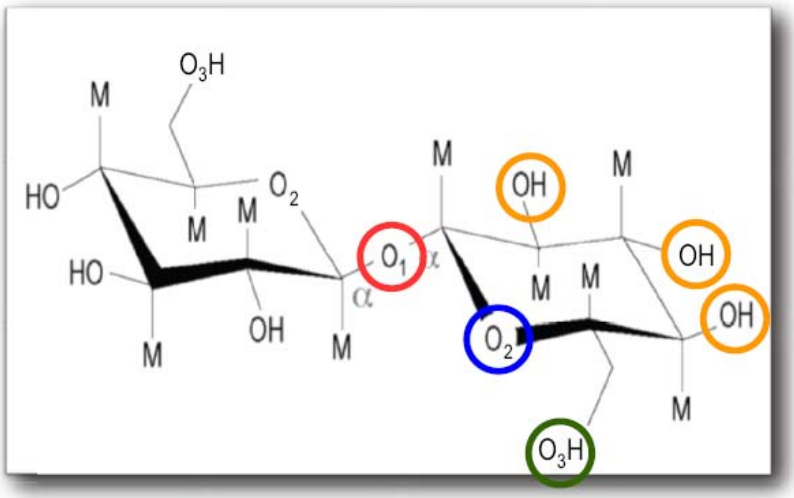

Fig.1 Trehalose is a disaccharide formed by a a 1-1 glycosidic bond between two glucose molecules, at site O1. M sites label not exchangeable hydrogens and H the exchangeable ones; OH labels the hydroxyl groups bonded to each ring carbon (three per ring, circled in orange); O2 the saccharide ring oxygens (one per ring, circled in blue); and O3H are the hydroxyl groups ending the CH2OH side chains (one per ring bonded to C6 site and circled in green).

Conclusions

Our study suggests that H bonding between trehalose and water is surprisingly limited, given the large number of sites on the trehalose molecule that in principle could interact with water. In addition, the influence of trehalose solvation on the water network is also surprisingly small. These conclusions are at odds with the accepted view that considers strong water-trehalose interactions as a key fact to explain the ability of trehalose to protect biological molecules from extreme temperatures and dehydration. The present experiment is the first direct and thorough study of the microscopic structure of water-trehalose solutions.

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WATER-STRUCTURE EFFECT OF SUGAR STEREOCHEMISTRY, AND ITS IMPACT ON PROTEIN THERMAL STABILITY

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Introduction

Water, the ubiquitous and powerful solvent, is probably the most unique and anomalous as well. Its peculiar structure and properties are most likely among the essential components of life formation, survival and evolution. The structure of liquid water and its modulation by solutes have far-reaching consequences in numerous fields of science and technology. In particular, the aqueous-solution stability and functionality of macromolecules, particularly of proteins, are essential in many fields of life sciences, biotechnology and food engineering. These attributes may be modulated to obtain better or worse solubility (salting in/out), by the addition of ionic [1] or non-ionic [2,3] cosolutes (or cosolvents), as in the famous Hofmeister series [1]. Some nonionic cosolutes, like sugars [4-6], act as protein stabilizers (soluting-out agents) by elevating their denaturation temperature, while others (soluting-in agents), such as urea, act as denaturants and decrease the denaturation temperature [7]. It is commonly accepted that the protein is preferentially hydrated in the presence of saccharides [4]. However the role of the sugar hydration in causing this preferential hydration, and the relationship between the stereochemical structure of the sugar and its hydration, or the effect on macromolecules in aqueous solution are still not clearly understood. A recent study of our group has shown a relation between sugar hydration numbers and poly n-isopropylacrylamide cloud point [8]. The objectives of the current work were to obtain molecular level insights on sugar hydration, its relation to sugar stereochemistry, and its consequential effect on protein thermal stability.

Experimental

We combined mathematical modelling and laboratory experiments. Using imitation modelling and atomistic Monte-Carlo molecular simulation based on the TIP5P water force field [9] and the GROMOS force field for carbohydrates [10], we studied stereochemical structure effects on saccharide hydration, and compared the in-silco results to density and ultrasound velocity measurements in dilute aqueous solutions of three isomeric aldohexoses: glucose, galactose, and mannose. We also used

isothermal titration micro-calorimetry (ITC) and differential scanning micro-calorimetry (DSC) to study sugar effects on a model protein in aqueous solutions.

Results

Our model predicted that the better a template a sugar molecule is, for cooperative hydration, the higher its hydration number will be. The atomistic simulation was developed and used to quantify the compatibility of each aldohexose molecule with ideal tetrahedral water structure as embodied in hexagonal ice (Fig. 1). We postulated that the better the compatibility, the better the template the sugar will be for cooperative hydration in the liquid state.

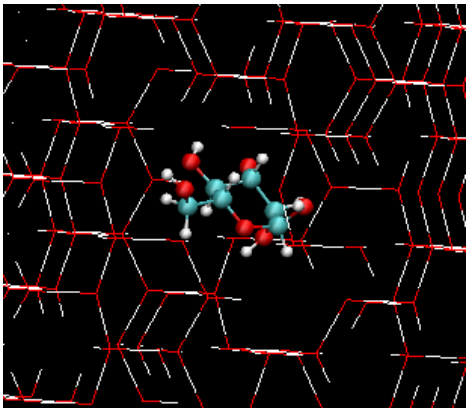

Figure 1. A snapshot from atomistic simulation of glucose in "hexagonal ice".

By energetic and spatial compatibility criteria, galactose was revealed to be most compatible, followed by glucose then mannose. The same ordering was found by ultra accurate density and sound velocity measurements in liquid aqueous solutions, indicating that galactose was most hydrated, followed by glucose, then mannose. We found the same order of effect also in aqueous sugar and protein solutions. The higher the hydration number, the stronger was the protective effect against protein denaturation. These results support our proposed templating concept, as the basis of explaining kosmotropic non-ionic solute effect on water structure, and may have an important role in the mechanism of the saluting-out effect of biopolymers by such cosolutes.

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RELAXATION IN PROTEIN SURFACE WATER NEAR THE RESOLUTION LIMIT OF NEUTRON SPECTROSCOPY

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Thin layers of water on biomolecular and other nanostructured surfaces can be supercooled to temperatures not accessible with bulk water. Chen *et al.* [1] suggested that anomalies near 220 K observed by quasi-elastic neutron scattering can be explained by a hidden critical point of bulk water.
Based on more sensitive measurements of water on perdeuterated phycocyanin, using the new neutron backscattering spectrometer SPHERES, and an improved data analysis, we present results that show no sign of such a fragile-to-strong transition. The inflection of the elastic intensity at 220 K has a dynamic origin that is compatible with a calorimetric glass transition at 170 K. The temperature dependence of the relaxation times is highly sensitive to data evaluation; it can be brought into perfect agreement with the results of other techniques, without any anomaly [2].
By the time of this conference, complementary results from a simpler model system, aquous LiCl solution, shall also be available [3,4].

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WATER AND POLYMER DYNAMICS IN HYDROGELS BASED ON POLY (HYDROXYL ETHYL ACRYLATE) – CO – POLY (ETHYL ACRYLATE) COPOLYMERS

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Hydrogels based on hydrophilic cross-linked polymers may absorb large amounts of water preserving their integrity. For several of their biomedical applications it is essential that mechanical stability is improved. One way to achieve that is by copolymerizing the hydrophilic polymer with a hydrophobic one. In this work we employ two dielectric techniques, thermally stimulated depolarization currents (TSDC) and broadband dielectric relaxation spectroscopy (DRS), to investigate water and polymer dynamics in hydrogels based on random copolymers of poly (hydroxyl ethyl acrylate) (PHEA) and poly (ethyl acrylate) (PEA) polymers, at the ratios of 90/10, 70/30, 10/90 PHEA/PEA components. PHEA is a hydrophilic polymer already employed in several biomedical applications, whereas PEA is a hydrophobic one. Additional information on the organization of the adsorbed water molecules within the copolymers is provided by gravimetric equilibrium sorption isotherms (ESI) measurements.

For the dry copolymers the dielectric results indicate that the systems rich in PHEA component are dynamically homogeneous, whereas for the 10/90 PHEA-co-PEA copolymer dynamic heterogeneity has been observed. The glass transition temperature, Tg, of the homogeneous copolymers decreases with increasing PEA content implying the plasticization action of the PEA phase. The secondary relaxation γ becomes faster with increasing PEA content. The conductivity is reduced with increasing PEA content.

The ESI data (Figure 1) are well described by the Guggenheim, Anderson and De Boer (GAB) model. For relative humidity, RH, higher than about 55% clustering of water appears in the form of water layers around the hydration sites. Water uptake is mostly controlled by the PHEA phase.

The results of dielectric DRS and TSDC measurements at several levels of relative humidity indicate that the segmental (α) relaxation associated with the glass transition is strongly plasticized by water, leading to a remarkable decrease of the glass transition temperature Tg. Conductivity increases with increasing water content.

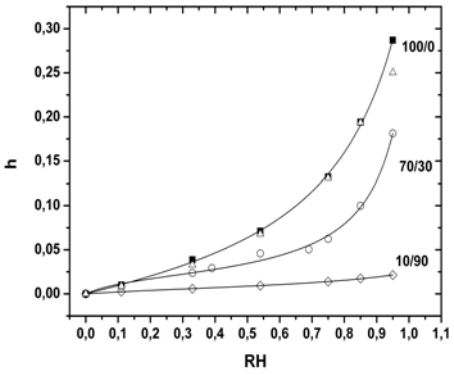

Figure 1. Water content h against relative humidity RH for the samples indicated on the plot. The lines are fittings by the GAB equation.

As an example of dielectric measurements, Figure 2 shows results for the frequency dependence of the imaginary part of the dielectric function, ε", for a copolymer in the region of the secondary relaxations at several water contents. We observe that the β_{SW} relaxation process emerges with the first traces of adsorbed water and is accelerated with increasing water content.

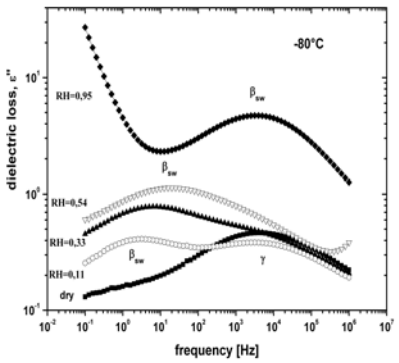

Figure 2. Dielectric loss, ε", vs frequency, f, for the 90/10 PHEA-co-PEA copolymer at several levels of relative humidity indicated on the plot.

Analysis of the various relaxation processes in terms of average relaxation time, dielectric strength and shape, by fitting model functions to the data, provides quantitative information on the modifications of molecular dynamics by the hydration process. An interesting result of the analysis is that the secondary relaxation β_{SW} exhibits different characteristics for RH > 55% (the critical RH for the creation of water clusters in ESI measurements).

Aknowledgements
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SOLUBILITY AND STABILITY OF β-CYCLODEXTRIN INCLUSION COMPLEX WITH TERPINEOL

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Introduction

Cyclodextrins are able to form inclusion complexes with hydrophobic molecules. Complex formation in solution is a dynamic equilibrium process and the stability of the complex can be described in terms of the equilibrium constant. α-Terpineol is a terpenic alcohol used as perfume and antifungal [1]. Since it is poorly soluble in water the complexation can increase its solubility. In this work, inclusion complexes of α-Terpineol (Terp) and β-cyclodextrin (BCD) were prepared. Physical properties and stability of the complexes were evaluated as a function of water content and storage time. The solubility of Terp was looked at by phase solubility studies.

Experimental

The inclusion complexes of Terp in BCD were prepared by coprecipitation and freeze-drying method, using different Terp/BCD molar ratios (1:1, 1:3). DSC was used to measured the glass transition temperature (T_g) an to confirm the formation of the complexes using the disappearance of the melting signal of Terp as a proof of real inclusion [2].

Sorption isotherms of BCD and of the complexes were obtained by the isopiestic method at 25°C [3]. The water content of the samples was determined as a function of time of storage until reaching the equilibrium condition.

For phase-solubility studies an excess of α-Terpineol was mixed in an aqueous solution containing increasing amounts of BCD under stirring at different temperatures (27 to 55°C) [4]. The amount of Terp in the solution was determined spectrophotometrically at 240 nm.

Results

The formation of the inclusion complex Terp/BCD was confirmed by DSC. Terp was completely encapsulated in BCD at the preparation conditions and studied molar ratios. The complex was stable at different relative humidities (RH) (11-97% RH) for at least 250 days of storage at 25 °C. The sorption isotherms for the Terp/BCD systems, in 1:1 and 1:3 molar ratios, were obtained and compared with the original cyclodextrin. The presence of Terp greatly modified the BCD sorption curves being the amount of adsorbed

water smaller in the combined systems. Since the “driving-force” of the complex formation is probably related with the substitution of high-enthalpy water molecules from the inner CD cavity by the guest molecule [5], these results confirm the encapsulation of the ligand at any of the studied molar ratio

Terp/BCD systems evidenced a well defined glass transition confirming their amorphous characteristics. For a certain water content, the T_g values dropped when increasing the Terp proportion in the CD matrix. This indicates that in the presence of Terp structural modifications take place affecting the physical characteristics of the matrix.

The plots of Terp concentration in the solution vs BCD concentration showed a linear trend confirming the 1:1 stoichiometry for the complex. Phase solubility studies were carried out at different temperatures and the correspondent equilibrium constants were calculated. The constants decreased with increasing temperature, as expected for an exothermic process. The Van’t Hoff plot (LnK_c vs 1/temperature) was a linear function and allowed to calculate thermodynamic parameters: enthalpy and entropy. The negative value of the enthalpy and of the Gibbs free energy demonstrated that the process is exothermic and spontaneous. Since complexation gives more ordered systems, the negative value obtained for the entropy change is another confirmation of the encapsulation of Terp.

The limited water solubility of Terp could be overcome by the formation of BCD inclusion complexes, which are stable at different storage conditiosns (RH 11-97 and 25°). The obtained phase solubility data are useful if Terp/BCD is required as water soluble additive for special products.

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WATER IMAGING: PHYSICAL CONCEPTS AND CURRENT APPLICATIONS IN THE PRE-CLINICAL AND CLINICAL PRACTICE

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Introduction

Magnetic resonance imaging (MRI) is a routine diagnostic tool in modern clinical medicine[1]. MRI has many advantages as a diagnostic imaging modality. It is noninvasive, delivers no radiation burden, and has excellent (submillimeter) spatial resolution. Soft tissue contrast is superb and MRI readily yields anatomical information. Moreover, there are many techniques that can provide contrast in MRI resulting in markedly different images from the same anatomical region. The most common used element for MR techniques is the hydrogen atom (1H) due to its high sensitivity that emerges from its magnetic properties and its high natural abundance in living organisms. Therefore, MR methods are often based on detecting proton signals of water and the influence of the environment on these signals. Such environmental influences include the local proton content, relaxation behaviour, tissue viscosity, flow, diffusivity, chemical composition and others.

Water as MRI imaging probe

Several clinical protocols were developed providing information not only on local anatomy with high resolution but also on blood flow, blood volume, tissue perfusion and cellular connectivity using water as imaging probe.
In particular major pathways in water translation processes can be identified in MRI images reporting on apparent diffusion coefficient (ADC) in a tissue[2]. Indeed water often do not diffuse along the space at the same rate but, in the presence of biological anisotropic structures, it display preferential motion directions. The patients presenting tissue abnormalities that change the water distribution in various cellular compartments, or change the ability of water to pass through cell membranes, show altered ADC values. Therefore the MR diffusion measurement offers a unique opportunity to obtain information about morphology otherwise inaccessibile to conventional MR imaging methods. Some examples of clinical applications of ACD maps will be given.

Paramagnetic contrast agents and their interactions with water

In order to increase the contrast-to-noise ratio in the clinical images sometimes exogenous contrast agents [3] are used to modulate the magnetic relaxation properties (longitudinal relaxation T_1, transverse relaxation T_2) of water protons in the tissues. These agents utilize paramagnetic metal ions and are evaluated on the basis of their ability to increase the relaxation rate of nearby water proton spins in dependence on the concentration of agent administered (i.e. relaxivity). Gadolinium(III), with its high magnetic moment and long electron spin relaxation time, is an ideal candidate for such a proton relaxation agent and is the most widely used metal center for such purposes [4].

However free Gd(III) is toxic and must therefore be administered in the form of stable chelate complexes that will prevent the release of the metal ion in vivo. The interaction of the Gd(III) complexes with water molecules, the extension of the solvatation shell as well as the dynamic of water exchange in the proximity of the paramagnetic complex are all important factors affecting the proton relaxation properties of water protons.

For the above reasons a lot of efforts were spent in the design of Gd(III)-based agents to optimize their interaction with water. The theory of the paramagnetic relaxation enhancement will be discussed and examples will be given where the computational characterization of water interactions with Gd(III) complexes can be used to describe experimental data.

Water inside liposomes as MRI imaging probe

The overriding challenge with MRI is its relatively low sensitivity. This technique requires an high concentration of imaging probes, indeed what is primarily observed, are hydrogen atoms from water that are present in tissue at ~90 M. The use of contrast agents can improve the signal-to-noise ratio but even with their use, the sensitivity of this technique is not comparable with other modalities (i.e. PET, SPECT, US) enabling molecular imaging procedures in vivo. Quite recently a new nano-sized system based on liposomial vesicles was proposed to provide a higher sensitivity with MRI [5,6]. In this case liposomes are uploaded with a paramagnetic shift reagent for water protons in the aqueous inner cavities producing the shift of the resonance of the water protons inside the liposomes from that of the external water. Thus, the ^{1}H NMR spectrum of such a suspension will display two signals for water that correspond to water confined in the liposome cavity and to the external solvent. The exchange of water molecules across the liposome membrane can be used to modify the signal intensity of the water protons through a saturation transfer process occurring through selective irradiation at the absorption frequency of the water protons inside the liposomes. The water signal intensity modification can be used to generate a negative image. Examples of these nano-sized systems and recent advances in their use in preclinical research performed from our group will be given.

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THE VARIED ROLES OF WATER IN STABILITY OF PHARMACEUTICAL SYSTEMS

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Stability has two rather different meanings in pharmaceutical systems. First, one may speak of thermodynamic stability, which normally refers to either thermodynamic stability of protein higher order structure, or in the case of small molecules, may refer to inter-conversion between solid forms. Secondly, we denote "pharmaceutical stability" as the resistance to irreversible change caused by chemical or physical transformations. The first part of this presentation deals with conformational stability of proteins in systems of low water content and the impact of water content on such stability. We find that denaturation does occur in the "dry" state, but only at temperatures roughly 100°C higher than in dilute aqeueous solution, but addition of water to the "solid" decreases the thermal denaturation temperature. Thus, from this viewpoint, water reduces the thermodynamic stability of proteins. We also suggest proteins in the solid state are thermodynamically unstable at room temperature, being generally below their cold denaturation temperatures at ordinary temperatures. Structural stability of dried protein formulations is controlled entirely by kinetics, not thermodynamics. Thus, thermodynamic stability of dried proteins is not practically relevant to problems in pharmaceutical stability.

The roles of water in determining pharmaceutical stability can involve one or more of three effects:

1. As a modifier of molecular mobility, with increased mobility meaning less pharmaceutical stability;
2. As a reactant in hydrolysis reactions;
3. As a stabilizer of polar or ionic transition states by increasing dielectric constant.

We present evidence that suggests the mobility effect is the most significant effect. However, the impact of small molecules, such as water, on molecular mobility may be complex in that such small molecules may increase some types of mobility and decrease other types, and it is not always obvious what type of mobility is critical to stability. Specifically, it is shown that sorbitol may plasticize the glass transition, and thereby increase global mobility, but at the same time anti-plasticize local fast dynamics, with a net result that at intermediate levels of sorbitol, pharmaceutical stability may be significantly increased for both small molecules and proteins. We also present evidence for a similar effect for intermediate levels of water. That is, we demonstrate

that in some cases, stability of a protein in the solid state is optimal at intermediate water content, and we suggest this effect is related to the anti-plasticization of fast dynamics by water.

