



How neutron scattering experiments can target the structure and dynamics of milk proteins?

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The powerful of neutron scattering techniques to characterize structure and dynamics of milk proteins is illustrated. Small angle neutron scattering and reflectivity are used to determine the structure and the interactions between milk proteins in solution, during gelation processes, or the protein structure at different interfaces. Experiments performed by inelastic and quasielastic neutron scattering allow one to observe the dynamics of water and proteins showing the major role of hydration on the dynamics of milk proteins.

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Introduction

Material science principles and concepts are being applied increasingly to study foods. Consideration of polymer structure and measurement of order-disorder transitions provide important insights about how food materials behave during processing and storage [1,2]. Glass transition temperature and the role of water as a plasticizer are key parameters to predict and control low hydrated products or powder stability [3]. Finally, an understanding of the biopolymer state at the molecular scale and the connection between the polymer structure, size and the texture of foods (macroscopic scale) still stays challenging [4].

The historical development of neutron scattering started from structural investigations and was further extended to the study of dynamical aspects. Being a young discipline, neutron scattering continues to grow and the domain of its application has extended a lot these last year's even to

biology and food science [5••]. Even if the neutron techniques will never be as convenient as a laboratory tool as the instruments are only accessible in large scale facilities, it has some advantages that make it very attractive and sometimes becomes the only technique ables to be used. At first, the wavelength and the energy of neutron are in the order of magnitude of the atomic and intermolecular distances in liquids and solids, and the range of translational, rotational and vibrational of the molecules. But the two main properties of neutrons that are unique are that they can penetrate matter very deeply and that isotopes have different scattering cross-sections values, so isotopic labeling can be used to select or highlight different molecules from mixtures.

The powerful of neutron scattering techniques on milk proteins structure and dynamics is illustrated in this work. The challenge is to increase understanding of the proteins functional capacities in foods and the stability of protein ingredients by widen the scope of the neutron techniques to food science [5••]. In a first part, studies which have used Small Angle Neutron Scattering (SANS) to observe the structure of milk proteins and their interactions in the casein micelle as well as, as well to monitor changes during milk gelation are selected. The second part illustrates the use of the neutron reflectivity to determine the structure of milk proteins at the air–liquid and liquid–liquid (polar/apolar phases) interfaces. In the last part, literature which allows for a better understanding of the relationship between protein dynamics and water hydration in milk proteins is discussed.

Structure and gelation of milk protein

The main physico-chemical and functional properties of proteins are linked to their three-dimensional conformation which in milk can vary according to the type of protein: such as globular (structure adopted by whey proteins), or micellar organization (the structure adopted by the main protein fraction of milk: the caseins) [6,7•].

Small angle neutron scattering

Small Angle Neutron Scattering (SANS) explores the average structure of materials at spatial scales going from 0.5 to 100 nm. This technique allows the determination of the main dimensions which characterize the size and shape of nano-objects, and their interactions. Neutrons can be scattered by all sorts of matrices whatever are their physical state, liquid, bulk solid, or dispersed solids (powders). The power of neutron scattering experiments essentially comes from the fact that the scattering event is

a sensitive function of the atom nature. Isotopic substitution and contrast variation methodology is being used more and more to match part of a molecule domain, or one constituent of an heterogeneous matrix for example. SANS is often preferred to X-ray or light scattering experiments because semi-diluted or concentrated samples can be used, which is often the case when studying food materials.

Casein micelles: internal structure and gelation

Milk comprises spherical particles of colloidal dimensions called casein micelles. The chemical composition of these particles is about 94% protein and 6% inorganic material such as calcium, phosphate and citrate ions. The radius range of casein micelles is typically 50–200 nm [6]. The substructure of casein micelles has been studied by small-angle X-ray and neutron scattering [7[•],8^{••},9]. From these studies, several models for casein micelles have been proposed. One of these models is a sphere comprised of spherical submicelles with an average diameter of 8–20 nm [10]. Other models suggest the micelles have an homogeneous structure of casein dispersed in the same micelle [7[•],8^{••}]. The structure of a casein micelle can best be described as a protein matrix in which calcium phosphate clusters (2 nm radius) are dispersed. A reasonable assumption is that the hydrophobic parts of the caseins form locally more dense protein clusters, and also that there is a contribution from a protein corona of the phosphor proteins adsorbed to the nano clusters of calcium.

During cheese production, chymosin, a proteolytic enzyme is added to the milk. This proteolysis leads to loss of steric stabilization as well as electrostatic repulsion and results in aggregation and subsequent gelation of caseins. In yoghurt and other acidified dairy products, the coagulation of casein is achieved by decreasing the pH. Whereas the micelle of casein is stable in the normal pH of the milk, and at room temperature, this supramolecular structure becomes unstable and led to the formation of a gel by slow acidification.