POLY-N-ISOPROPYL ACRYLAMIDE AS A PROBE FOR WATER-ALCOHOL INTERACTIONS

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Poly-N-isopropyl acrylamide (PNiPAM) is a neutral amphiphilic polymer. As such it is sensitive to hydrogen bond formation and hydrophobic hydration, the balance of both determining its solubility in aqueous media. We exploit this fact to explore the interactions of PNiPAM in water - alcohol mixtures. While PNiPAM is soluble in both pure water and pure alcohol, it is insoluble in mixtures of the two over a certain range of solvent compositions. The insolubility region depends on the size and geometry of the hydrocarbon chain of the alcohol, and shifts to lower alcohol mol fractions in the series methanol, ethanol, isopropanol and propanol. We find that the insolubility region coincides with the minimum in the excess enthalpy of mixing of the two solvents themselves, which indicates that the solubility of PNiPAM directly depends on interactions among the solvent molecules. This correlation appears to generally apply to solvents that enhance the local hydrogen-bond network of the water molecules around them, the so-called structure-makers. These results suggest that simple experiments probing the solubility of PNiPAM in aqueous solvent mixtures can be used to determine thermodynamic properties of the solvent mixtures themselves.

TENSIOMETRIC VERSUS SKIN (TVS) MODELLING AS IMPROVED APPROACH FOR THE DIRECT EVALUATION OF SKIN HYDRATION

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It is well know that skin hydration plays an important role on the epidermal barrier functioin and on the skin aging critical level.
Skin hydration is usually evaluated by a low frequency capacitance measurement of the skin (corneometry).
The physical principle of the method is based on the changes in stratum corneum of the electrical properties that occur when the skin is hydrated. The dry stratum corneum is a medium of weak electrical conduction, hydrated stratum corneum is more sensitive to the electrical field. This phenomenon is involved in the chains of keratin, which have a dipole moment, the ions in the intercellular spaces, which may react to the application of an electric field, and the same water molecules that are capable of forming a network of hydrogen bridges. Based on the strength of bond that forms with the keratin, the water can be defined as tightly linked (0÷7%), bound (7÷35%) and free (>35%).
The relationship between electrical conductivity and water content is not linear and depends from the arrangement of the water molecules in the chains of keratin and the bond strength the keratin itself.

TVS modelling

Biosynthetic activity and epidermal cell turnover cause interactions that at the surface are reflected in form of free energy (mN/m). This energetic reflex is induced by the unbalance of internal attractive and repulsive forces having polar and dispersed nature that origin in different skin layers.
By an improved tensiometric approach (TVS modelling), the AA measured for the first time directly *in-vivo* and in *ex-vivo* the surface free energy (SFE) and its disperse (DC) and polar component (PC), and developed a reliable and reproducible test able to evaluate in a objective way the epidermal functional state and its hydration level (TVS skin test).
TVS modelling (i) is based on the permutability-principle of tensiometry, (ii) is founded on the system structure-surface correlation, (iii) is performed by tenskinmeter-apparatus and contact angle method using pure water and the biocompatible TVS skin fluid as reference liquids.
By discriminating from the PC component the polar fraction due to the present-relative humidity, the epidermal hydration (CPW%) can be objectively evaluated at native level.

Applicative exemples: Figure 1, TVS skin test monitoring on large scale (n=1560); Figure 2, specialist evaluation of epidermal hydration.

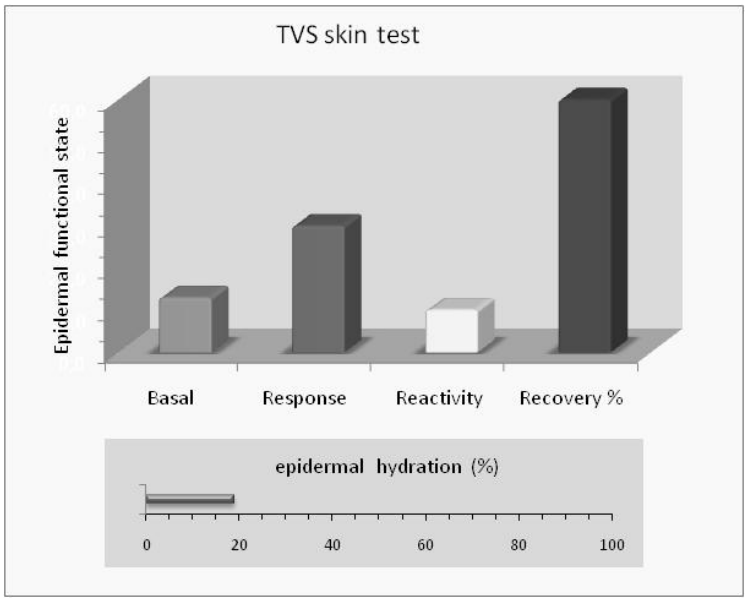
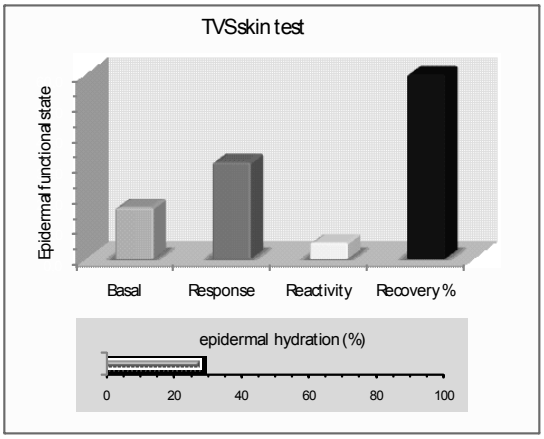


Figure 1. Monitoring on large scale

dry skin



oily skin

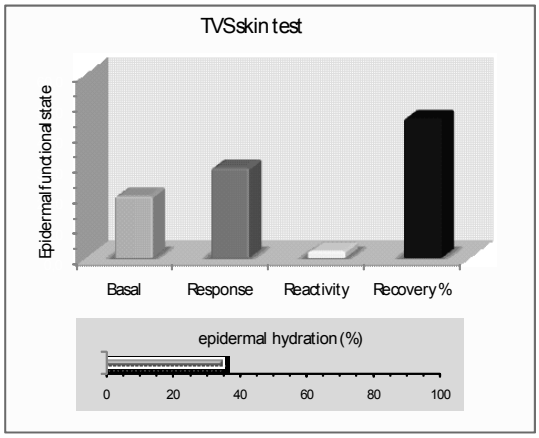


Figure 2: epidermal hydration

PANEL SESSIONS

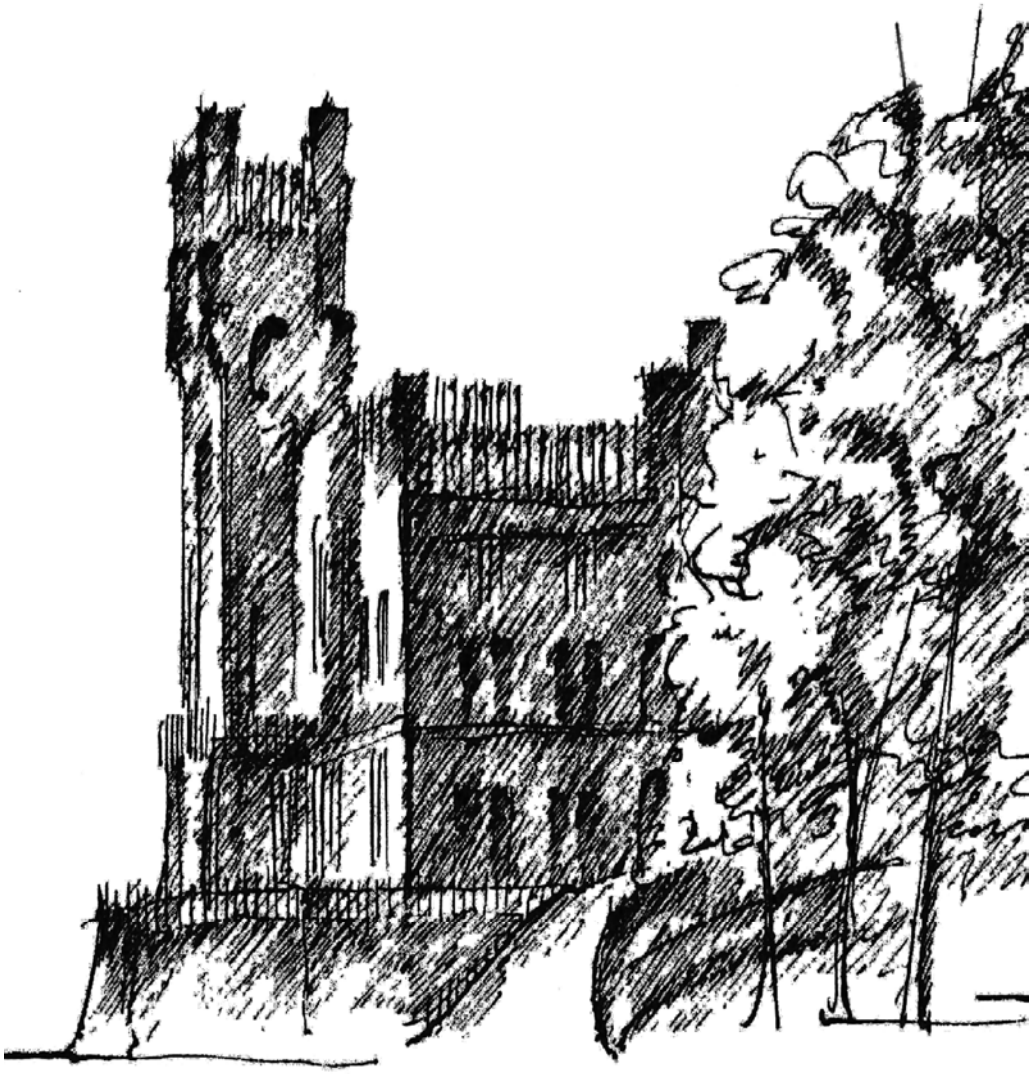
PANEL DISCUSSION A

Water and macromolecules

PANEL DISCUSSION B

Hydration water

POSTER PRESENTATIONS



A POLARIZATION-INDUCED RHEOLOGIC BEHAVIOUR IN WATER AND POLAR LIQUIDS

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It was observed that, when polarized by an intense electric field, water is able to self-arrange into macroscopic cylindrical wires which can hang up floating against gravity [1,3]. This phenomenon is now known as "water-bridge". Several attempts have been made to give an explanation of this apparently unusual behaviour of water. A number of experiments have been carried with the aim of probing any possible structural change of bulk water, after the application of the electric field. None of the available clues looks at the moment conclusive. Here we report the results of the first Raman scattering experiment on floating water-bridges. The intermolecular OH-stretching band has been investigated and the results have been compared with those from bulk water. Some changes in the scattering profiles after application of the electric field are shown to have a structural origin. The bridges have been obtained, for the first time, in a vertical geometry and under application of an alternating field. The adopted geometry has allowed revealing an asymmetry between opposite direct current biases, which can be related with the nature of the charge carriers. In addition, similar effects have been observed in other polar liquids. Finally, analogies and differences with water are discussed.

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MONITORING THE SLOW DYNAMICS OF WATER IN CRYOPROTECTANT MIXTURES BY DIELECTRIC SPECTROSCOPY

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Introduction

The study of dynamics in water-sugar mixtures from the liquid to the supercooled and glassy states can be suggested by practical considerations (like their use as cryoprotectants for biomaterials [1,2]) but it could be potentially a subject of interest for fundamental research [3]. In particular, mono-, di- and poly-saccharides, that as pure components are often good glass-formers, are also quite soluble in water due to the large number of hydroxyl groups. These systems were shown to be able to prevent ice formation down to very low temperatures, so that it has been possible to vitrify even mixtures where a considerable amount of water is present (up to more than 50% and almost 90% as weight and molar fraction respectively) [4]. The mechanisms that prevent ice formation from water in these systems are mainly due to hydrogen bonding, to the intrinsic disorder of the sugar component, or, in the case of hydrated samples of poly-saccharides with high T_g, to the possible confinement of water molecules at the nanometer or sub-nanometer level between domains of the saccharide macromolecules. The renewed interest for studying the amorphous state of water in these kind of mixtures is suggested by the recent observation that the reorientational relaxation times of water in aqueous mixtures and hydrated systems are very similar for many materials (with quite high hydration level) and show a marked crossover from a Vogel-Fulcher temperature behaviour to an Arrhenius one on cooling, resembling the Fragile to Strong transition already postulated for confined and bulk water [5-7]. This crossover was alternatively ascribed to the consequence of the confinement, as the finite size would stop the growth of a cooperatively rearranging region of the water relaxation, interpreted thus as an α-process [6] but other explanations were proposed [7-8]. The strong dipole moment associated to water molecule allows to study its orientational dynamics by means of dielectric spectroscopy, a broadband technique able to cover more than 13 decades in timescale and that can be applied from very low to ambient temperature and even at very high pressure. In this work dielectric spectroscopy was extensively used to

investigate the dynamics of different amorphous aqueous systems both at ambient and, for the first time, high pressure conditions.

Experimental

Dynamics of water in glass-forming mixtures with fructose (monosaccharide), trehalose (disaccharide) and glycogen (Polyglumyt@, ACRAF, a polysaccharide) were studied by dielectric spectroscopy in the frequency range from 10 mHz to 10 MHz. Mixtures of sugars with different concentrations of water (20-30% wt. in fructose, 4-15% wt. in trehalose, 23-77% wt. in glycogen) were obtained and the dielectric study was carried out in the temperature range from liquid to the glassy state on cooling by isothermal steps, from 350 K down to 110 K. In the case of water-fructose and trehalose mixtures also high pressure measurements from 0.1 MPa up to 650 MPa were done. Differential Scanning Calorimetry measurements were also carried out to check miscibility, vitrification and absence/presence of crystallization in the mixtures.

Results and Discussion

The spectra of the studied water-fructose and water-trehalose mixtures always exhibit two relaxation peaks: the slower (α-process) and faster (*v*-process), as already reported in previous studies [8-10]. The slow process is related to cooperative motions of water with sugar molecules and it is related to the calorimetric glass transition, since $\tau_\alpha(T_g)$=100 s, where T_g coincides with the specific heat step. The fast *v*-relaxation time, related to water, showed a marked crossover from a Vogel-Fulcher temperature behaviour to an Arrhenius one on cooling. The activation energy in the glassy state is nearly 50 kJ/mol, that is the energy required to break two hydrogen bonds. Examples of Arrhenius plot for water-fructose at ambient and elevated pressure are shown in fig. 1. It was found that the crossover temperature was increasing with the applied pressure and it always occurred just below T_g of the mixtures (see inset in Fig.1.b). A similar crossover at T_g was found also for the temperature dependence of dielectric strength [8]. This behaviour was demonstrated to be common to the secondary relaxation in mixtures of common van-der-Waals liquids. All the dynamic scenario can be interpreted in the framework of the interrelation between α- and β-relaxation in glass-forming systems.

In the case of water-glycogen mixtures dielectric spectra revealed also two relaxation peaks (see Fig. 2a), both moving at low frequency on decreasing temperature. The slowest peak is more intense and it is less sensitive to temperature than the faster one. The latter is visible only at higher temperatures, when its strength increases. Roughly, the peak positions (and therefore the relaxation times) did not change too much with water concentration. An overview of the dynamics is shown in Fig. 2b where data for the frequency of loss peak of glycogen mixtures and some literature data are also compared [7, 11].

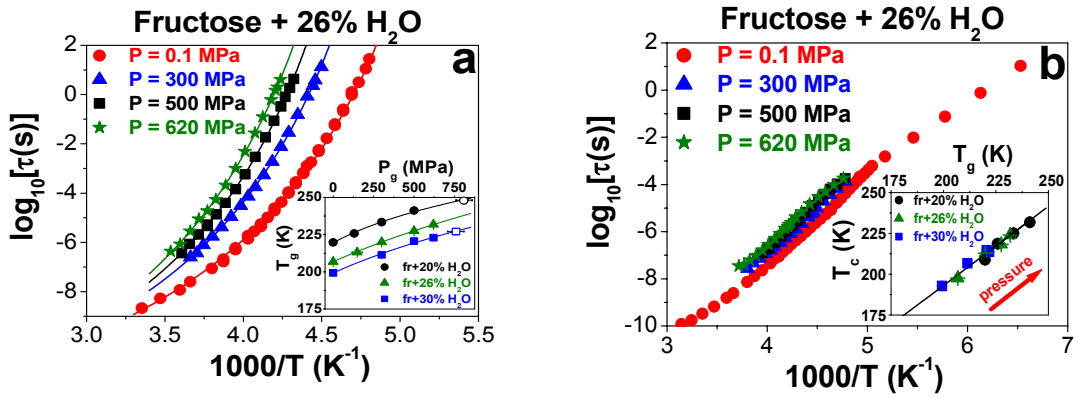

Figure 1. Plot of logarithm of relaxation times against reciprocal temperature of the a) *α*-process and b) *ν*-process of fructose-26% water mixture for various isobars: 0.1 MPa (circles), 300 MPa (triangles), 500 MPa (squares), 620 MPa (asterisks). Lines represent VFT fitting curves.

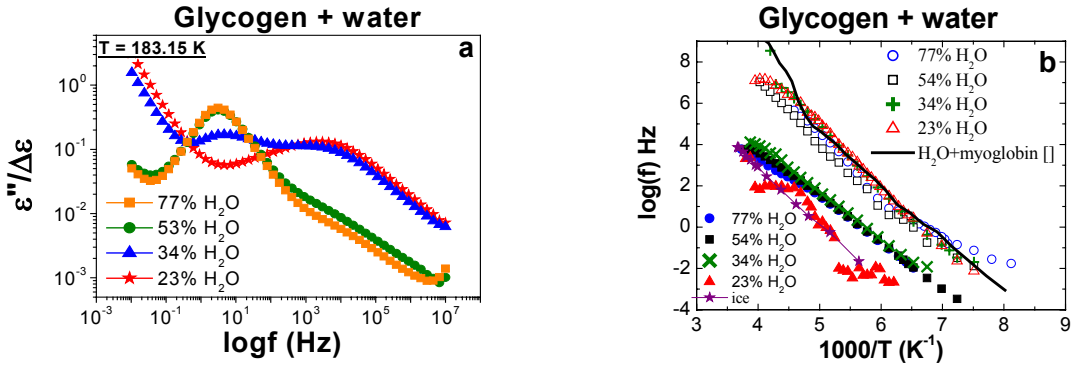

Figure 2. a) Log-log plot of normalized imaginary part of dielectric function, $\varepsilon''/\Delta\varepsilon$, vs. frequency for water-glycogen mixtures with 23% (asterisks), 34% (triangles), 53% (circles) and 77% (squares) of water at T = 183.15 K; b) Plot of logarithm of frequency maximum against reciprocal temperature of the slow (close symbols) and the fast (open symbols) processes for water-glycogen mixtures with 23% (triangles), 34% (crosses), 53% (squares) and 77% (circles) of water. Data from ref.[7] of water relaxation in hydrated myoglobin are also reported.

From the comparison it is clear that the slow relaxation is related to the reorientational motion of water in ice lattice, whereas the faster relaxation is nothing but the ubiquitous *ν*-relaxation process, related to amorphous form of water. It is remarkable that there is a strong similarity also with the relaxation time measured for hydration water in myoglobin [7]. Also the relative strength behavior of the two processes can be explained in terms of crystallized and uncrystallizable water as already found in ref.[11]. The amount of uncrystallized water is almost the same for all the mixtures, being

located about 2-3 layers around the glycogen macromolecules but, on increasing the water fraction, the additional water all contributes to the fraction of crystalline water and so the relative strength of the slower process increases, up to mask the *v*-relaxation process. On the contrary, the ice relaxation is almost absent for water weight fraction of 23%. Glycogen is therefore a good system for obtaining large fraction of amorphous water. In these systems, characterized by high T_g, there is absence of dynamic crossover for the *v*-relaxation in the T range investigated.

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EFFECTS OF THE PROTEIN NET CHARGE AND STERIC HINDRANCE ON THE GLASS TRANSITION OF PROTEIN-TREHALOSE-WATER SYSTEMS AND ON THE PROTEIN THERMAL DENATURATION

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Introduction

Biopreservation is a relevant topic for its technological implications in food industry, pharmaceutics and medicine; indeed, glassy saccharide matrixes protect proteins against denaturation under extreme environmental conditions as extreme drought, high or low temperature. Among biopreservants, the disaccharide trehalose is the stabilizer most used in nature [1]. The molecular mechanisms behind this effect are not yet fully understood and are, at present, matter of challenge. Among the suggestions proposed to explain the trehalose peculiarity are i) the water replacement hypothesis [2], according to which stabilization, in the dry state, occurs via the formation of direct H–bonds between the disaccharide and the biostructure, and ii) the preferential hydration hypothesis [3], according to which trehalose, rather than directly binding to biomolecules, entraps the residual water at the biostructure interface by glass formation. Recently [4], in ternary myoglobin–trehalose–water systems, a linear correlation has been found between the matrix glass transition (T_g) and the denaturation temperature (T_{den}) of the embedded protein. This leads to infer that the collective properties, which determine the matrix glass transition, are correlated to the properties in the protein surroundings, which determine the denaturation of the protein in the liquid phase. This indicated that the protein–matrix interactions, which govern the enhancement in protein stability, extend over a long range, evidencing deep aspects not inferable by simply studying binary sugar–water matrices.

To better understand the thermodynamic aspects of ternary protein–trehalose–water systems, we studied by DSC the effects of the protein charge and steric hindrance on T_g and T_{den} in protein–trehalose–water systems. In particular, we studied at pH 7 lysozyme (positively charged, small size), BSA (negatively charged, large size) and hemoglobin (no charge, large size). The water/trehalose ratio is such that samples span from dilute solutions to dry plasticized amorphous solids.

Experimental

DSC measurements were performed in the temperature range ~100K to ~400K. Glasses were obtained by drying trehalose–protein aqueous solutions to a water/sugar molar ratio lower than 20. Calorimetric upscans were measured with a rate of 10 $Kmin^{-1}$, after quick cooling (500 $Kmin^{-1}$) to liquid nitrogen temperature; as usual, the glass transition temperature of the samples was identified from the stepwise increase of the specific heat; an irreversible endothermic peak (at $T > 330$ K) indicated the protein denaturation.

Results

The systems were found to obey the Gordon–Taylor formula both in the presence and in the absence of the embedded protein. Results suggest that in high water systems the sugar barely affects the protein denaturation temperature, while an increase exceeding 70 K (with respect to the value in dilute trehalose solutions) is observed at very low water/trehalose ratio. Results show that the presence of the protein lowers the T_g with respect to binary systems suggesting that the protein, acting as a chaotropic component, reduces the strength of the H–bond network encompassing the sample.

While both protein charge and volume seems to affect the T_{den} value, the protein charge does not influence the glass transition, which depends strongly on the protein volume. The T_{den}–T_g correlation can be extended to each protein, but it is valid only below given water/trehalose ratios specific for each protein. This enable to formulate a sugar specific T_{den} dependence on hydration that can be of great relevance for biotechnological applications.

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ORAL VACCINES FOR AQUACULTURE: DEVELOPMENT OF A VACCINE MICROENCAPSULATION SYSTEM BASED ON BIODEGRADABLE POLYMERS.

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Aquaculture represents a fast growing sector of food industry, providing one half of the fish consumed by humans. The intensive production of fish farming results in stress for fish and in increased vulnerability to disease outbreaks. The widespread use of antibiotics for the control of epidemic infections led to the development of antibiotic-resistant bacteria strains and to environment and consumer unsafe products. The main objective of modern fish farming is the production of traceable, green and healthy food. In this context, the applied research is currently oriented towards the development of new preventive strategies based on fish vaccination.

The present work has been carried out in the frame of a project entitled *Oral vaccine carrier for fish farming of Friuli Venezia Giulia* in which a network of scientific Institutions and fish farming plants are sharing their competences to develop an oral vaccination protocol for lactococcosis in trout, based on the administration of the inactivated bacteria microencapsulated in biodegradable polymeric matrices. Lactococcosis by *Lactococcus garvieae*, is one of the most relevant fish diseases in intensive aquaculture, causing substantial economic losses to rainbow trout farming (*Oncorhynchus mykiss*). The oral vaccination appears to be promising because the first contact with pathogens usually occurs through the mucosal surfaces of the gut at the level of the gut-associated lymphoid tissue (GALT). Compared with intraperitoneal injection of vaccine, oral administration is simple, cost-effective and suitable for mass fish immunization.

Presently, just few commercial oral vaccines are available, due in part to the necessity to protect antigens (to avoid digestive hydrolysis) and to enhance their uptake by the hindgut, in order to induce an effective protective immune

response. The set up of a microencapsulation system for large scale production of vaccine loaded microcarriers represents a technological challenge. Polysaccharidic biopolymers have been chosen among the candidate biomaterials because of its cost-effectiveness. In particular, alginate, chitosan and a cellulose derivative (hydroxypropyl methyl cellulose, HPMC) have been used for the encapsulation, based on an ionotropic gelation combined with a polyelectrolyte complexation. Lysozyme, whose immunomodulating properties have been largely demonstrated, has been selected as adjuvant and co-encapsulated with the antigen. Furthermore, the development of a pilot plant has been implemented with the selection and the characterization of the most suitable materials for vaccine embedding. In parallel, a model system based on capsules of 2 mm diameter has been studied in order to quantify the relationships between polymeric matrix composition and carrier performances. The full characterization (yield, loading and encapsulation efficiency, release profile) of the carriers has been achieved and parallel morphologic (SEM), calorimetric (DSC) and infrared spectroscopic analyses have been performed. A rheological study has been also carried out on the mixed polymer solutions and hydrogels to rationalize the operative conditions for the microencapsulation. Data obtained from preliminary *in vivo* trials, support these polysaccharidic microcarriers as a new and promising system for oral fish vaccination.

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PREFORMULATION STUDIES ON ALGINATE-BASED DELIVERY SYSTEMS: SOLUTIONS AND HYDROGELS.

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Introduction

Biopolymers have been extensively employed for the development of several delivery systems for pharmaceutical and biomedical applications. In particular, alginate has been established as a very versatile material for preparing microcapsules for the entrapment of therapeutic biomolecules due to its gel-forming capability in presence of divalent cations such as calcium. The rational design of an ordered polymer network with well-defined physicochemical properties and reproducible drug release profiles is based on the identification of the key parameters and mechanisms that govern the rate and extent of drug release [1]. As known, diffusion, swelling and erosion are the most important rate-controlling mechanisms of controlled release systems [2]. In the case of porous hydrogels, if pore sizes are much larger than the molecular dimensions of the drug, the diffusion coefficient can be related to the porosity and the tortuosity of the hydrogels. As a consequence of tortuosity, release from swollen hydrogels of macromolecules such as peptides and proteins can be sustained due to their significant hydrodynamic radii. If designed appropriately, the structure and mesh size of swollen hydrogels can be in principle tailored to obtain the desired rates of macromolecule diffusion. Therefore, understanding the mechanisms and identifying the key parameters that govern drug release from hydrogels are the first step toward accurately predicting the entire release profile.
In this study, release profiles of a model protein (lysozyme) from the mixed alginate/biopolymers beads were carried out in a release medium in which the relevant mechanisms are diffusion and swelling, while erosion is limited. The experimental approach is based on the characterization of thermal and rheological properties of the systems.

Results

A model system based on alginate gel beads has been prepared. In order to design a complex encapsulation system resulting from the entanglement of polymer chains, alginate has been mixed with other biopolymers such as

hydroxypropylmethylcellulose (HPMC) and chitosan. HPMC is one of the most important hydrophilic carrier material used for the preparation of oral controlled drug delivery systems, and chitosan is commonly used in encapsulation system to reinforce the alginate matrix.
The evaporation rate of the solvent water has been taken as a functional parameter that depends on the composition of the gel bead and the evaporation process behavior has been studied to relate solvent diffusion with the process of solute release. DSC has already been used by us to characterize the gel system where water is entrapped in within the nano-porous three-dimensional polymer matrix [3].
Finally, a rheological investigation has been carried out on alginate gel disks. From the mechanical spectra only small differences were found on the linear viscoelastic properties among the considered systems. For both the storage and loss moduli an apparent rank order resembling water evaporation was not evident, while it slightly appears considering the phase lag, that means the relative weight of the viscous component. However, due to the small differences among the values, this study will need further considerations.

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THERMODYNAMICS OF WATER MEDIATED ANION – LYSOZYME ASSOCIATION

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Addition of simple electrolyte can dramatically change the properties of protein solution [1]. The phenomenon is most often explained by variation of the solvent mediated interactions between ions and proteins. However, the corresponding thermodynamic parameters, essential for understanding of ion - protein association at the molecular level, have not yet been determined. Presented work provides first complete thermodynamic study of ion - lysozyme association. The thermodynamic quantities are discussed in the light of Hofmeister series.

In the present study we employed isothermal titration calorimetry to measure the heat effects coming from salt-to-lysozyme and lysozyme-to-salt titrations in aqueous buffer solutions (pH = 4.0, lysozyme mean net charge = +11). The calorimetric binding isotherms obtained for NaCl, NaBr, NaI, $NaNO_3$, NaSCN, KCl, $CaCl_2$, and $BaCl_2$ at different temperatures were described simultaneously by a model assuming independent binding sites for anions (counter-ions). Since the influence of cations (co-ions) on the measured heat effect appears to be weak, the presented global thermodynamic analysis allowed reasonable determination of the thermodynamic profile ($\Delta G°$, $\Delta H°$, $\Delta S°$, $\Delta C_P°$) and number of binding sites for the anion - lysozyme association. The resulting binding constants $K \approx 10^2\ M^{-1}$ increase in order $Cl^- < Br^- < I^- < NO_3^- < SCN^-$ [2]. This association is entropy driven accompanied by a small favorable enthalpy contribution and positive change in the heat capacity.

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MUCOADHESIVE BEHAVIOR OF HYDROPHILIC POLYMERS: MUCINE-CHITOSAN INTERACTION IN AQUEOUS SOLUTIONS.

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Introduction

The development of bioadhesive materials represents a crucial goal for pharmaceutical research and industrial applications. Such materials can be employed for the preparation of drug delivery systems capable to prolong the drug residence time at the sites of absorption. The application ranges from topical preparations to mucoadhesive formulations for oral administration of drugs such as insulin or heparin. The mucoadhesive behaviour of a material is directly related to the attachment capability to a mucosal surface. Mucus is a viscous and heterogeneous biological product that coats many epithelial surfaces. It consists mainly of water, inorganic salts, carbohydrates, lipids and glycoproteins. Mucus glycoproteins are also called mucins and consist of a protein core with branched oligosaccharide chains. Approximately 80% by weight of the glycoprotein consists of oligosaccharides, which make the mucin more hydrosoluble and also protects the protein core from proteolytic degradation [1,2].

Experimental

In this study chitosan, a polysaccharide derived from the exoskeleton of crustacean and insects, characterized by intrinsic mucoadhesivity, has been selected. Native and engineered (glycosylated and methylated) [3] chitosan samples have been employed. The effect of the peculiar polysaccharide-mucin (from porcine stomach) interaction in blends of polymer aqueous solutions has been studied by merging the results of turbidimetry and DSC calorimetry measurements, implemented with data derived from a viscosimetric characterization.

Results

The characterization of mixtures of chitosan and chitosan derivatives with mucin showed different types of polymer-mucin interaction, responsible of the excellent mucoadhesive properties of such polymers (when tested *in vitro* and *ex vivo*). The combination of the results of different macromolecular characterizations allows to highlight the mechanisms underlying the physico-chemical nature of the system.

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DETACHMENT OF HYDRATION WATER FROM BIOMOLECULAR SURFACES UPON HEATING

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Structure and functioning of biomolecules depend crucially on the properties of hydration water near their surfaces. Experiments evidence a rapid change of hydration water preceding denaturation of biomolecules upon heating [1-3]. Molecular simulations show that connectivity of hydrogen (H) bonds in hydration shell changes drastically in the temperature interval where many biomolecules undergo heat-induced structural transition [4]. At low temperatures, a biomolecule is covered homogeneously with a spanning network of hydration water. This network breaks upon heating onto small water clusters and this process may be described as a percolation transition in finite system [5,6]. This transition was found to be related with the onset of random chain conformational behavior of elastin-like peptide [4]. The thermal expansion coefficient of hydration water at the surface of amyloidogenic peptide Aβ_{42} shows a sharp increase by about 30% at the percolation threshold [7].

In the present study, we examine the temperature dependence of the components of the potential energy and specific heat C_p of hydration water in relation to the connectivity of H-bonded network in hydration shell by simulations of hydrophobic and hydrophilic peptides in water. The temperature dependence of C_p of hydration water of both biomolecules is similar to that in the bulk (Fig. 1). C_p in hydration shell exceeds (C_p^h) the bulk value (C_p^b) on ≈1.1 J/(mol K) near hydrophilic peptide and on ≈ 5.3 J/(mol K) near hydrophobic peptide. The latter value indicates that the surface of hydrophobic peptide is close to those of the Lysozyme and Ribonuclease. The specific heat C_v of water in hydration shell decreases upon heating stronger than in the bulk, so that at T > 320 K, C_v of hydration water is smaller than that of bulk water, even near hydrophobic peptide. Therefore, the strong increase of C_p near hydrophobic peptide in a wide temperature range should be attributed mainly to the high thermal expansion coefficient of hydration water.

Two constituents of the total potential energy of hydration water, the interaction within hydration shell (E_{hh}) and interaction between hydration and bulk water (E_{hb}) were studied separately. It was found, that both constituents show sharp change close to the percolation transition of hydration water (Fig. 2). Thus, C_p of hydration water undergoes qualitative changes due to the thermal break of H-bonded network in hydration shell: the contribution of water-water interactions within hydration shell to C_p sharply decreases, whereas the contribution of interactions between hydration and bulk water to

C_p sharply increases. The improving connectivity between hydration and bulk water at high temperatures makes the surface of biomolecules effectively more hydrophobic and may affect dynamics of biomolecules and their aggregation.

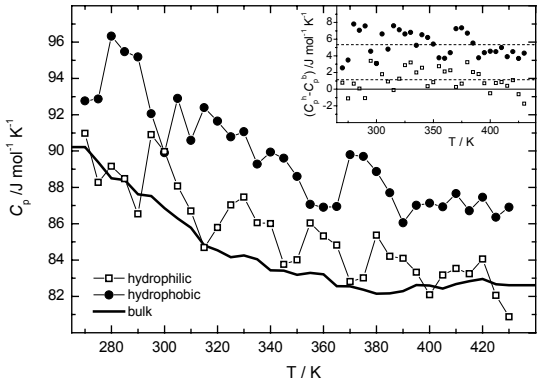

Figure 1. The temperature dependence of the specific heat C_p of water in the bulk (C_p^b) and in the hydration shells of two peptides (C_p^h).

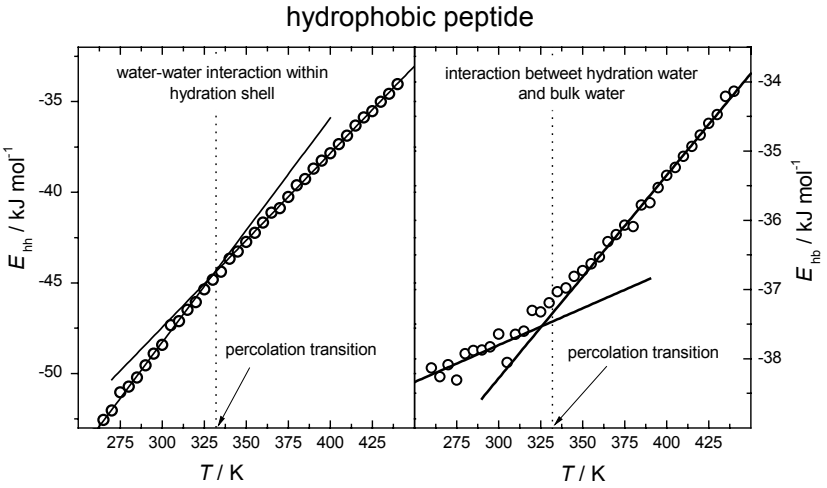

Figure 2. Potential energies of interactions of hydration water with neighbors in the hydration shell (E_{hh}) and with the bulk water (E_{hb}).

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INFLUENCE OF THE AQUEOUS ENVIRONMENT ON PROTEIN RELEASE FROM ALGINATE HYDROGELS

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Introduction

Alginate is an extremely suitable biopolymer that is increasingly used in the pharmaceutical, biomedical and biotechnological industries, thanks to its rheological properties, the absence of toxicity and the fact that it easily produces hydrogels. It is particularly appealing as an encapsulating material for drugs, cells, vaccines and proteins. Encapsulation may be applied to all biologically active materials as an entrapment matrix that provides protected and controlled release. In the pharmaceutical field, encapsulation technology has been exploited in order to create systems able to preserve an active ingredient's stability and to modulate the release profile in vitro and in vivo [1]. The aim of the present study was to investigate the stability and solvent response of the beads in relation to the protein release. Release of two representative proteins, lysozyme and human insulin, from alginate beads was studied in different aqueous environments in order to understand how the pH and the ionic strength of the medium influence the beads behaviour. Furthermore, the proteins pI is another important parameter involved in release processes since proteins can have an interaction with alginate or not, depending on the aqueous environment pH [2].

Experimental

Beads with dimension of a few millimeters were prepared by drop-wise ionotropic gelation of alginate/protein solution into a Ca2+ solution. The encapsulation efficiency and the amount of protein entrapped in the beads were investigated. In protein release studies, a known quantity of beads was resuspended in 10 ml of dissolution medium and aliquots were withdrawn at different intervals. Then, the amount of released protein was determined. The bead behaviour in different buffers was also investigated by light scattering at fixed angle in order to measure the erosion process. In fact, drug release through alginate hydrogel matrix is modulated by diffusion to the aqueous environment during a swelling process of the beads and erosion of the same matrix [3].

Results

In acid conditions of aqueous media (pH 1.2), alginate-based beads tend to shrink due to the decreased electrostatic repulsion between protonized carboxylate groups which prevents the swelling process [4]. In gastric

conditions proteins are released very slowly as a result of a compact and less permeable polymer matrix [5].
By increasing the pH value to 7.4, the drug release depends on the protein charge, the ionic strength of the medium and the presence or not of an erosion process. In phosphate buffer, protein release is completed in 2 hours due to the erosion of the alginate-calcium matrix. Release test in TRIS HCl buffer at the same pH demonstrates a lack of an erosion process and the process is mainly due to diffusion. Proteins with a high pI interact with the negative charge of alginate polymer and the diffusion turns out to be very low [1]. Increasing the ionic strength of Tris HCl, the interaction between proteins and alginate fails.
In summary, in this study the behaviour of two proteins was therefore investigated to understand the influence of pI on the release rate and the interaction of the proteins with alginate hydrogels in different aqueous media.

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HYDRATION PROPERTIES OF CYCLODEXTRIN-WATER SOLUTIONS BY BROAD BAND DEPOLARIZED LIGHT SCATTERING

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Broad Band Depolarized Light Scattering (DLS) measurements performed on aqueous solutions of α-cyclodextrin (α-CD) are here used to explore the hydration properties of these natural cyclic oligosaccharides. Their peculiar toroidal shape, in wich water can locate both inside and outside, charaterizes them in having a relatively-high water-solubility thanks the hydrophilic outer surface and a renowned ability to form inclusion complexes with a large variety of guest hydrophobic molecules [1-5]. The spectral analysis od DLS spectra allowed us to single out two different solvent relaxation processes of picosecond time-scale assigned to hydrating and bulk water molecules [6]. This dynamics is related to the continuous hydrogen bonding in agreement with molecular dynamics simulations [7]. Structural information about the number of water molecules in the hydration layer is also obtained. These findings constitute a key step for understanding the role played from water in determining the properties of different forms of cyclodextrins and their derivatives, in turn related to complexation ability of these macrocycles.

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ANOMALOUS BEHAVIOR OF WATER DYNAMICS IN CONCENTRATED TREHALOSE SOLUTIONS

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Concentrated aqueous solutions of the three well-known homologous disaccharides, namely, maltose, sucrose and trehalose, have been systematically studied in a unexplored frequency region by Brillouin ultraviolet light scattering, as a function of temperature and number of water molecules per disaccharide (Figure 1). Upon decreasing the water content, these measurements reveal two regimes for the collective dynamics of the hydrogen bond of water molecules. The change of regime corresponds to the onset of the percolated hydrogen bond network found for the sucrose system. Still, for trehalose an anomaly appears: the hydrogen bond lifetime of proximal water molecules measured on trehalose solutions becomes faster than the lifetime measured on maltose and sucrose solutions, presaging a different reorganization of the sugar matrix in the percolated regime (Figure 2). Whether this effect could help in reducing both desiccation stresses and ice formation in anhydrobiotic organisms is to be assessed.