Tromp and Bouwman [11^{••}] performed Spin-Echo Small Angle Neutron Scattering (SESANS) experiments to measure the static structure of milk and yogurt. SESANS uses spin-echo to probe density correlations in real space directly from 20 nm up to 20 μm , overlapping the SANS range but observing much larger molecules. The SESANS setup consists of a monochromatic, polarized neutron beam traveling through two magnetic field regions, in between which the sample is placed. Experiments are performed by varying the magnetic field strength. The direct relationship between the measured polarization and structure has been shown experimentally on colloidal systems [12].

The method has been applied to study the structure of milk and yogurt [11^{••},13]. The authors simulated the

acidification process by adding glucono- δ -lactone (GDL) to D₂O-milk. GDL slowly hydrolyses and decreases the pH of the system. They also simulated the manufacture of cheese products (curd) by adding chymosin to the milk. The decrease of the polarization is different and depending of the product. It is close to 300 nm for milk and extended to 3 μm for yogurt and curd. The results obtained show that the initial slope of the measured polarization of all samples is identical. Indeed, the composition of the initial product is the same, milk which consists of casein micelles with size below 300 nm. The signal of the yogurt continues to decrease up to 3 μm . The micelles aggregates form larger structures than the original size of the casein micelles. The curd has a structure that also extends to 3 μm , but the decay at the shorter length scales is faster, which means that a structure is formed, which is characterized by longer length scales than those observed during the formation of yogurt. The authors interpreted this difference as a locally denser packing in curd resulting from a weaker electrostatic repulsion.

Milk proteins at interface

Milk proteins have excellent capabilities for stabilizing foams and emulsions and are widely used for this purpose in food applications [14]. To understand their adsorption mechanism in more detail, information about their conformation at the microscopic level is necessary. One of the techniques well suited for this task is neutron reflectometry (NR) [15,16]. The first attempts to apply neutron reflection (NR) to protein adsorption was conducted in early 1990, for the determination of β -casein adsorption at air–water interface by Atkinson *et al.* [17] and Fragneto-Cusani [18].

Neutron reflectivity

By neutron reflectivity studies, it is possible to characterize the protein structure at interfaces. One can determine layer thickness (from 10 to two thousands Å) and its chemical composition. Different types of surface and interface can be studied: air–liquid, solid–liquid and liquid–liquid. In a neutron reflectometry experiment, the intensity of the neutron beam reflected by the surface is measured as a function of the scattering vector Q . The intensities of both incoming and exiting beams are monitored and the ratio of the two is defined as the neutron reflectivity, R . The reflected intensity shows finite-size fringes that can be analyzed to determine total thickness of the layer, material composition and roughness. The periodicity of the fringes is related to the layer thickness [19].

Milk protein adsorbed at the air–water interface

Using the technique of neutron reflectometry, Well and Brinkhof [20] determined the volume fraction profile of the milk protein β -casein, adsorbed at the air–water interface in great detail. The total thickness of the

adsorbed layer was measured as approximately 15 nm. It consists of a very dense region at the interface with a volume fraction of about 45%. The thickness of both region increased by increasing the protein concentration. These results were very similar from previous results obtained by Atkinson *et al.* [17] on adsorption of β -casein and β -lactoglobulin at the air–water interface.

Milk protein adsorbed at the lipid interface

Junghans *et al.* [21^{••}], used tethered bilayer lipid membranes as a model system to mimic the interactions between whey protein: β -lactoglobulin and lipids at an interface. The interaction of native and denatured milk whey protein with membranes of different compositions was studied [21^{••}]. The data obtained indicated that the protein adsorbed on top of the monolayer with a thickness of 26 Å, suggesting that the protein remained in its native state or only partially unfolded. In fact, the gyration radius of the native β -lactoglobulin in solution is about the same size. The authors demonstrated that the native protein was only able to penetrate the lipid bilayer, if the membrane was not very densely packed.

Effect of hydration on milk proteins dynamics

Among foods, dried products are extremely attractive for convenience and stability. Indeed, at room temperatures they can have a shelf life longer than liquid foods, and in a minimum volume and weight, thus facilitating their packaging and transport. However, although the problems are not comparable to those found in liquids, there are many changes modifying both their appearance and functionality during dehydration and storage. These changes are often induced by the presence of residual water in the system [22,23]. A hydration layer around proteins exhibits dynamical properties markedly deviating from those of bulk. It is of a large interest to elucidate the mechanisms responsible in determining the peculiar features of the hydration water in the proximity of the protein surface and to assess if these mechanisms could play a role in regulating the protein dynamics and functionality [24,25,26^{••}].