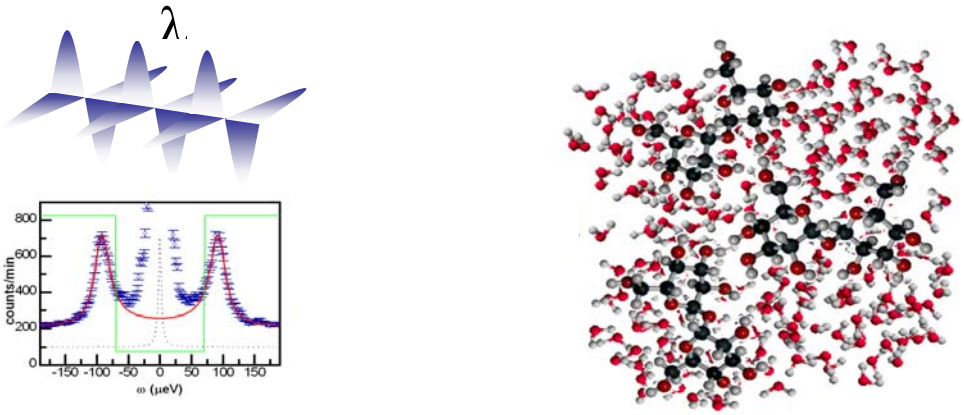

Figure 1. Representation of the Brillouin scattering experiment in the aqueous trehalose solution.

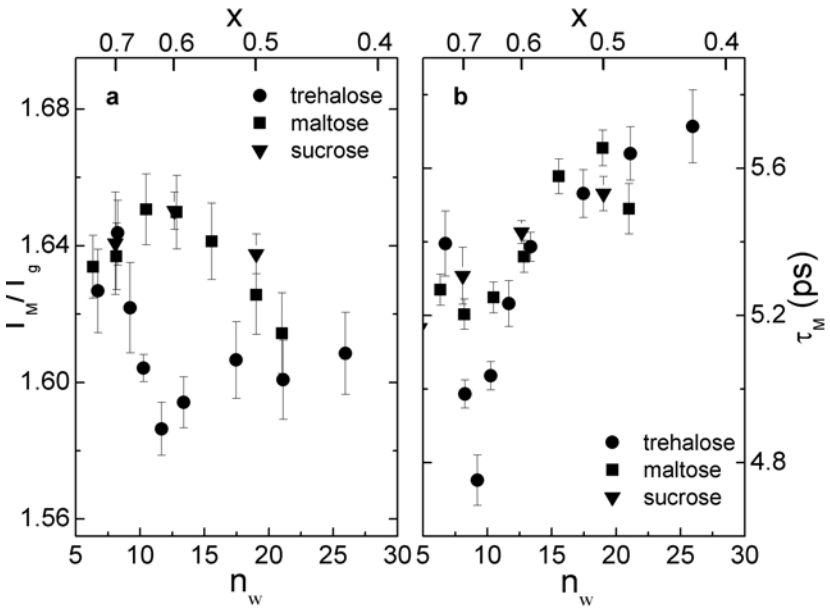

Figure 2. Concentration dependence for the three sugar solutions (trehalose, maltose and sucrose) of TM /Tg (a), and of the relaxation timę tM =1/wM (b). TM and Tg are maximum relaxation and glass transition temperatures, respectively; wM is the frequency at the maximum width, Gb, of the inelastic peaks.

INTERFACIAL BEHAVIOUR AND TECHNOLOGICAL FUNCTIONALITY OF OLIVE POLYPHENOLS IN O/W OLIVE OIL EMULSIONS AS AFFECTED BY DIFFERENT SURFACE ACTIVE AGENTS

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Introduction

Olive oil represents one of the most important food and ingredient among the European productions; largely used as seasoning, it is also getting more interest in the preparation of formulated and processed foods like pesto and sauces thanks to its peculiar sensory, nutritional and functional properties. Oxidative deterioration, which in emulsified systems can be mainly considered an interfacial phenomenon, is one of the causes that can deeply affect the shelf life of multiphasic systems; one of the strategies adopted by the food industry is to add protective compounds.

Previous studies carried out in our research group on o/w emulsified systems made by olive oil, Tween 20 or b-lactoglobulin and added with polyphenols (gallic acid, catechin and quercetin) showed a different effect exerted by these bioactive compounds on the dispersion degree of olive oil emulsions as well as on the evolution of the oxidation that could be related to their different polarity and anphiphylicity [1,2]. In the case of olive oil, its high content in natural antioxidants could represent a natural way to avoid or reduce the rate of lipid oxidation provided that these polyphenols can locate nearby the interfacial layer.

The aim of this work was to evaluate the interfacial behaviour and the ability of some olive oil polyphenols in olive oil o/w emulsions to influence the stability to lipid oxidation as affected by the composition of the nature of the surfactant agent.

Experimental

Emulsions were prepared using refined olive oil (20% w/w) and PBS (50mM, pH 7). Oleuropein, tyrosol and syringic acid were chosen as olive oil antioxidant compounds to add to the systems in order to test their protective activity in emulsions. Tween 20 and whey protein isolate were used as surface active agents in the concentration of 0.5% and 2.5% (w/v) respectively. Surface and interfacial tension measurements, dispersion degree, antioxidant partition, lipid hydroperoxide and TBARs contents were carried out on the emulsified systems over storage time (30 days at 50°C).

Results

All the olive polyphenols were proven to have interfacial activity in olive oil/buffer systems; among them oleuropein showed an increasing interfacial activity with the increasing of its concentration. In emulsions, the addition of the antioxidants under investigation differently affected the dispersion degree but the influence was strictly dependent on the interfacial composition. In the systems stabilized by the whey protein isolate, the antioxidants showed no effect on the dispersion degree while in the Tween 20 stabilized emulsions the antioxidants, especially oleuropein and syringic acid, caused a general improvement of the dispersion state allowing the formation of more dispersed systems.

The oxidative stability of the differently prepared emulsions were affected by both the surfactant agent and the presence of the polyphenols.

This research could find obvious practical application in the development of innovative formulated multi-phased food with the beneficial effects deriving from the presence of naturally occurring antioxidant compounds.

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TRACKING INDIVIDUAL WATER MOLECULES PREFERENTIALLY BOUND TO PROTEINS

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High-resolution crystallography provides information on the precise 3D structure of proteins, ligands and several water molecules; in general, however, only a maximum of one third of preferentially bound water molecules are identified by crystallographic techniques. In the case of small proteins, NMR techniques may be equally purposive. By contrast, the data from low-resolution solution techniques, such as small-angle X-ray scattering (SAXS) and hydrodynamics, inherently contain hydration. Knowledge of hydration details is essential for various reasons, including understanding manifold protein interactions as crucial prerequisites for flexibility, dynamics and functionality, and the construction of tailor-made proteins, e.g. in context with drug-design projects and the development of functionalized surfaces and polymers by mimicking proteins.

Development of Different Hydration Strategies

A critical inspection of anhydrous and hydrated protein models obtained by crystallography with models derived from quite different experimental techniques and calculation approaches allows a scrutinized comparison of the models under analysis. Among a variety of problems, the amount of hydration and the position of the individual water molecules turned out to be the most crucial points. To meet this challenge, a variety of techniques and approaches were examined and both models and molecular parameters were analyzed: (i) Conventional and *ab initio* modelling approaches signify satisfactory agreement between crystal- and SAXS-based protein models, provided hydration contributions and other precautions are taken into account [1]. (ii) Recourse to crystallographic or model data also allows hydrodynamic modelling; in the case of multibead structures novel modelling refinements (e.g., efficient bead reductions) have to be adopted [2-4]. (iii) The creation of hydrated models from cryo-electron microscopy data necessitates qualified assumptions regarding hydration, e.g. in terms of voxel densities [5]. (iv) Combining the exact surface topography (molecular dot surface; derived from atomic or amino acid coordinates of proteins or

appropriate models) and our recent hydration algorithms (programs HYDCRYST and HYDMODEL) allows the prediction of individual water molecules preferentially bound to certain amino acid residues [6-10]. In this context, various approaches and procedural methods were tested: sequence of assignment to accessible residues, atomic vs. amino acid coordinates, original vs. coarse-grained models, fine-tuning of input parameters, variation of channel characteristics (e.g., width) etc. A critical comparison of the water sites on the surface, in active centres, ligand binding sites, crevices, channels, contact areas etc. proves far-reaching identity of crystallographic data and our predictions.

Application of Advanced Hydration Algorithms to Various Proteins

The good agreement of the results found for hydrated models by crystallography, SAXS, and other techniques offers the possibility to complement different techniques and to predict details such as the localization of potential water sites - even in those cases where no crystallographic waters, water clusters or water channels have been identified. Examples presented include proteins ranging from simple to complex, multisubunit, liganded proteins (e.g., lysozyme, myoglobin, apoferritin, bacteriophage capsids, giant haemoglobins, antifreeze proteins), and water-channels in membrane proteins (e.g., aquaporins) as well. Visualization of protein sites of special concern (charged, hydrophilic and hydrophobic residues and patches, radiosensitive groups, active centres of enzymes, ligand binding sites, docking sites and contact areas) together with individual waters provides the basis for a much deeper understanding of the mechanisms of biological action and effective biotechnological application.

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STRUCTURAL HETEROGENEITIES IN EXTREMELY DILUTED ALKALI HALIDE WATER SOLUTIONS

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Solutions of LiCl or NaCl in water were studied through Rayleigh/Brillouin scattering as a function of the weight concentration of salt from 1% (10^{-2}) to extremely low concentration (10^{-30}). It is remarked that a concentration of 10^{-30} corresponds practically to the absence of alkali or halogen ions in water. Thanks to the dynamically controlled stability of the used Fabry-Perot interferometer and using a counting system where photons spuriously scattered by macroscopic impurities or bubbles are sorted out, reliable Landau-Placzek ratios could be evaluated. The solutions were shaked after each centesimal dilution. It was observed that the intensity of elastic light scattering compared to the intensity of the Brillouin lines decreased with the dilution by going from the concentration of 10^{-2} down to 10^{-6}. It increased in the interval of concentrations from 10^{-8} down to 10^{-14}, and was approximately constant between 10^{-14} and 10^{-30}. In these solutions, the light is elastically scattered by sub-microscopic heterogeneities. These heterogeneities were interpreted as being isolated or aggregating nanobubbles. Their lifetime was observed to be longer than two months.

HYDROPHOBIC HYDRATION: A BRILLOUIN SCATTERING STUDY OF DILUTED WATER- TERT-BUTYL ALCOHOL SOLUTIONS

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The high interest for aqueous solutions of hydrophobic solutes stems from the important role of hydrophobic hydration and interaction in many fundamental biochemical processes. We have measured longitudinal viscosity of diluted water - tert-butyl alcohol solutions in the 10GHz frequency range by means of Brillouin light scattering and inelastic ultra-violet scattering. The main advantage of our hypersonic investigation with respect to more traditional ultrasonic measurements is that the GHz frequency range is completely unrelaxed for those processes involving water-alcohol and alcohol-alcohol interactions, so that the viscosity measurements here obtained originates mainly from breaking and formation of hydrogen bonds. In contrast with previous determinations, here we show an activation energy which is independent from alcohol mole fraction up to x=0.1, and comparable to that of bulk water, attributed to breaking and formation of hydrogen bonds. This finding is in favour of a dynamic scenario, where the slowing down of hydration water is due to a reduction of configurational entropy, without the need of icelike structures [1].

A simple two-component model is used to describe the steep increase of viscosity with increasing alcohol fraction, and a retardation factor 1.6 is found between the relaxation times of hydration and bulk water, suggesting that the slowing down of water mobility can be explained by hydrophobic hindrance effects. In fact this value is consistent with that recently obtained by numerical and analytic investigations [2] of the solute excluded volume effect. This effect, together with a molecular large-amplitude jump mechanism of water reorientation [3], has been proposed as the key ingredient for understanding hydrophobic hydration.

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HYDROPHYLIC AND NON-POLAR INTERACTIONS IN HOMOLOGOUS IODINATED CONTRAST MEDIA SOLUTIONS

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The Iodinated Contrast Media (ICM) studied in this research work are used in intravenous injection and classified as non ionic monomeric contrast media. The main molecular skeleton (Fig.1) of these compounds consists of a triiodinated aromatic ring that is substituted by three side aliphatic chains linked to aromatic ring by amidic bonds at position 1, 3 and 5. Iodine atoms at position 2, 4 and 6, in addition to absorb X-ray, are responsible for the atropisomerism phenomenon and thus for the thermodynamic stability of concentrated solution of ICM.

Carbonyl groups are hindered to rotate around sigma bond CO-Ar, Ar-N bond too (atropisomerism phenomenon). The solubility can be modified varying the moieties bound to the side chains. ICM systems are characterized by aggregation phenomenon due to the interaction solute-solute through hydrophilic and hydrophobic interactions. The aggregation, in aqueous solutions of ICM solutions, causes a lowering of osmolarity.

This research is focused on the effect of the specific hydrophilic and hydrophobic interactions between solute-solute on the solution properties.These interactions are investigated at nanoscale level to understand and control the aqueous solution aggregation phenomena by both thermodynamic and spectroscopic measurements.

Heat of dilution measurements were performed by direct calorimetric experiments on Iopamidol, Iomeprol and Iopromide solutions. Dilution series were carried out from concentrations of about 1.5 m down to the limit of experimental accuracy. Heats of dilution data were elaborated to calculate the apparent relative enthalpy at infinite dilution. The most important result lies in the clear evidence that the dilution of Iopamidol and Iopromide in aqueous solutions is an exothermic process, while the Iomeprol in aqueous solution the dilution is endothermic. This difference must be explained in terms of different balance of the aqueous solutions energetics, where both solute-solute and solute-water interactions are involved.

Assignment of conformers and their population in solution has been assisted by recording 13C-NMR and 1H-NMR spectra of precursors of ICM. Experimentally, NMR spectroscopic technique was used mainly on aqueous solution of ICM to study the atropisomerism: 13C and 1H monodimensional spectra, hetero-related spectra (HSQC) omorelated spectra (NOESY) and diffusion spectra (DOSY).
For each nucleus (C and H) were measured the relaxation times T1 and T2 as a function of concentration whose relationship has been interpreted in terms of local mobility to identify the functional groups involved in intermolecular interactions. The variation of chemical shift as a function of concentration is not equal for all nuclei of C and H. The nuclei of C and H which show greater dependence with concentration are contained in the side chains of the molecules. The dependence of chemical shift with concentration allowed to say the side chains are responsible for the ICM intermolecular interactions.
In addition to evidence given from elaboration of the osmotic coefficient data, size of aggregates in solution was calculated by processing the results of diffusion experiments (DOSY) which provided information on the global mobility of these systems.
Work is in progress to correlate these data with MD simulation studies carried out on systems in a wide range of concentration.

COLD DENATURATION OF PROTEINS. THE ROLE OF WATER

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Introduction

Heat denaturation of proteins have been extensively studied, however cold denaturation process has not received similar attention. The interplay between temperature and pressure is still not completely understood and some controversies have been generated about the importance of water structure on the protein stability.

Pressures around 2 kbar strongly affect bulk water properties as viscosity [1], tracer diffusion [2], and rotational and translational diffusion [3]. In the surrounding of the mentioned pressure these properties change their trend showing profound modification in the structure, essentially its hydrogen bond network. At certain pressure the water behave as any 'ordinary' liquid. The results of Preimer *et al.* [3] shown that under pressure the rotational diffusion is enhanced more strongly than translational diffusion. A straight forward explanation is that the pressure weakens the hydrogen bond network allowing faster rotation of water molecules as tetrahedrical structure is broken down. The decreasing of hydrogen bonds under pressure was also observed by neutron scattering [4] and low angle X-ray diffraction [5]. Neutron diffraction experiments [6] at 268 K have observed continuous transformation from low density water species to high density water when the pressure is increased. The high density water has no tetrahedrical O-O-O angles and collapse of the second coordination shell. The decreasing on the number of hydrogen bonds by effect o pressure have been also confirmed recently [7].

These effects of pressure on the water lattice will alter the hydrophobic interaction, that relays on tetrahedrical coordination of water, altering the protein stability. Irrespective of other factors of denaturation it seems clear that the weakening of the main driving force of folding will be the most important effect of pressure on denaturation of proteins.

In this presentation we will show some results of molecular dynamics simulation of proteins under pressure and discuss the interpretation of cold denaturation based on the modification of the water hydrogen bond network on the hydrophobic interaction.

Methods

We carried out the Molecular Dynamic (MD) simulations using the GROMACS 3.2.1 package using all-atoms force field for the minimization

process, as well as for all the MD simulation. LINCS and SETTLE algorithm were used for bond constrain on proteins and water respectively. For the electrostatic forces we applied the Reaction Field method. Lennard-Jones interactions were calculated within a cut-off radius of 1.4 nm. Simulations were done at constant pressure and temperature using the Berendsen's thermostat and barostat.
The SPC/E water model [8] was used throughout. Besides its simplicity recent test for the hydrophobic effect have been successfully [9]. Relevant to the present study, PVT properties have been tested under a wide range of temperature and pressure conditions, results deviate in less than 1.0% from recent experimental data [10].

Results and discussion
Molecular dynamics simulations (MD) on lysozyme [11] and apomyoglobin [12] at 300 K and 3 kbar show the denaturation process with good agreement with the NMR experimental result. The lysozyme does not show a complete unfolding, probably due to the disulfides bridges, but clearly shown a large increment in the hydrophobic exposed area. In apomyoglobin the unfolding process start after around 20 ns and still is incomplete after 200 ns of simulation. An extrapolation considering a two state process suggests that the complete unfolding will be produced around the microseconds [13] which agree with the experimental expectation. In both cases the increasing in the solvent hydrophobic accessible area is remarkable when the system is submitted under pressure. We may ask what the mechanism that produces such effect is. Whatever the cause of denaturation will be, we expect the increasing of hydrophobic exposed area, both hydrophobic and hydrophilic. There are reasons to believe that the increasing of hydrophobic exposed area is not just a mere consequence of unfolding, and the driving force of denaturation is the weakening of hydrophobic interaction as a consequence of the gradual loss of the hydrogen bond network. Therefore, the increasing of the hydrophobic surface area is an essential element in the process. The above argument is reinforced by fluorescence experiments [14] in which the acting of pressure and chaotropic agents are compared. These effects are similar to the cleavage of hydrogen bonds produced by such compounds.
The pressure up to the range of 1-2 kbar does not affect the functionality of the proteins but over those values the denaturation start to be noticeable. This range is the one in which is observed the transition of the water structure from its particular properties to the regular behavior of most liquids; it looses the capacity to form terahedrical structures, even induced by a non polar surface. Above this limit the hydrophobic interaction begin to disappear, with the consequent changes in the protein structure.
Increasing temperature at normal pressure induce protein denaturation although protect it at some moderate pressure. This protection can be explained since increasing the temperature will keep the entropic term (-

$T\Delta S$) within the necessary value. This phenomenon has been seen clearly studying water order near to hydrophobic walls [15].
As regards the cold denaturation, lowering temperature decreases the favorable entropic contribution. Moreover, the tetrahedrical structure of water increases, making smaller the difference between the regular structure and those close to the hydrophobic solute. The thermodynamics of cold denaturation also deserves a more detailed treatment than the ones available nowadays; we will may some comments about that.

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HYDROGEN BOND PROPERTIES OF SACCHARIDE MATRICES STUDIED THROUGH INFRARED WATER ASSOCIATION BAND

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Introduction

Saccharides, and in particular trehalose, are known for their high efficiency in protecting biostructures against adverse environmental conditions [1], although the preservation mechanism is still debated. Experiments and simulations on carboxy-myoglobin (MbCO) have shown that the protein dynamics is highly inhibited in a low-water trehalose host medium, the inhibition being markedly dependent on the amount of residual water. Furthermore, different protein↔matrix coupling has been observed in dry saccharide systems and the peculiar effects of each saccharide have been related to its hydrogen-bonding (HB) properties [2,3]. The properties of the HB networks in saccharide-water have been studied by Infrared Spectroscopy (FTIR); in particular the so-called Water Association Band (WAB), at ~2100 cm^{-1}, has been found particularly useful to study the properties of water-containing HB networks, as this band is attributed to a combination of the water bending mode with intermolecular vibrational modes [3]. In saccharide matrices, at low hydration, the band is highly structured. We suggest that the sub-bands arises from different classes of water molecules, according to their interactions with other components in the system. The study of the effect of the salts of the Hofmeister series on the WAB in these samples gave hints for the assignment of the sub-bands to different water micro-environments.

Experimental

Here we report a FTIR study on solid amorphous matrices, at low water content, of five saccharides (trehalose, sucrose, maltose, lactose, raffinose) containing myoglobin at various protein/sugar mole ratios from 0 (no protein) to 0.1. For each sample the water content is assessed from the area of the intramolecular water combination band at 5200 cm^{-1}.

In these samples the WAB is structured in five recurring sub-bands. We studied the effects on these components of various salts, with either structure-maker or structure-breaker properties on the HB network [4], on trehalose matrices with either no protein or myoglobin/sugar mole ratio 0.025.

Results

The alteration in the shape and population of the WAB components, induced by the salts, enabled us to roughly assign the sub-bands to different classes of water molecules. Then, this classification turns to be useful to assess the effects that different sugars, as well as different sugar/protein mole ratios, have on the water molecules' environment, i.e. on the HB network of the system.

Results showed that, despite similarity in molecular structure, the five saccharides show noteworthy differences in the WAB shape. Upon addition of protein, some saccharides matrices (e.g. sucrose) present only a little alteration of the WAB, indicating a small perturbation of the HB network, while others (e.g. trehalose) show strong protein effects, with alteration of the band shape and of the population of the components. Moreover at definite protein/sugar mole ratios, a “collapse” has been identified for all the sugars, which divides a regime in which the band looks similar to the one in a binary matrix from a different one, in which it appears dominated by the presence of protein. The different features of the HB networks present in the five sugar matrices, as probed by water molecules, and their response to protein addition, are helpful in clarifying the mechanism of saccharide based bioprotection and trehalose peculiarity.

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ION-SPECIFIC EFFECTS ON THE STRUCTURE AND DYNAMICS OF ELECTROLYTE SOLUTIONS CONFINED IN NANOPORES

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Introduction

Due to global climate changes and increase in the world population, access to drinkable water has become one of the most important issues for the next years. In this context, nanofiltration membranes are promising candidates for desalination as they allow a great volume of water to pass while retaining the ions and as they have proven to be very selective and to enhance the flow of certain species[1, 2, 3, 4].

Computational details

We performed a Molecular Dynamics study of aqueous electrolyte solutions confined in hydrophobic nanomembranes modeled as a single-walled carbon nanotube. We examined the effect of the size and polarizability of the ions[5, 6, 7] on the structure and dynamics of the confined electrolyte solution by considering the series of sodium halides (NaX with X = F, Cl, Br and I). We also address the effect of pore size by varying the diameter of the carbon nanotube.

Results

As far as structural properties are concerned, the behavior of the NaF electrolyte solution significantly differs from that of the other sodium halide solutions. Due to their small size, Na and F in NaF are found to be significantly solvated by water. On the other hand, due to hydrophobic effect [8], Cl, Br, and I tend to be repelled from the regions where the density of water is large.

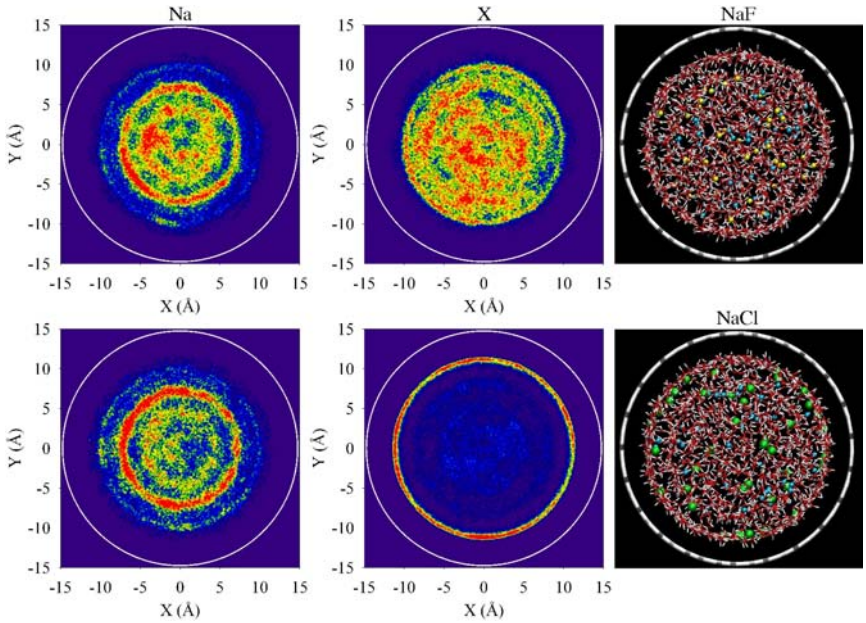

Figure 1 Density contour plots and typical molecular configuration for NaF and NaCl electrolyte solutions confined in a carbon nanotube of a diameter D = 3nm. The density in the contour plots increases from purple, blue, green, yellow, orange, and red. Grey segments in the molecular configuration are bonds between the carbon atoms of the nanotube while the white and red segments are the water molecules. The blue, yellow, and green spheres are the Na, F, and Cl ions, respectively.

Ion-specific effects on water and ions dynamics are found to be diminished when the electrolyte solution is confined at the nanoscale. For instance, both the data for water and the ionic species indicate that the ratio of the self-diffusivity for the confined material to that for the bulk is independent of the nature of the anion F, Cl, Br, I. Moreover, while the average solvation times for Na in NaF and Na in NaX (X = Cl, Br, I) significantly differ for bulk electrolyte solutions, they are very similar when considering the confined solutions. Such an attenuation effect of confinement on the dynamical properties of the electrolyte solutions is also observed on the pairing of the anions and cations.

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ION TRANSPORT THROUGH A HYBRID MEMBRANE MADE OF GRAMICIDIN-A CONFINED IN A NANOPOROUS POLYMER FILM

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Introduction

Recent advances in membrane sciences allow the use of membranes in many fields such as food and agriculture, potable water production, industrial water treatment and biotechnology. Among the newly developed technologies, nanofiltration for liquids is the most recent one. One of the scientific challenges is to develop nanofilters with both high permeability and selectivity, which are often considered as antagonist features. Today, the number of solid state membranes that are readily useable is limited and their performances, i.e. permeability and selectivity, are restricted. New materials for nanofiltration are thus clearly requested. The building of a hybrid membrane made of a thin nanoporous polymer film in which ionic channels, known for their high selectivity and permeability properties, are confined can therefore be thought as a challenging way to develop successful nanofilters. However, as far as we know, no investigation on that topic has ever been reported. In this paper, we present the first extremely encouraging results obtained on a hybrid membrane made of a track-etched polycarbonate thin (i.e. 5µm) film whose the nanopores are filled with gramicidin-A. Diameter of nanopores is 15nm. The ionic channel studied here is gramicidin-A, a protein that allows the transport of K^+ [1] and Na^+ [2] but totally hinders that of divalent cations when it is inserted in a living cell membrane. This ionic channel is one of the simplest and hence one of the most studied by physiologists [3].

Experiments and results

The first essential step of this work is to control the insertion of gramicidin-A into the nanopores of the polymer thin film. The membrane is first treated in ethanol and then poured in a solution which contains gramicidin-A. The latter is either pure or labeled with alexa fluor 594. In order to check whether gramicidin-A is properly inserted into the nanopores and not simply deposited at the polymer surface, the membrane is studied by mean of confocal fluorescence spectroscopy [4]. We report in fig.1a the diffusion and

fluorescence intensity measured across the membrane at the vicinity of a nanopore.

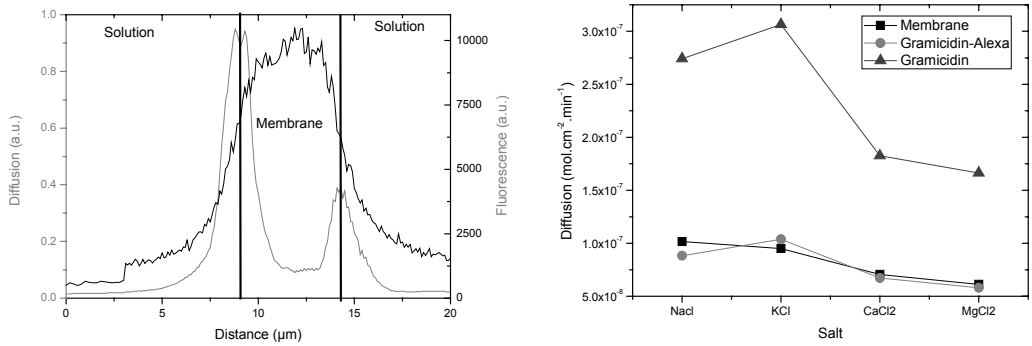

Figure 2. (a) fluorescence spectra measured on the 15nm nanopores hybrid membrane obtained after 72h of impregnation in a gramicidin-Alexa fluor 594 solution. Maxima of the diffusion signal (full gray line, right axis) correspond to the membrane/solution interface. The fluorescence intensity (full black line, left axis) shows that gramicidin-A is localized inside the nanopore. (b) ionic diffusion: raw (black square), impregnated with pure (dark gray triangle) and labeled gramicidin-A (gray round) membranes.

Then, we investigate diffusion of 10^{-2}M Na^{+}, K^{+}, Ca^{2+} & Mg^{2+} chlorine aqueous solution through the membrane. Ionic charge diffusion determined at the beginning of the diffusion process are reported in fig.2 for the raw membrane and the membranes impregnated with gramicidin-A. When pure gramicidin-A is inserted into the polymer thin film nanopores, we observe that (i) ionic diffusion is significantly enhanced (ii) diffusion of divalent cation solution is much lower than that of monovalent cation and (iii) a slight inversion between NaCl and KCl solution. Moreover, we can see the "blocking" effect due to the alexa fluor 594.

Conclusion

For the first time an ionic-channel, i.e. gramicidin-A, is successfully confined into the nanopore of a thin polymer film and the effect of this confinement on ionic diffusion is clearly identified. Of course, much more insights into the microscopic mechanisms involved in this phenomenon are requested. Nevertheless, these primary results open an extremely promising field of research in the domain of nanofiltration.

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THE PROTEIN GLASS TRANSITION AND ITS RELATED DYNAMICS

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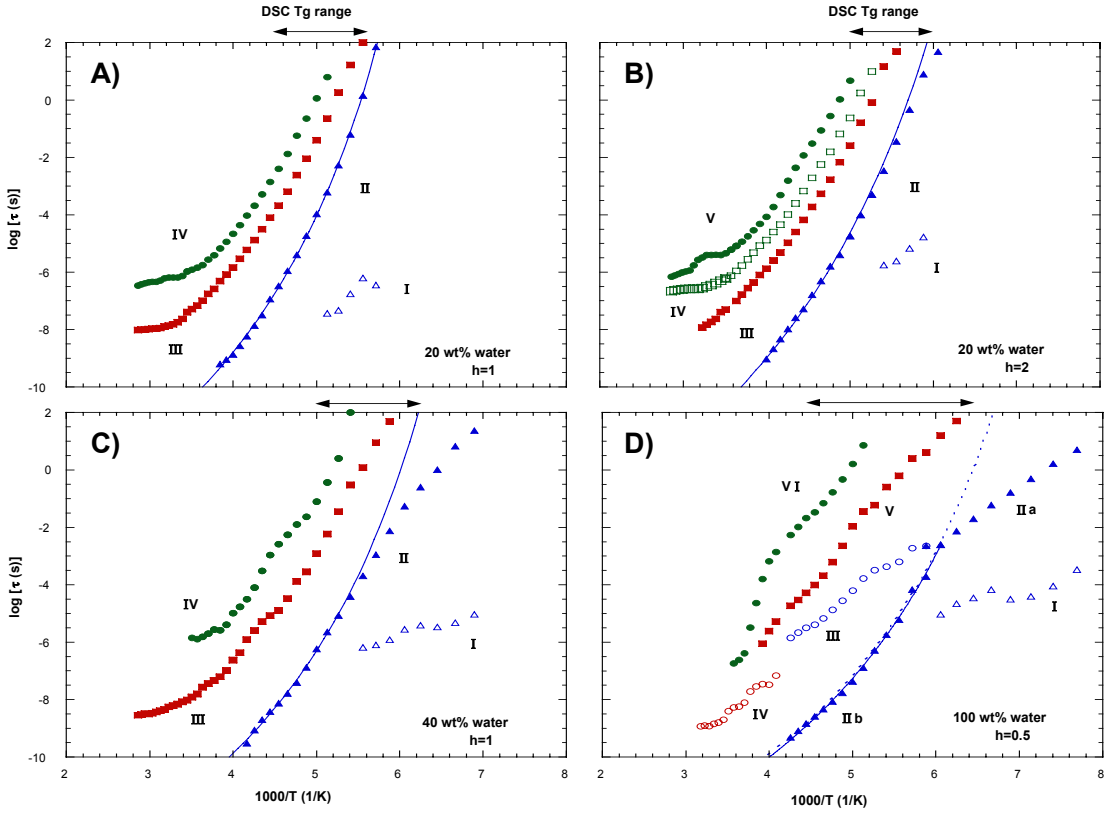
The dynamics of a protein is determined by its energy landscape, but due to the complex and continuously changing structure of a protein this energy landscape is very difficult to determine. The situation is very similar to that of ordinary glasses (for proteins at low temperatures) and supercooled liquids (for proteins above the glass-transition-like onset of large-scale motions around 200 K) [1,2], with the exception that for proteins the increase of fluctuations with increasing temperature is not directly caused by the increasing thermal energy. Rather, the protein motions are promoted by temperature induced increasing solvent motions. Thus, the motion of a protein from one substate to another seems to be driven by the solvent.

The glass transition of hydrated proteins has been investigated by calorimetric and rheologic measurements, see e.g. [3-6], and it has been shown that, apart from that this transition can be tricky to observe, it is exceptionally broad [4-6], i.e. the protein glass transition is not characterised by one specific temperature or a narrow temperature range. The broadness of the transition has e.g. been suggested to be due to a large distribution of relaxation times within the protein-water system [4] or be caused by a size distribution of water clusters on the protein surface [6].

In this study we have investigated myoglobin in different water-glycerol solvent mixtures by use of broadband dielectric spectroscopy (DS) and differential scanning calorimetry (DSC) with the aim to understand the role of hydration water for the dynamics and glass transition of proteins. In particular the aim has been to relate the, in general, very broad calorimetric glass transition ΔT_g in the protein-solvent system to the relaxation processes obtained by dielectric spectroscopy.

From our results we are able to show that a number of processes, due to both solvent and protein relaxations, participate in the glass transition region of proteins, and that the broadness of the transition depends on the water content in the solvent [7]. More specific, for water rich solvents we find that below a crossover temperature at roughly 170-180 K no α-relaxation is observed in the solvent, and under such conditions no clear protein processes are observed. Thus, this crossover in temperature dependence (and the corresponding change in physical nature of the water dynamics [8]) seems to be responsible for the glass transition of hydrated proteins [7]. Likely, it is the onset of long-range cooperative water motions at the

crossover temperature that initiate the larger-scale protein motions present above the glass transition temperature.



Dielectric relaxation times and the DSC glass transition range ΔT_g for some of the measured samples. Total solvent contents and water fractions (given in wt%) in the water-glycerol mixture solvents as given in the figure.

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AGGREATION-PRONE REGIONS OF THE STRUCTURED F1-ATPase IN AQUEOUS SOLUTION.

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There is considerable interest in understanding the dynamics of protein association to form in aqueous solution organized structures, such as neurofibrillary tangles (NFT) and amyloid fibrils. These structures are composed of protein molecules stacked in a cross beta-sheet conformation with parallel strands that stretch perpendicular to the fibril elongation axis. Their formation in water solution from structured globular proteins likely requires partial unfolding leading to adoption for an aggregation-competent conformation, i.e. by exposure to water of regions previously buried inside the proteins, and do not involve severely denaturating conditions. Because seeding with preformed fibrils accelerates the process, this is considered resembling crystal growth from a aqueous solution.

Accumulation of aggregated structures originating from around 25 different proteins (often specific for each disease) and the associated impairment of cellular function and cytotoxicity is common to many human and animal degenerative diseases. In particular, in Alzheimer's disease (AD), it was unexpectedly detected the association of the ATP synthase alpha subunit with the NFT, which are located in cytosol and are composed of modified microtubule-associated Tau proteins. As alpha subunit accumulation has been observed even at early stage of AD, it has been proposed that it is a molecular event related to neurodegeneration and may be a possible target for therapeutic strategies [1].

ATP synthase is a large multi-subunit complex which is localized in the inner mitochondrial membrane, where it catalyses the aerobic synthesis of ATP by rotational catalysis [2], but also at the plasma membrane level in different tissues [3], including neurons and myelin sheath [4], where it makes different additional functions. Alpha-chain is a 55 kDa globular subunit of the F1 hydrophilic part of the enzyme, named F1-ATPase, where it is present in three copies associated with three copies of the catalytic beta chain.

The aim of this work is to define the aggregation-prone regions of ATP synthase and to provide mechanistic insight into protein stabilization and prevention of protein aggregation in aqueous solution, thus contributing

to the development of NFT inhibitors, which are still lacking despite of the enormous effort devoted to these studies.

The computational analysis performed according to [5] confirms that only alpha subunit has a high propensity for aggregation, in accordance with [1]. Interestedly, the beta chain of F1-ATPase, which has around 45% homology with the alpha subunit [2], does not show tendency to aggregation, suggesting that a limited number of alpha chain regions are determinant for the process. An *in vitro* model is developed, which consists in the aqueous solution of the whole F1-ATPase complex containing the structured alpha and beta chains together with gamma, delta and epsilon subunits. The effects of different physicochemical factors on protein partial unfolding and association are studied, such as altered pH, temperature, anions in solution with different valences, cosolvents such as glycerol, and exposure to air-water interface. The results show that the structural context of F1-ATPase is crucial for determining the aggregation process.

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HYDRATION OF CATIONIC POLYELECTROLYTES

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Ionenes are cationic polyelectrolytes with quaternary ammonium groups located in the backbone of a polyion (Figure 1). Analogues with different values of *x,y* can be prepared [1], as well as solutions with different counterions. Thermodynamic and transport properties depend on both factors: charge density of the macromolecule determined by x,y, and on chemical nature of counterions [2-6]. Ionenes show some pharmaceutical and medicinal potentials but more fundamentally they present an ideal study compounds, since systematic studies of effects of polyion's charge density and nature of counterions on the solution's properties can be performed. The findings can be helpful in understanding more complex biologically important polyelectrolytes (proteins, nucleic acids).

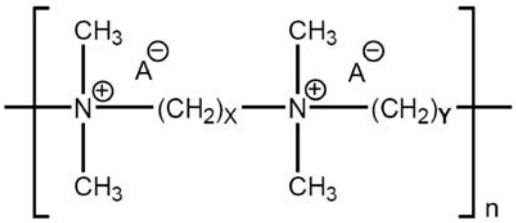

Figure 1. Repeating unit of *x,y*-ionene

In this contribution we present some experimental and theoretical evidence for different hydration behaviour of 3,3; 4,5; 6,6; and 6,9-ionene bromides and fluorides. We mesured the transport numbers of a ionene polyion-constituent and of counterions (bromides, fluorides) in water solutions. Together with our experimental data for the conductivity of ionene solutions in question we were able to calculate the fractions of the "free" counterions in the solution, *f*. Markedly different behaviour in trends of *f* on charge density of the ionene was observed in regard with the type of the counterions. For ionene bromides, the fraction of "free" counterions increased with the charge density of the ionene (3,3 < 4,5 < 6,6 < 6,9), while the values of *f* for fluoride counterions were approximately constant. This implies different nature of interaction of bromide and fluoride ion with the polyion. We state that the nature of interaction of bromide counterion with the ionene's backbone is predominately electrostatic, while the fluoride counterion interacts, so to say, "hydrostatically". Bromide ions are only weakly hydrated and loose their hydration shell waters when interacting with the charges of the macromolecule. On the other hand the hydrated fluoride

ion behaves like a charged hard sphere and stays hydrated even upon interaction with the polyion, the fluoride counterion shares its water with the polyion. This picture is consistent with our recent research on dielectric relaxation properties of the above-mentioned ionenes [4,5].
To supplement the picture of structuring of the water near the polyelectrolyte chain we performed an NpT Monte Carlo simulation experiment. The model of a polyion was a chain of 2D discs (charged or interacting only through Lennard-Jones potential). Such polyion was simulated together with MB-dipole waters [7, 8]. Figure 2 shows a 2D distribution function of water molecules around a model segment of a "3,3-ionene". Left, middle, and right discs are positively charged, and we see that the density of water molecules is increased in their vicinity. Water is slightly pushed away from the hydrophobic disks. From snapshots from the simulation we observed that around hydrophobic units water organizes itself in a way that enables formation of cavities. This effect is more pronounced for polyions with lower charge density. We think that such cavities can be occupied by fluoride ions. The picture is supported by a more realistic molecular dynamics study of a 3,3-ionene [9].