Quasielastic and inelastic neutron scattering experiment

The molecular dynamics of a macromolecule is described by some relaxation processes which vary according to the type of movement observed [23]. An α -relaxation or a main relaxation is defined to characterize the cooperative motions of a whole molecule or a structural entity with the other molecules or entities which constitute the material. This main relaxation is related to the glassy transition corresponding to the passage of the liquid into noncrystallized solid (or glass) by dehydration or fast cooling. This molecular arrangement somehow freezes the dynamics of molecules at large scale and preserves a structure similar to the one that the macromolecules have in the liquid state. Whilst glass formation certainly slows some kinetic events in the system, it is now understood

that glasses are not stable, and during the slow processes of drying, rehydration and storage, can age [22]. These organization changes are induced by motions at a local scale (vibration of atoms or reorientation of small groups of atoms), which are still possible below the glass transition temperature (T_g), even if long range cooperative motions are restricted. These sub- T_g relaxations are called β or γ depending on their relative position to α relaxation and are probably associated to hydration layer mobility. Numerous food products are constituted by glassy and amorphous materials such as sugars and/or proteins, or protein-sugar mixtures. Milk powders contain amorphous solids, which can undergo a glassy to rubbery transition [23].

For studies of the β relaxations, the measured neutron scattering intensity takes also in account the energy variation of the neutron. It is usual to simplify the interpretation of data by separating the different kinds of contributions to motion according to their time scales and amplitudes, and subsequently making the hypothesis that these contributions are essentially not coupled between each other. Thus vibrational motions occurring in the 10-fs time scale in a protein may be considered as independent from diffusive-type motions (occurring in ps to ns) due to changes in conformational states of its side chains. This differentiation leads to the separation of a spectrum between inelastic scattering (due to vibrational motions), quasielastic scattering (arising from diffusive motions), and elastic scattering in the case of confined scatterers (related to the limited volume sampled by each scatterer) [27].

Orecchini *et al.*, performed experiments on the interplay between water and protein dynamics. The low-frequency dynamics of the globular protein β -lactoglobulin has been investigated by inelastic neutron scattering, on both dry and D₂O-hydrated samples [28]. Hydration water is found to affect these low frequency motions. To establish which protein structural elements give origin to these vibrational modes, the authors studied different samples submitted to suitable H/D exchange procedure, which allowed them to highlight the contribution of different hydrogen classes of the protein. Mainly, they concluded that hydration water is found to affect the low-frequency motions of both buried and solvent-exposed protein atomic groups. Therefore, the plasticizing effect of water, which is known to trigger large amplitude molecular motions, acts over the whole protein, rather than on its external surface. The respective vibrations propagate through the same spatial domains thus suggesting a picture of hydrated proteins as homogeneous media.

In another study, Gaspar *et al.* [29] investigated the repercussions that the absence of the secondary structure of caseins have on motions occurring in time scales of the order of pico-seconds to nano-seconds, accessible by

time-of-flight quasi-elastic neutron scattering experiments. The interplay between structure and dynamics was investigated in a series of experiments, performed on hydrated powders of different proteins up to a hydration level of 0.4 g of D₂O per g of protein. The differences observed for the different proteins such as casein, as well as on three well-folded proteins, with distinct secondary structures, namely, myoglobin (α), lysozyme (α/β) and concanavalin A (β), suggest that the secondary structure of proteins plays a relevant role in determining the internal dynamics, probably not so much concerning its characteristic time, but rather concerning the fraction of protons actually involved in those motions, as well as the amplitudes of those motions. In any case, proteins composed mainly of β -sheets appear to be less flexible, followed by proteins composed mainly of α -helices and then by those composed of a mixture of α and β -motifs, the unstructured proteins being in fact the most flexible ones.

Conclusion

Regarding the increasing consumer's demand to have high level quality foods, it is more necessary than ever to understand the complex structure of food and food ingredients and how they are affected by processing. This has increased the need for more sophisticated non-destructive analysis techniques, such as neutron scattering which can yield information on the structure and the dynamics of the food ingredients.

Neutron scattering experiments performed on milk proteins (isolated proteins or on mixtures) allowed to clarify the structure of these proteins, and to better understand the interactions they have with other constituents of the milk, to envisage better control of their functionalities (gel, emulsion, powder). Small angle neutron scattering or reflectometry are two tools of choice for a better characterization of their structure in solution or at interfaces (solid–liquid, air–liquid or liquid–liquid). Inelastic or quasielastic neutron scattering have been used to study the dynamics of unordered caseins or the globular whey beta-lactoglobulin in the form of hydrated powders. These studies underlined the particular interplay between hydration water and milk proteins.

Neutron techniques have been increasingly used in the last years to study foods and their ingredients (proteins, polysaccharide, fat, sugars, vitamins, ...). The powerful of these techniques comes from the fact that concentrated matrices can be observed under processes with different conditions of T, HP, RH, shearing, etc. and the broad range of scales that can be covered. The difficulty, as many others biophysical methods arises from the fact that the samples must be very well characterized before neutron used in order to be able to totally interpret the data obtained. However, in the search of new ingredients or new functionalities, neutron often brings a critical

understanding on the relationship between structural organization and physicochemistry of the matrices or allows a better knowledge on the impact of applied process to the material and interaction between molecules.

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