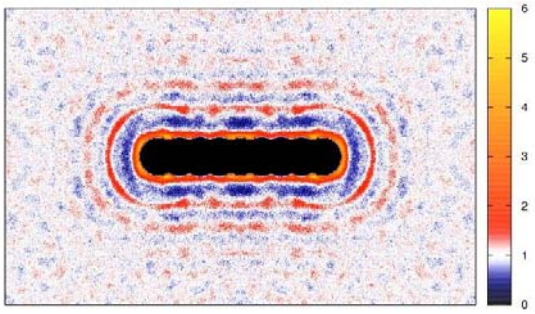

Figure 2. Distribution function of water molecules around a model polyion.

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THE DENSITY AND THERMAL EXPANSION OF HYDRATION WATER NEAR BIOSURFACES

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The volume and the thermal expansivity of biomolecules are highly sensitive to their structure and conformation, thus, making volumetric and pressure perturbation calorimetry measurements able to detect and characterize the conformational behavior of biomolecules [1].The available experimental methods measure the total changes of the solution properties due to the presence of biomolecules, giving information about their *apparent* volumetric properties which include both the intrinsic properties of biomolecules and the difference of the properties of hydration water from those in the bulk. Both experiments and simulations evidences that various properties of hydration water are markedly different from the bulk [2]. In particular, the difference between the density and thermal expansion of hydration and bulk water makes an important contribution to the apparent volumetric properties of biomolecules measured experimentally. Knowledge of the volumetric properties of hydration water allows evaluation of the intrinsic properties of biomolecules [3].

The density of hydration water ρ_h near various bimolecules was studied in a wide temperature range by all-atomic simulations [4]. In all cases, ρ_h was found to be lower than the water bulk density (Fig. 1). The temperature dependence of ρ_h is close to linear, that is strongly different from that in the bulk water and agrees with theoretical expectations [5].

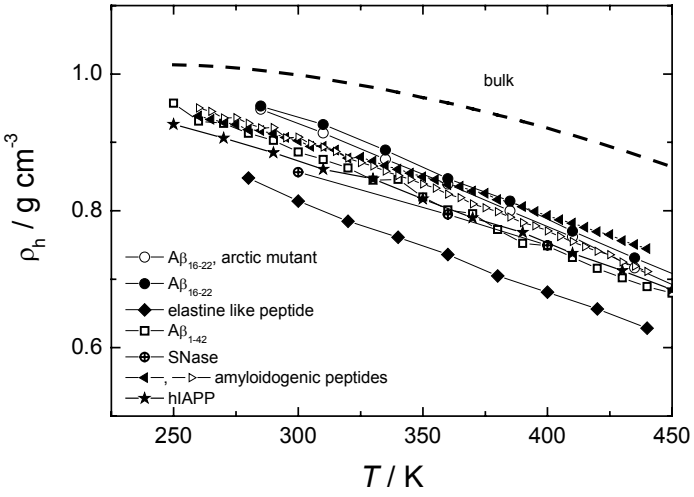

Figure 1. Density of hydration water near the surface of various biomolecules

Thermal expansion coefficient of water near biological surfaces (α_h) was found to be notably larger than that in the bulk (Fig. 2). In case of small or relatively hydrophobic biomolecules, the contribution to volumetric properties arisen from the hydration water masks completely the intrinsic behaviour of biomolecules. We propose a method which allows estimation of the intrinsic volumetric properties of biomolecules in simulations [6,7] and in experiment [3].

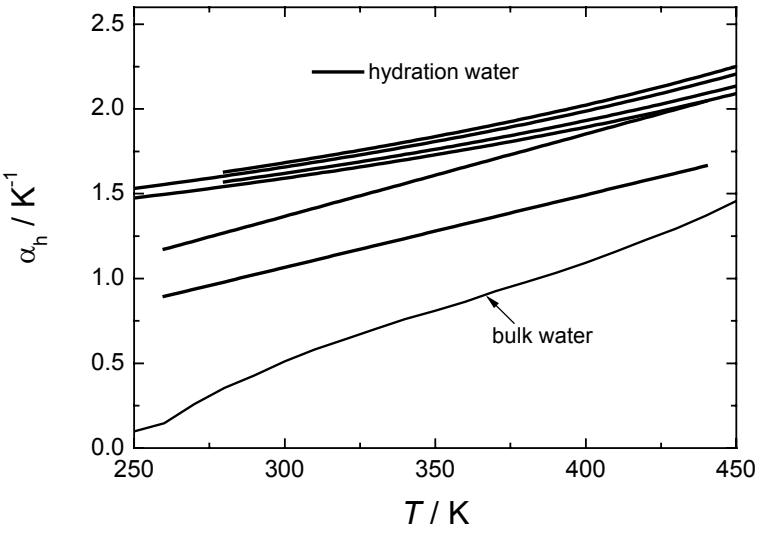

Figure 1. Thermal expansion coefficient of hydration water near the surface of various biomolecules listed in Fig. 1.

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COLLECTIVE DYNAMICS OF INTRACELLUAR WATER IN LIVING BACTERIA

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Water dynamics plays a fundamental role for the fulfilment of biological functions in living organisms. Decades of studies show that water in hydrated protein powders displays peculiar dynamical properties with respect to pure water, as concerns both collective and single-particle dynamics. These results tend to suggest that water is "tamed" by the interaction with the macromolecule and thus serves the needs of biological functionalities [1]. On the other hand most results concern simplified model systems, while very few studies of water in real living organisms at physiological condition exist to-date. By means of coherent inelastic neutron scattering on fully deuterated *Escherichia coli* cells, we present here an *in vivo* study about the collective dynamics of intracellular water in living bacteria. The results are discussed and compared with existing literature about both pure [2] and protein hydration water [3].

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STRUCTURE OF PROTEIN AND DNA HYDRATION WATER FROM ITS VIBRATIONAL FEATURES.

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The nature of the hydration water of biomolecules has been investigated through inelastic neutron scattering experiments to shed light onto its structural properties. Here we report the results found at low temperature for DNA disordered fibers and deuterated maltose binding protein (MBP) powders. In both cases, below a certain hydration degree, the solvent density of states shows significant similarities with that of high- and low-density amorphous ice. Only at rather large water content, some vibrational features reminiscent those of Ih ice-like ordered structures begin to appear. From the comparison between the cases of DNA and MBP some speculations are made on the specific interaction of nucleic acids and polypeptides with water. The possible relationship of the disordered amorphous-like character of the biomolecule hydration water with functionality and cryoprotection is also discussed [1].

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BSA AGGREGATION IN TREHALOSE-WATER SYSTEMS

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Introduction

Recent studies [1] on ternary protein-trehalose-water samples have shown that the protein thermal denaturation temperature is linearly correlated with the glass transition temperature of the system, despite the quite large temperature difference between the two processes. In such studies it is stated that the collective, long range properties of the matrix that regulate the glass transition are strictly correlated with local features on which the denaturation of the protein depends. In order to ascertain whether an analogous correlation exists between the effects of trehalose on the protein's aggregation process and the glass transition temperature of the system, we performed Light Scattering measurements on thermal aggregation of Bovine Serum Albumin (BSA) in presence of trehalose at various concentrations.

Experimental

We used Static and Dynamic Light Scattering to study the thermal aggregation of BSA in buffered aqueous solution, at 0.1% w/w protein concentration and in presence of trehalose, whose weight concentration was varied from 0 to 50 % trehalose/(trehalose+water). Measurements were performed in the temperature range 50 °C – 80 °C.

Results

Addition of trehalose appears to cause temperature shifts of the entire process towards higher values. In particular, the effects of trehalose on the temperature of the aggregation process appear to be linearly correlated with the effects of the sugar on the glass transition temperature. The latter quantity was estimated through the Gordon-Taylor equation [2] with already reported parameters [1]. Addition of sugar is also responsible for shape changes in plots of scattered light intensity versus temperature during temperature scan. Such changes reflect differences in the aggregation process, which can be sorted out through a suitable analysis of the increase of the hydrodynamic radius during the temperature scans. In particular, we determined the fractal dimension of the aggregates, which gives information on their packing.

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AGGREGATION PROPERTIES OF PROTEINS UNDER HIGH PRESSURE

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Fourier transform infrared spectroscopy (FTIR) coupled with High Pressure (HP) techniques is a suitable tool to investigate unfolding/misfolding processes providing useful information on the kinetics of aggregation of proteins[1]. Since HP affects only the volume contribution to the Gibbs free energy, it is able to perturb the structure of proteins in a reversible way [2,3]. The principle governing pressure effects is that it tends to shift a system towards the state that occupies the smallest volume, it causes the electrostriction of charged and polar groups, the elimination of packing defects, and the solvation of hydrophobic groups. Cavities and packing defects are expected to be major contributors to volume changes and their presence will make the system more susceptible to pressure unfolding/dissociation. Because high pressure allows stabilization of folding intermediates such as molten-globule conformations, this method provides an unique opportunity for their characterization. We present here latest developments in the set up of a high pressure infrared facility for the study of protein folding misfolding and aggregations at the SISSI beamline at Elettra.

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LIQUID-VAPOUR PARTITION OF AROMA COMPOUNDS IN SUCROSE AND TREHALOSE SOLUTION

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Introdution

The flavor perception is mainly related to the release in the gas phase of volatile aroma compounds present in the food. Kinetic and thermodynamic mechanisms control the rate of release and the concentration of the volatile in the head space that, in turn are affected by intrinsic characteristics of the aroma and the food matrix (chemical, physical and physico-chemical properties), environmental parameters (e.g. temperature, pressure) as well as by the interactions with non volatile compounds occurring in the system. In the last decades many studies have been carried out to understand the effects of these interactions and in particular those with macromolecules (proteins, carbohydrates, etc.) and mainly in simple model systems[1]. On the contrary, scarce attention has been given to small solutes (i.e., salts, mono- and disaccharides) in affecting the liquid-vapor partition of aroma compounds even if their presence in a food is known to influence some important physico-chemical and physical properties of the system and in particular those of the water phase.
Thus, aim of this study was to investigate the effect of nature and concentration of two disaccharides (sucrose and trehalose) on the liquid-vapour partition of aroma compounds in binary aqueous solutions.

Experimental

Liquid-vapour partition coefficient of four esters (ethyl acetate, ethyl butyrate, isopentyl acetate and ethyl hexanoate) was determined in aqueous binary (water-disaccharide) solutions made with sucrose and trehalose at solute concentration 0- 50% w/v.
The phase ratio variation (PRV) method [2] was used to determine the partition coefficients *k* of the flavors under thermodynamic equilibrium conditions, by HS-GC analysis;
Water activity, surface tension measurements and FTIR spectroscopy were also carried out on the sugar solutions with or without the aroma compounds.

Results

Results evidenced an influence of both the nature of the aroma compound as well as the sugar type and its concentration on the partition coefficient (*k*).

In water and in sugar solutions up to 20 % w/v, according to other authors, at increasing number of the carbon atoms of the ester, a higher *k* value was observed [3]. The lower *k* value was determined for the more polar compounds (ethyl acetate, ethyl butyrate) whilst the highest k was that of ethyl hexanoate.
Moreover, in general, the values of the partition coefficient of the volatile esters in the trehalose solutions were lower ($p < 0.05$) than the sucrose one at similar solute concentration even if the water activity values of the same solutions were not significantly different.
At sugar concentrations above 20%, a increase of the partition coefficient was observed in particular for the more hydrophobic esters. A non linear variation of *k* with a "belt shape" trend was observed for ethyl hexanoate, showing its maximum in the sucrose solution at 40% (w/v) followed by a significant decrease of the partition coefficient in the more concentrated solution of this disaccharide (45% w/v). In the trehalose solutions a similar trend was observed but the highest k value occurred in the solution at 45%w/v.
FTIR spectra of the sugar solutions with or without aroma compounds did not show meaningful changes index of possible sugar-aroma interactions even at the very high solute concentrations investigated. These results could be explained taking into account changes in the physico-chemical and physical properties of the sugar solutions (i.e. formation of hydrophobic micro-regions in the more concentrated solutions[4]) that could favor the retention in the matrix by physical entrapment.

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DIELECTRIC STUDIES OF MOLECULAR MOBILITY IN PROTEIN-WATER MIXTURES

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In this work we employ broadband dielectric relaxation spectroscopy (DRS) and thermally stimulated depolarization currents (TSDC) techniques to investigate water and protein dynamics in mixtures of water and bovine serum albumin (BSA) over wide ranges of composition, from dilute solutions (20 wt% protein) to practically dry protein pellets.

Figure 1 shows TSDC thermograms obtained with hydrated solid samples and with solutions. In terms of the more familiar DRS, a TSDC plot corresponds to measuring dielectric losses as a function of temperature at a fixed low frequency in the range of 10^{-2}-10^{-4} Hz. The pronounced peak at about 150 K for the dilute solutions with 20, 40 and 53.8% protein is attributed to the relaxation of crystallized water (ice). The broad peak in the same temperature range for the high protein content samples contains dipolar contributions from the protein, plasticized by water (shift to lower temperatures) and possibly dipolar contributions from uncrystallized (bound) water. The peak at higher temperatures for the relatively dry solid pellets, clearly observed for the 90.2% protein content, has been associated with the displacement of protons on the surface of the protein molecules. At water contents higher than 20% (curves 1-6) there is a peak (double for some compositions) in the range of 180-200 K, which is associated with the hydrated protein glass transition.

The complex DRS spectra of the various compositions were analyzed by fitting appropriate model functions to the experimental data. The analysis provides for each of the relaxations information on the time scale, the magnitude (relaxation strength) and the shape. Time scale can be best discussed on the basis of the Arrhenius plot (activation diagram) shown in Figure 2 for the three processes I, II and III observed in the dilute solutions of 20, 30 and 40% BSA, where results are more clear. Process I, which shows distinct changes in time scale (as well as relaxation strength) at about 240 K (partial melting of ice) and 200 K (protein glass transition) is attributed to relaxation of uncrystallized water. Process II originates from crystallized bulk water, whereas process III, which has the same time scale in the three mixtures of Figure 2, is associated with the protein glass transition.

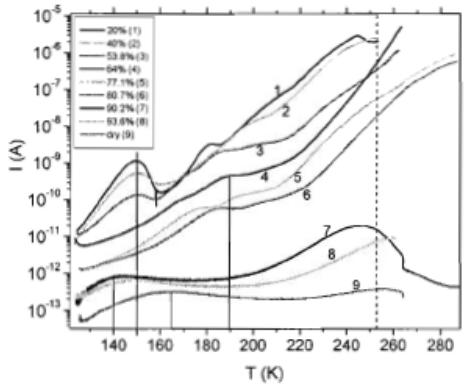

Figure 1. TSDC thermographs of BSA/water mixtures. The vertical lines correspond to the local maxima of the thermographs at low temperatures. The dashed vertical line highlights the polarization temperature T_p = 253K.

Extrapolation of the contours of the three processes to a relaxation time of 100s gives temperatures very close to characteristic temperatures determined by adiabatic calorimetry in similar BSA aqueous solutions, providing further support for the interpretation of the DRS data. DRS spectra obtained with hydrated solid samples are more complex comprising several contributions. Results obtained with the solutions and results of gravimetric water sorption measurements provide assistance in their analysis.

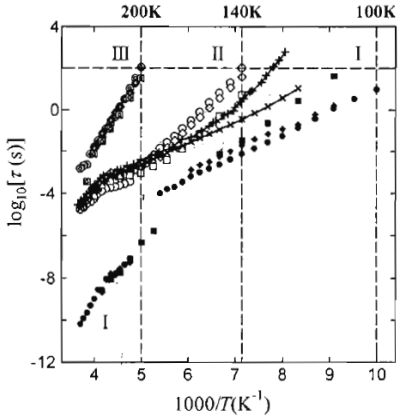

Figure 2. Arrhenius plot of processes I (solid symbols), II (open symbols), and III (double symbols) for the 20 (circle), 30 (diamonds), and 40% (squares) BSA-water mixtures. Crosses and pluses connected by lines are data of pure ice. The horizontal dashed line corresponds to τ=100s.

Aknowledgements
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USE OF SYNCHROTRON X-RAY MICROTOMOGRAPHY TO INVESTIGATE MICROSTRUCTURAL PROPERTIES OF COFFEE BEANS AS AFFECTED BY WATER STATE

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Introduction

Water is the most effective plasticizer in food matrices, decreasing glass transition temperature (Tg) and mechanical resistance and determining a softening effect with the increasing of its concentration [1]. An opposite effect (i.e. hardening, toughening), referred as 'antiplasticisation' has been observed upon hydration in polymers as well as in several low moisture, glassy food matrices (i.e. freeze-dried meat, vegetables, seeds) [2] as well as in green and roasted coffee beans [2, 3]. Some hypothesis on this phenomenon have been identified but antiplasticisation appears as a complex phenomenon. There is a lack in studies aimed to a better understanding of the relationship between the state of the water, the structural (micro- and macro-) and compositional properties of the matrix and the antiplasticisation effect of water [2]. In the case of coffee beans, DSC and NMR studies showed that plasticization occurs only after the completion of the monolayer hydration, where water behaves as a bulk-like agent [3]. Synchrotron X-ray computed microtomography (mCT) was in this study used as a non-destructive imaging technique to investigate the microstructural properties of green and roasted coffee beans at different levels of hydration.

Experimental

Evaluations were carried out on single, entire beans of *C. Arabica (*Brazil) at their native (green beans) or original (dark roasted beans) moisture content as well as on samples at different hydration degree obtained by dehydration and further rehydration to cover the range of hydration in which antiplasticisation and plasticisation occur in coffee beans [2]. Experiments were carried out on samples at the following water activity values: 0.1 (dried samples), 0.6, 0.76 0.84 and 0.91. The mCT data were collected at the SYRMEP beamline of Elettra in Trieste (Italy). Projections images have been recorded at beam energies of 19 keV and 20 keV for green and roasted beans, respectively. A 12 bit water cooled CCD camera with a pixel size of

9x9 microns^2 has been used as detector. The reconstruction of tomographic slices was performed using a software developed at Elettra [4].

Results

The use of the X-ray mCT with a monochromatic and parallel beam allowed us to obtain images with beam artefacts strongly reduced with respect to images obtained by conventional X-ray sources. Thus it is possible to perform precise morphological and textural analyses of the coffee beans and as affected both by processing and moisture content by means of a non-destructive methodology. The quantitative morphological analysis has been carried out by the *Pore3D* software library custom implemented at Elettra [5].

The results of 3D investigations of green and roasted coffee at their original hydration degree (a_w = 0.6 for green beans and a_w = 0.1 for the roasted ones) evidenced the main changes induced by the roasting process. The green beans showed a structure with closed cells full of fat and proteins and scarce porosity (9-15%). Roasting leads to a porous structure (up to 44-47%) with large pores generated by the heat-induced degradation of the organic matter and the high pressure induced by water evaporation and CO_2 production during the process. The increase of hydration degree in both green and roasted coffee beans leaded to a progressive increase of the pore size and in the case of the dark beans at the highest a_w value (0.91) a maximum diameter of 70 μm was measured, significantly larger than that observed in the correspondent sample at low hydration degree (a_w: 0.1).

The results of these analyses could improve the understanding of the role of water in affecting the mechanical properties of the cellular food products and in particular those related to the hardening (antiplasticisation) and softening (plasticisation) effects due to hydration.

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HYDRATION PROPERTIES OF CYCLODEXTRIN NANOSPONGES INVESTIGATED BY VIBRATIONAL SPECTROSCOPY

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Cyclodextrin nanosponges (CDNS) belong to an important class of polymers obtained by reacting cyclodextrins with suitable cross-linking agents [1-3]. The final nanosponge structure contains both cyclodextrin lyphophilic cavities and carbonate bridges, leading to a nanoporous network of more hydrophilic channels. A given chemical (organic, inorganic, molecular or ion) substance may in fact diffuse inside the three-dimensional network formed by the polymer (polymer swelling) and remain confined within the pores of CDNS lattice, modifying or amplifying its physico-chemical properties, to be finally released in a controlled way. The considerable inclusion/release properties of CDNS make them highly attractive as nano-vehicles in many technological fields, such as drug delivery, biocatalisys, agricoltural chemistry and absorbant for polluting wastes in environmental control [4-6].

Recently we focussed our attention on 3 types of βCDNS obtained varying the molar ratios of β-cyclodextrin and pyromellitic dianhydride, in order to modulate the pore size of the polymers which is, in turn, related to their swelling ability [7]. Raman and IR spectra of dry and water treated βCDNS were collected and compared in a wide spectral range (1-4000 cm^{-1}) and the changes observed in the spectral features have been associated with structural changes in the polymer network. In particular, the hydration-induced changes of the O-H and C-H vibration modes in the CDNS spectra have been discussed, providing important information on the state of water inside the nanoporous network of swollen CDNS, with particular emphasis on the diffusion phenomena in the gel-like state.

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INFLUENCE OF WATER ACTIVITY AND MOBILITY ON PEROXIDASE ACTIVITY IN MALTODEXTRIN SOLUTIONS

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Introduction

Water availability in biological systems is related to water activity (a_w), which describes water 'freedom' in terms of relative water vapour pressure [1].

In dry solid foods, the enzymatic activity is inversely related to a_w, whilst in liquid models this relationship is not always valid [2,3] because the a_w depletion is reached by increasing the concentration of solutes and this affects both the water content of the system and its molecular mobility.

Molecular mobility in foods system is often couched in terms of glass transition temperature (Tg) and bulk viscosity. Viscosity and Tg are physically related among them through the WLF equation; thus, in some studies, T'g is taken as the reference temperature instead of Tg since the former is exclusively related to the molecular weight of the solute.

Tg/T'g dictated mobility seems to take part in the regulation of the rate of enzymatic reactions [4,5]; however, the studies on the effect of water state and molecular mobility on enzyme activity are still scarce and the individual effect of these variables has not been disclosed. Some authors [1,6] suggested that enzyme activity should be related to both solvent characteristics, described by a_w, and molecular mobility as described by viscosity and Tg.

This study was aimed to investigate the effect of a_w, bulk viscosity and T'g on the activity of horseradish peroxidase (HRP) in buffered solutions added with maltose and maltodextrins of different molecular weight.

Experimental

Concentrated buffered solutions with the same bulk viscosity (40 mPa s) were prepared using 0.1 M potassium phosphate buffer, maltose and maltodextrins ($\overline{M}_W$: 994, 1266, 2500, 8000).

Peroxidase activity was tested in buffered solutions. Viscosity was measured using a falling ball viscometer. Water activity was measured using a dew point hygrometer. All measurements were carried out at 25 °C. Differential scanning calorimetry was used for the T'g determination. Thermograms were obtained after annealing at T'g + 10 °C.

Results

Maltose and maltodextrins additon inhibited the HRP activity. Viscosity being equal, solutions added with low molecular weight maltodextrins inihibited the HRP activity more than maltose in despite of their higher a_w value (Figure 1). This could be explained by a decrease of the system molecular mobility due to a T'g increase. On the other hand, HRP activity increased with the increasing of maltodextrins molecular weight even though the T'g dependent mobility decreased. The a_w increase could explain this result but not in the case of high molecular weight maltodextrins (Figure 1).

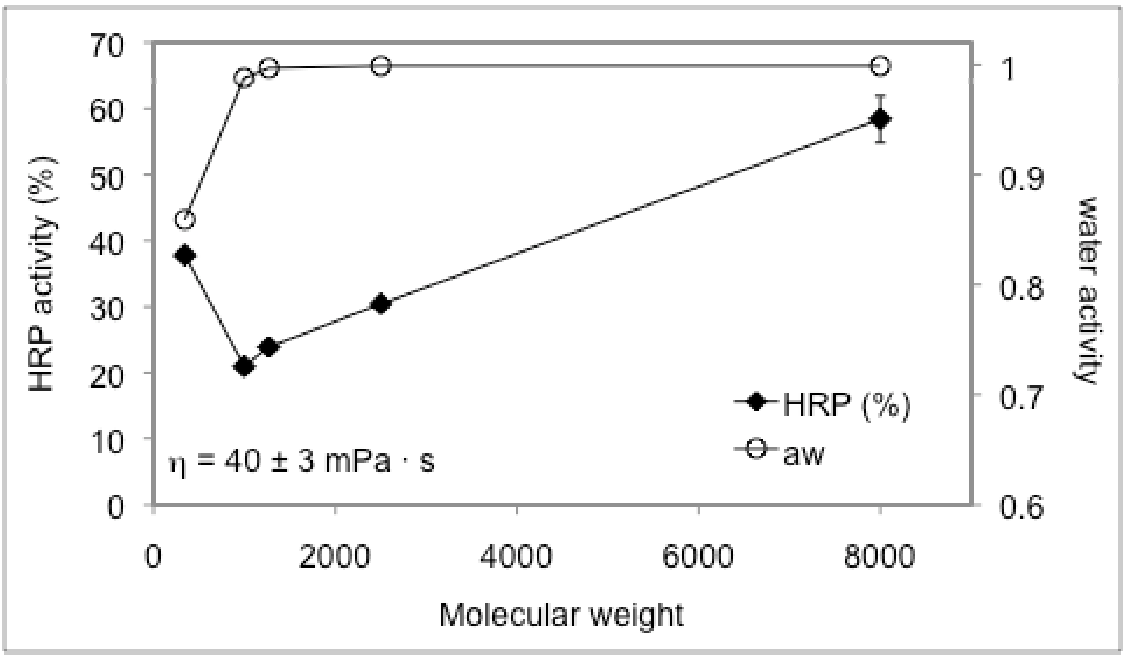

Figure 1. HRP activity and a_w as a function of solutes molecular weight.

High molecular weight maltodextrins do not form ideal solutions in water but physical dispersions. Eventhough a_w and T'g has been proven to describe the effect of water state and mobility on enzyme reaction rates as determined by the molecular size of solutes, they could not explain mobility of discontinuous systems where the bulk viscosity is different from the viscosity in the vicinity of the protein due to solvent composition.

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CONFORMATIONAL CHANGES IN THE UNFOLDING PROCESS OF LYSOZYME IN WATER AND ETHANOL/WATER SOLUTIONS

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The study of the structural properties of proteins is fundamental in order to access the comprehension of their biological function. In the present study, the thermal unfolding of lysozyme in D_2O and D_2O/CH_3CH_2OD solutions is analyzed by means of FTIR spectroscopy. The detailed investigation of the conformational rearrangement of the protein upon heating is achieved from inspection of the amide bands [1,2]. The effects of partial and total deuteration on amide groups are also investigated for they suggest important information about the exposure to the solvent, especially in the pre-melting region [3]. Besides, the comparison between the characteristics of the unfolding process of lysozyme in water and in ethanol/water solutions are performed to clarify some misinterpretation about the possible formation of intermediates [4].

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HENRY´S LAW CONSTANT OF SULFIDES AND DISULFIDES

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Henry's Law constant (HLC) is a fundamental property describing air-water partitioning of solutes at low concentrations (typically less than 0.001-0.01 mole fraction). However, it is difficult to measure reliably, in particular due to the low concentrations involved, which e.g. result in problems with the adsorption of small amounts of solute on the wall of the apparatus. Here, we present data obtained with a dynamic stripping method combined with Proton Transfer Reaction Mass Spectrometry (PTR-MS) for sulfides and disulfides which overcomes the mentioned difficulties. Theoretical QSAR models are compared in view of our results and one of them is improved based on the present results.

Keywords
Sulfur compounds, Henry's Law Constant, Proton Transfer Reaction Mass Spectrometry, Quantitative Structure Activity Relationships QSAR

Introduction

Henry's Law constant is a fundamental parameter. It reflects in particular the unique properties of water as a solvent, which are based on the polarity of water and its ability to form hydrogen bridges. This in turn has a tremendous influence on entropy effects in water, in particular for solutes in water.
Sulfides and disulfides are important in environmental sciences (pollution, emission and waste treatment; cloud formation), geochemistry, petro chemistry, agriculture, biology (e.g. disulfide bonds in proteins), and medicine. Furthermore, in food sciences, because of their low perception thresholds, many of them have an important effect on the sensory profiles of several foods with a positive or negative impact on consumer acceptability. However, despite the interest in sulfur compounds and despite the fundamental relevance of Henry's Law constant, there still is a lack of information on the Henry's Law constants of sulfur compounds and sulfides. In fact, the published data on sulfur compounds are scarce, and if available, often inconsistent [1].

Experimental
Henry's Law constant was measured with a dynamic stripping technique combined with Proton Transfer Reaction Mass Spectrometry (PTR-MS) [2], [3], which allows for the accurate determination of Henry's Law Constant of sulfides and disulfides.

Results
Data for Henry's Law constants for sulfides and disulfides are presented, as well as the comparison of experimental data with literature and a selection of presently available models (modeled vapor/solubility; group and bond contribution additivity methods (Henrywin and [1]); LSER; SPARC).

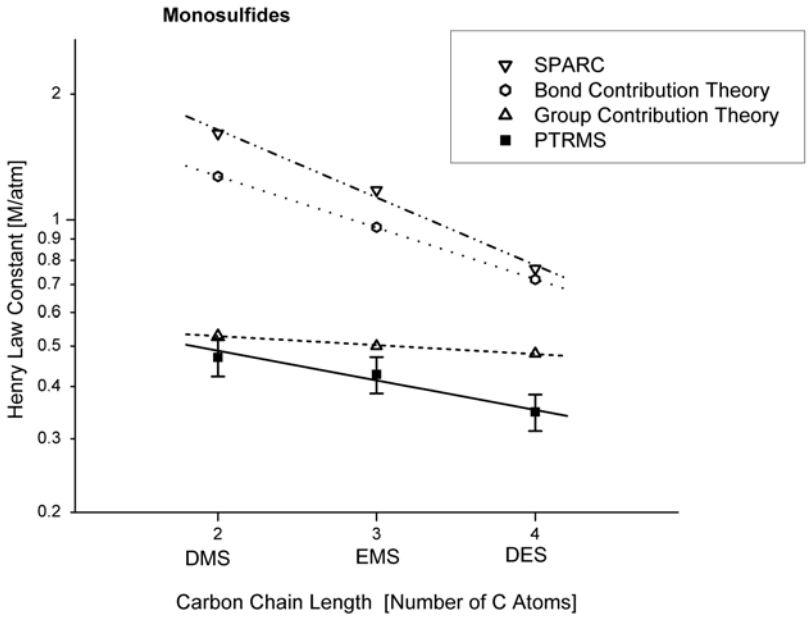

Figure 3 Henry´s Law constants for monosulfides (DMS=Dimethylsulfide, EMS=Ethylmethylsulfide, DES=Diethylsulfide): comparison of PTR-MS data to modelled results from Henrywin and SPARC.

Furthermore, the presently available model by Plyasunov and Shock [1] was adapted to include our results from PTR-MS measurements, which thus allows for more reliable HLC estimations.

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THE ROLE OF WATER DURING BEVERAGE PROCESSING IN DISPENSING SYSTEMS

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Water is used in beverage dispensing machines to prepare dispensed products via numbers of physico-chemical processes, such as extraction, mixing, and dissolution. Water content in liquid beverages varies significantly and could be as high as 99%, for example. Water quality is critical, therefore, to deliver consistent, high quality and safe food products.

Scaling in dispensing machines is a significant problem caused by water hardness. Heating of hard water often leads to limestone scale deposits, causing a host of problems such as inadequate water temperature and reduced quantity of water delivered. These affect extraction/dissolution processes, change product concentration and reduce temperature leading to undesirable taste, texture and mouthfeel of the dispensed beverages.

In the field of water treatment for commercial, industrial and domestic use, a number of methods to reduce/eliminate scaling have been proposed, some or most of which have certain undesirable characteristics, drawbacks or disadvantages associated therewith. The present work overcomes the undesirable characteristics, drawbacks and disadvantages of known methods without affecting water quality.

Water treatment with polyphosphates or metals alone significantly improved food service dispensing machine performance in terms of consistent quantity and temperature of the beverage delivered. We found that the combination of polyphosphates and metal treatment has a synergetic effect on scale control. The developed water treatment method utilizes this effect and provides water to consistently prepare high quality beverage.

Mechanisms of water treatment using the metals, polyphosphates and their combination were studied. Among many approaches used to understand the mechanisms of water performance under the treatment conditions, the morphology of crystals formed in untreated treated waters was evaluated. Zinc and copper alloys are preferred among other metals tested because they are more effective than other metals or metal combinations, and are suitable for applications that utilize drinking water. The effect of metals and polyphosphates, alone or in combination, on treated water quality will be discussed.

INTERACTION OF WATER WITH SURFACE STRUCTURES OF *BACILLUS CEREUS* SPORES

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The bacterial spore is composed of a set of protective structures arranged in a series of concentric shells; each component contributes in some essential way to spore stability [7]. The fate and transport of spores are influenced by their interactions with the medium matrix, e.g. an aqueous medium, which were defined by their surface thermodynamics [5,6]. Microbial surface thermodynamic properties are a reflection of physico-chemical properties of bacterial surface, which are determined by macromolecular components such as proteins, lipopolysaccharides and phospholipids, etc. [11]. The role of the outermost spore layers, the coat and the exosporium (the latter encasing spores belonging to *Bacillus* species such as *B. cereus* and *B. anthracis*) in determining their surface thermodynamic properties is poorly understood. However several studies indicated that the hydrophobicity of certain spores is attributable to the presence of an exosporium [1,13]. The occurrence of *B. cereus* spores with damaged exosporium has been recently demonstrated when spores were produced under various sporulation conditions or were subjected to shear stress such as those encountered by the mature spores during processing [8].

In this research we used *B. cereus* spores with damaged exosporium (a naturally occurring modified phenotype) in comparison to a type strain, to determine structural and physico-chemical properties involved in interaction with water.

Adsorption isotherms were determined to reveal chemical affinity of *Bacillus* spores for water. The low affinity of spores for water was demonstrated by the low average amount of water (0.034 g H_2O g^{-1} dry matter) on binding sites, calculated as the GAB monolayer. The subsequent molecules were not found structured in a multilayer (k » 1), but have characteristics comparable with the molecules in the bulk water. The full wettability of spores was clearly evident from a water contact angle of 0° immediately after removal from an aqueous phase. Since at least 96% of spore water exchanges with the external water, whereas the water exchange across the inner membrane is very low [12], the bound water might be “entrapped” in the spore core.

Spores are capable of relatively rapid expansion/contraction in response to increased/decreased relative humidity [14], possibly in relation to exosporium/coat/cortex flexibility [7]. The flexibility of exosporium has been

found to promote adhesion of the spores by increasing of the area of spore contact with the surface [3].
The reduced affinity for water is mainly related to the inability of microorganisms to exert strong, direct electron donor-electron acceptor (acid-base) interactions with water [10], and it is usually assayed testing the adhesion of microorganisms to hydrocarbons (microbial adhesion to hydrocarbons, MATH). Although MATH is usually said to assay hydrophobic interactions, it has long been known that most hydrocarbons in aqueous solutions bear a negative charge [9]. In consequence MATH is an interplay of hydrophobic and electrostatic interactions [2]. Through the hexadecane/aqueous phase partitioning system, a mutant with a gross defect in the assembly of the exosporium on the surface of the *B. cereus* spore coat has been isolated, indicating that the lack of affinity for hexadecane could be attributed to the lack of exosporium [1]. MATH experiments showed that about 7% of *B. cereus* damaged spores were partitioned into the aqueous layers, less than we expected on the basis of morphological (TEM) data, which showed mostly spores lacking an intact exosporium layer. Similar results (9%) were obtained for the type strain.
Since *B. anthracis* spores devoid of BclA were found markedly less water repellent than wild-type spores, it has been suggested that the specific structural component that contributes to the hydrophobic nature of *B. anthracis* spores is the exosporium glycoprotein BclA [4]. Essential components of the exosporium surface of *B. cereus* are the glycoproteins BclA and ExsJ [13]. In the exosporium-damaged spores, a 200 kDa BclA band was retained, while the ExsJ band was not evident, suggesting that only the exosporium glycoprotein BclA contributed to spore surface hydrophobicity.
The ATR-FTIR spectra from exosporium-damaged spores were different in carboxyl and amide groups from control spores, providing evidence of their involvement in spore surface properties.
This work shows how a combination of techniques allows a more comprehensive characterization of spore surfaces in interaction with water. These results may help in the development of strategies to reduce spore persistence on a variety of substrata.

Acknowledgement
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INVESTIGATION OF WATER IN KIWIFRUIT TISSUE AND QUALITY EVALUATION BY MAGNETIC RESONANCE IMAGING

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Introduction

Magnetic Resonance Imaging (MRI) is a technique known essentially for its medical applications, and produces non invasively high quality images of internal volume or section of any sample containing molecules with short correlation times, mainly water, fatty acids and sugars. MRI images provide information about the distribution of molecules and, through relaxation times, account for the interactions between the observed spin and the surrounding environment. In this work kiwifruits were investigated by MRI to study the tissue evolution with post-harvest storage [1]. The effects of plant growth regulators (PGR) were also addressed. T_2-weighted images were able to distinguish PGR treated fruits from untreated and to identify the specific PGR used. The use of PGR, increasing fruits dimension and accelerating cellular division, is forbidden in organic farming and often also in the conventional one. Nevertheless PGR detection is difficult with common chemical techniques in kiwifruit for the long ripening period, so that other analytical approaches are highly demanded.

Experimental

MRI experiments were carried on Hayward untreated, auxin-treated and cytokinin-treated kiwifruits, at harvesting and after storage with normal refrigeration or modified atmosphere. A Bruker AVANCE 300 MHz spectrometer equipped with cylindrical birdcage single-tuned nucleus (^{1}H) coil probehead with diameter of 70.0 mm was used. The water signal was monitored and used for the image reconstruction.

Results

MRI images of untreated, auxin-treated and cytokinin-treated kiwifruits are shown in Figure 1. The untreated one displays five zones of different contrast: four concentric spherical crowns and the columella at the centre; the concentric areas are maintained in the auxin-treated, but dark channels extending from the columella through the entire mesocarp are visible. These channels are longer, larger and more abundant than in untreated. A deep

asymmetry of the concentric areas is observed in the cytokinin-treated, with dark zones staining the outer bright area.

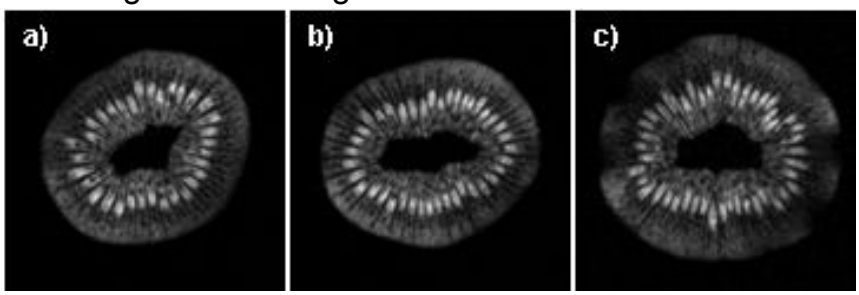

Figure 1. MRI image of a) untreated, b) auxin-treated, c) cytokinin-treated kiwifruit.

A quantitative assessment of the images is required for discriminating treated from untreated samples; so that we applied the greyscale analysis to convert images into measurable quantities. A histogram is obtained, reporting the population of pixels for every value of the greyscale on the digitalized image. The obtained histograms are shown in Figure 2: the auxin-treated samples have more pixels at high intensity (100-170) compared to untreated, while cytokinin-treated have more pixels at low intensity (20-75). These results account for more “free” water in tissues of auxin-treated, and more “bound” water in cytokinin-treated samples with respect to untreated.

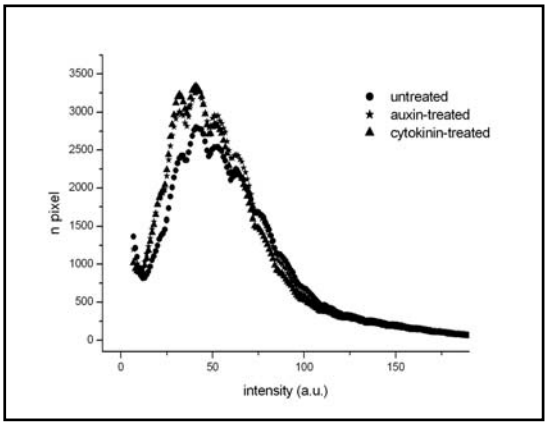

Figure 2. Histograms of untreated, auxin-treated and cytokinin-treated fruits.

The same method was used to study the influence of PGR on storage in refrigeration or controlled atmosphere: the application of the two storage protocols displays that untreated and cytokinin-treated are nearly unaffected by storage conditions, while auxin-treated have more pixels at high intensity when stored in refrigeration conditions, which is a sign of advanced ripening.

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COMPUTER SIMULATION STUDIES OF THE INTERACTION OF GLUCOSE WITH CAFFEINE IN AQUEOUS SOLUTION

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Although both caffeine and glucose have been extensively investigated both for their solution behavior and their relevance in many biological systems, studies of the interactions between them seem to have been completely neglected. Molecular dynamics (MD) simulations have been used to model the structure of a complex aqueous solution of caffeine and glucose. These simulations consisted of a periodic cubic box 32.02 Å in length containing a single caffeine molecule and 36 glucose molecules, giving a caffeine concentration of 0.1 m and a sugar concentration of 3 m. Two separate simulations were performed, one for each of the two anomers of the sugar. Previous work has found a tendency for the H1-H3-H5 non-polar face of the β-anomer of glucose to stack against the hydrophobic face of the indole side chain group of the amino acid tryptophan. For this reason, tryptophan residues are usually found in the active sites of proteins that bind sugars. The similarity of the planar and moderately hydrophobic caffeine molecule to indole suggested that there may also be a weak affinity of glucose for this molecule as well. The results of the simulations indeed found a tendency for the hydrophobic face of the β-D-glucopyranose to stack against both faces of the caffeine molecule (Figure 1). The effective concentration of caffeine used in the simulation is close to the saturation limit at 25 °C, which in a real solution would lead to significant caffeine-caffeine aggregation. With only one caffeine molecule in the simulation, such caffeine stacking cannot happen in these calculations, which limits the possibility of comparison with experiment. Future simulations will include multiple caffeine molecules in a larger primary simulation box for the purposes of direct experimental comparison.

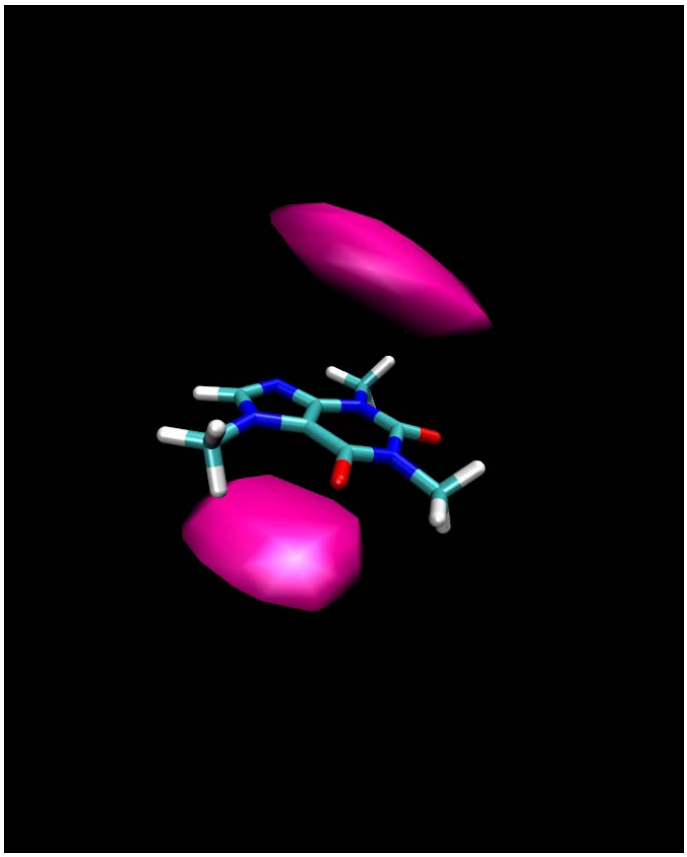

Figure 1. Distribution of the excess glucose density around a caffeine molecule.

Inasmuch as the heterotactic interactions should be primarily dependent on the solute type, one may foresee that in caffeine-carbohydrate interactions the enthalpic contribution to the pair-wise interaction can be small if compared to that of caffeine with alcohol [1]. Direct calorimetric experiments are underway to confirm this view.

[1]J. H. Stern and E. Lowe *Journal of Chemical and Engineering Data, Vol. 23, No. 4, 7978* 341-345.

WATER MOBILITY AND DISTRIBUTION IN GREEN COFFEE PROBED BY TIME DOMAIN NUCLEAR MAGNETIC RESONANCE

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The knowledge of the water content in green coffee is a parameter of great importance to develop a quality end product and to help to control undesired effects such as microbial growth and off-flavours. As a consequence, the knowledge of how water is distributed inside the raw bean is of interest. To date, not many works deal with this topic, which still remains quite challenging [2].
Time Domain NMR spectroscopy (TD-NMR) can provide non-destructive information on mobility of water by measuring the transverse (spin-spin) relaxation time constants in a multicomponent system.
In this poster, the results of an exploratory study addressed to shed light on the distribution of water within the green coffee bean as seen by TD-NMR are presented.
Intact and ground green coffee beans were previously desiccated and then gradually hydrated adding increasing amounts of water up to 50% w/w [1].
NMR measurements at each hydration level were performed collecting relaxation curves with the CPMG sequence by means of a low field NMR spectrometer operating at 20 MHz. The spin-spin relaxation times (T_2) were then evaluated with the help of a suitable inversion algorithm of Laplace transform (UPEN).
Four main relaxation components were generally observed, two of which attributable to water in different physical states. The most evident effect of the wetting process was an increase of the intensity of the proton population at the lowest (1-3 ms) relaxation time as a function of water added.
Further investigations were carried out by re-hydrating desiccated beans with deuterated water, in order to estimate the contribution of non-water matrix protons to the hydration mechanism [3,4].
To visualize the pore size distribution and the hydration sites present in the microstructure of the green coffee seed, scanning electron microscopy (SEM) images were acquired on native, partially and fully hydrated green seeds.
Additional diffusion experiments were also performed on native and completely wetted green coffee beans to characterize the different water phases in different biological compartments.

T_2 values and the corresponding populations intensities found by NMR were compared with SEM micrographies.
A possible interpretation of the T_2 of each different proton species (free and bound) belonging to water in relation to particular pore structures of the matrix is presented.

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AN EXPERIMENTAL-NUMERICAL STUDY OF CHIRAL RECOGNITION PROPERTIES OF β-CYDS VS. IBP

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Cyclodextrins (CyDs) are macrocyclic sugar molecules formed by glycosidic links between D-glucose monomer units obtained by the enzymatic conversion of starch [1]. They have often been employed as reference hosts in supramolecular science due to their capability of including a wide range of molecules into their hydrophobic cavities via noncovalent interactions, typically hydrogen bonds, Van der Waals and hydrophobic interactions [2]. Due to their importance in pharmaceutical research, environmental protection and food industry, cyclodextrin complexation process has been widely investigated by using both experimental and theoretical approaches. Numerous attempts have been made in order to define the relative contribution of forces that govern the complexation. The driving forces for complex formation have been attributed to steric factors, extrusion of water from the cyclodextrin cavity, relief of ring strains in the cyclodextrin ring and by interactions between host and guest molecules [3]. Besides interest focusing on CyDs' ability to form inclusion complexes, great attention has been paid on their chiral recognition ability as well. The chiral recognition properties of CyDs are of great importance especially in the pharmaceutical industry, since most drugs are chiral, and enantiomers of many drugs have been shown to possess different pharmacological activities [4]. Although cyclodextrins are frequently used for chiral separation of racemates, the mechanism of chiral recognition has not yet been fully characterised.
Here, we studied how the vibrational properties are affected by the chiral recognition process, upon selection of the non-steroidal anti-inflammatory drug Ibuprofen (IBP) in its chiral (R)- and (S)-, and racemic (R, S)- forms, as model guest, and native and modified β-cyclodextrins (β-CyDs) as model host. The usefulness of β-CyDs to form inclusion complexes with IBP has been investigated, in pure water, by UV absorption. Phase-solubility diagrams allowed the determination of the stoichiometry and the association constant between IBP and β-CyDs. The changes induced, as a consequence of complexation, on the vibrational spectrum of IBP, have been studied, in solid phase, by attenuated total reflection Fourier transform

infrared FTIR-ATR and Raman spectroscopies. The recorded spectra have been compared with the wavenumbers and IR and Raman intensities as obtained by quantum chemical and classical computations for the free and complexed guest molecule. This combined experimental-numerical approach gave crucial information on the expected different “host-guest” interactions that drive the chiral recognition process, helpful to put into evidence differences in the conformational properties of the complexes, that are retained a prerequisite for chiral recognition.

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WATER-TRIACYLGLYCEROL INTERACTIONS AFFECT OIL BODY STRUCTURE AND SEED VIABILITY

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We are investigating interactions between water and triacylglycerols (TAG) that appear to affect oil body stability and viability of seeds. Dried seeds are usually stored at freezer temperatures (-20°C) for long-term conservation of genetic resources. This world-wide genebanking practice makes the assumption that low moisture and temperature slows chemical reactions associated with aging according to kinetic models of amorphous solids [1,2], and that TAG transitions within seeds are completely reversible. Increasingly, we are discovering exceptions to these kinetic models in species of seeds that contain TAG which crystallize and melt at temperatures greater than -25°C. The most dramatic effect of water-TAG interactions is massive cellular disruption and consequent seed death that results when fluid water is added to crystallized TAG [3,4]. A more subtle effect is observed in seeds containing proportions of monounsaturated: saturated fatty acids giving TAG crystallization temperatures between -5 and -25°C. These seeds tend to deteriorate faster in the freezer compared to higher storage temperatures and we hypothesize that the temperature anomaly is associated with TAG mobility as oil bodies restructure during crystallization induced by freezer storage. Thermomechanical analysis (TMA) shows major contraction and expansion of TAG-filled seeds during TAG transitions and dimensional changes are directly correlated with water content [5]. Differential scanning calorimetry (DSC) measurements show that increasing water content tends to increase the rate of TAG crystallization [3]. Electron micrographs show that temperature cycling through TAG transitions can lead to restructuring of oil bodies and

incorporation of cytoplasmic materials [4,6]. Collectively, these observations suggest that oil and water may not mix, but they do interact. The interaction contributes to the instability of seeds during long term storage at freezer conditions. Seeds containing so-called tropical oils are prone to these temperature anamolies. Cooling at appropriate rates to liquid nitrogen can avoid TAG crystallization and prolong seed shelf life.

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WATER PLASTICIZATION OF POLY(LACTIDE-CO-GLYCOLIDE): A FTIR INVESTIGATION

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Lactic and glycolic acid copolymers, as well as their homopolymers are among the few biocompatible and biodegradable polymers approved by the Food and Drug Administration for parenteral use [1]. In the last years these polymers have been extensively used for the preparation of drug delivery systems, such as microparticles, aimed at controlling the release of the embedded compounds [1]. Even though water plays a fundamental role on the active release [2], then on the device performances, a complete molecular based understanding of its interaction with such hydrophobic matrices is still lacking.

One of the main water effects on completely amorphous polymers, such as poly(lactide-co-glycolide) (PLGA), is the reduction of the glass transition temperature (T_g), a phenomenon known as plasticization [3]. In the specific case of implantable drug delivery systems, a T_g lowering below 37°C increases of several order of magnitude small molecule diffusion coefficient [4], switching the release mechanism to a simple Fickian diffusion [5,6].

In order to gain molecular insights into the water plasticization mechanism of PLGA, the effects of temperature and water activity have been investigated by means of Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC).

Different spectral regions have been studied in the 20-70°C temperature range. In particular, the vibrational signals in the 1000-2000 cm^{-1} range, essentially related to C=O and O-C-O stretching, CH_3 and CH bending and CH_3 rocking modes [7], have shown to be sensitive to the thermal variations in the investigated temperature frame. Moreover, spectral changes, more consistent at higher temperatures, evidenced a non-linear behaviour. An inflection point in the T_g region, as determined by DSC measurements, has been observed together with the effect of the enthalpic relaxation on the first heating ramp (Figure 1). These findings are consistent with those obtained for a poly(L-lactide) polymer [7].

In the completely hydrated polymer, a frequency shift towards lower frequencies of the C=O band has been observed, suggesting the involvement of this group in concomitant hydrogen bond interactions.

Interestingly, this effect was continuously reduced by the temperature increase.

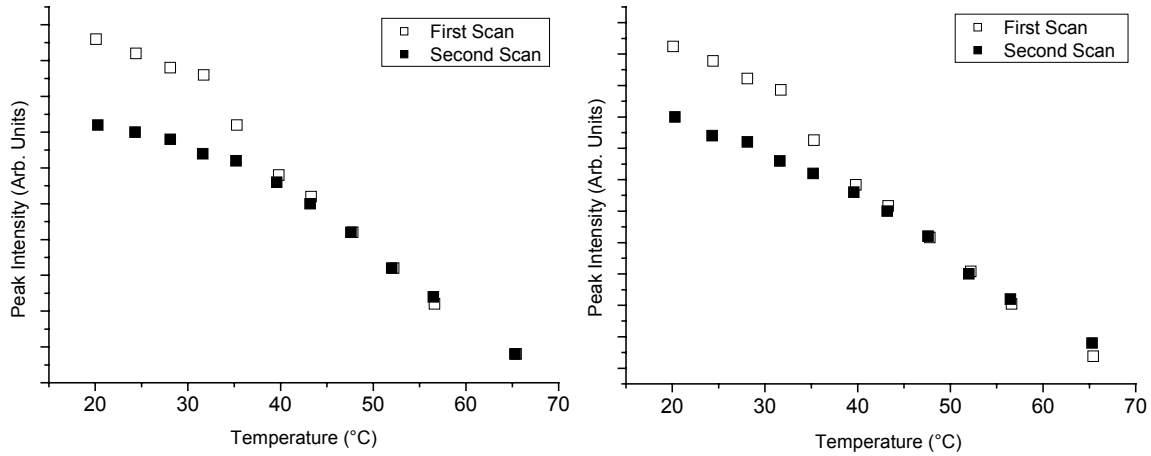

Figure 1. Peak intensity as a function of temperature for the CH_3 rocking band at 1133 cm^{-1} (left) and the C-O-C symmetric stretching band at 1094 cm^{-1}(right).

The performed analyses shed some light on the molecular mechanism responsible for the enhancement of the polymer chain mobility induced by both temperature and water. Overall results show that the FTIR may represent a suitable technique to detect the glass transition region in amorphous polymers.

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H/D ISOTOPIC EFFECTS IN PROTEIN THERMAL DENATURATION: THE CASE OF BOVINE SERUM ALBUMIN

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The present work studies the H/D isotopic effects on the structural and thermodynamic stability of Bovine Serum Albumin (BSA) within the temperature range 5°C-90°C by comparing the protein unfolding pathways in H_2O and D_2O using far-UV circular dichroism (CD). The experiment has shown that the BSA molecules possess similar conformations in H_2O and in D_2O at temperatures lower than 50°C, but follow different unfolding pathways at higher temperatures. The presence of D_2O retards the occurrence of the irreversible thermal denaturation of BSA by demonstrating a higher onset temperature at 58°C, in contrast with 50°C in H_2O. However, as the temperature is increased further, the irreversible transition becomes more significant in D_2O than in H_2O. We estimate that in this experiment the heat treatment up to 90°C irreversibly denatured 58% of the protein secondary structures in H_2O, and 63% in D_2O. The reversible conformational transformation of BSA is also discussed. It is shown that the experimental single-wavelength CD data corresponding to the reversible unfolding of BSA in H_2O and D_2O can be reproduced by a simple two-state transition model with the same thermodynamic variables, suggesting very similar reversible transition behaviors in the two solvents. This experiment directly tests the commonly used assumption that substituting D_2O for H_2O as the solvent does not affect the structural stability of proteins in solutions. In a variety of experimental techniques (IR, Raman scattering, NMR, and neutron scattering etc.), it is a standard procedure to dissolve proteins in D_2O instead of H_2O to avoid the large background from H_2O. This is based upon the assumption that the protein molecules are indistinguishable in H_2O and D_2O, an approximation that must be justified carefully.

CHANGES OF WATER STATE ON GREEN AND YELLOW KIWIFRUIT DURING OSMOTIC DEHYDRATION

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Introduction

Osmotic dehydration or water impregnation soaking permits the partial removal of water from plant tissues by their immersion in hypertonic solution [1]. In the optic of fresh-cut fruit production, the osmotic dehydration (OD) is a valid mild stabilizing process if combined with other partial preservative technologies (e.g. modified atmosphere packaging, refrigeration). The OD treatment can both increase the overall quality of the product and extend its shelf-life by decreasing the water mobility [2]. The main aim of this research was to study the mass transfer kinetics and the water state evolution during the OD treatment of two different species of kiwifruit (*Actinidia chinensis* var. Hort16A and *A. deliciosa* var. Hayward), following a multianalitycal approach by the joint use of DSC and LR-NMR techniques.

Experimental

The OD treatment was applied on two species of kiwifruit (*A. deliciosa* var. Hayward and *A. chinensis* var. Hort 16A), having a refractometric index of 12 ± 0.4°Brix. The OD was carried out by dipping the samples in 61.5% sucrose solution with a product/solution ratio of 1:4 (w/w); the samples were submerged in the OD solution at 25°C, 35°C and 45°C for different times (0, 5, 15, 30, 60, 120 and 300 min). The moisture content of each sample was determined according to AOAC 920.15 and soluble solids content by measuring the refractive index with a digital refractometer. OD kinetics of kiwifruit were evaluated by calculating net change of fruit slices mass (DM^0_t), water (DM^w_t) and solutes (DM^{ST}_t) adopting the equations purposed by [3]. Data relative to mass transfer were modeled to the equation proposed by Peleg [4]. This kinetic model offers the advantage that by calculating the inverse of the constant (k_1 e k_2), it is possible to obtain the initial rate of mass transfer parameters values at the equilibrium condition [5]. DSC curves were obtained by cooling samples to - 60°C and then heating at 5°C/min to 110°C after an isothermal hold for 5 min at - 60°C. Proton T_2 of the samples were analyzed at 24°C with the CPMG pulse sequence using a Bruker Minispec PC/20 spectrometer operating at 20 MHz.

Results and conclusions

For both the studied species, the treatment temperature positively influenced the water loss and solute gain parameters. *A. deliciosa* showed an higher temperature dependence in terms of solute gain compared with *A. chinensis.* According to the Peleg model, the main differences between the two kiwifruit species performances were detected during the first phase of the treatment. DSC measurements showed that the end point of freezing and the unfreezable water content decreased together with the moisture content, following a logarithmic behaviour. In the optic of industrial processing, the time needed to reach the water content corresponding to the absence of freezable water has been estimated by the interpolation of experimental data. This time value was much higher for *A. chinensis* compared with *A. deliciosa*. NMR evaluation permitted a deeper understanding of the OD process, enabling to study the modification of cellular compartmentalization. The cytoplasm showed a leakage of water contemporaneous with solute gain, the vacuole underwent to probable shrinkage of its native structure, while the cell wall showed destructuring phenomena similar to those detected during kiwifruit ripening.

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INVITED SPEAKERS

ORAL PRESENTATIONS

POSTER PRESENTATIONS

AUTHOR INDEX

FRONTIERS IN WATER BIOPHYSICS