STRUCTURE OF PROTEINS-POLYELECTROLYTE COMPLEXES

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We wish here to show how useful can be the combination of a "in-house" work, which involves synthetic polyelectrolyte material allowing deuteration labelling, with the work of an external team, at INRA, on natural polyelectrolytes, namely polysaccharide.

Understanding the mechanisms driving the formation of complexes of polyelectrolytes and proteins of opposite charges complexes is of a fundamental importance as such complexes are often encountered in biological or industrial situations [1]. The sum of interactions in the system is generally dominated by electrostatic interactions and their screening by salt, but hydrophobic interactions or Van der Waals interactions can also be involved. This results in a large variety of morphologies for the macroscopic structures of complexes solutions. The system can be monophasic or diphasic. Such phases can be either clear, either turbid, either form a solid-like precipitate. From a rheological point of view, phases can be either fluid, either form a gel.

There is nevertheless a lack of knowledge on the local structure of both polyelectrolytes and proteins in the inner complexes, *i.e* at the protein scale, as complexes are essentially experimentally studied by means of rheology, pH titration, titrimetry, turbimetry, light scattering. We show here that Small Angle Neutron Scattering (SANS) is a powerful relevant technique to reveal the structure of the complexes as it provides a direct access to the different length scales of the system sizes.

As a protein, we used here lysozyme, a small globular protein positively charged in acidic medium (its isoelectric point is 11.2). As polyelectrolytes, we used two different negatively charged polyions:

(i) first we use a synthetic deuterated polyelectrolyte (PSSNa) to unravel the main features of the structures that can be obtained. The contrast method allows to get the specific structures of the individual components within the complexes. *(ii)* In a second time, we focus on the influence of the electrostatic parameters that control the inner structures of complexes made with biological polysaccharides (pectin) that are of an industrial interest for food formulation.

Revealing the main features of complexes: PSSNa and lysozyme: using deuteration to play on contrasts.

In this first part lysozyme is mixed with PSSNa, a polyelectrolyte with an hydrophobic backbone and a small stiffness. At the pH of our study (4.5), each repetition unit bears a negative charge. In all experiments described, PSSNa chains are in semi-dilute regime and the negative charges provided by PSSNa are in excess compared to the positive charges provided by lysozyme.

PSSNa chains are fully deuterated. Solutions of lysozyme and PSSNa are firstly prepared by dissolution in an acetate/acetic acid buffer (5.10^{-2} mol/L) that maintains the pH at 4.5. Both solutions are then mixed and homogenized with a vortex for few seconds and left at least two days before SANS measurements. For each lysozyme/PSSNa ratio two samples were prepared : one in D_2O that matches the neutronic PSSD contrast and one in a 57%/43% H_2O/D_2O mixture that matches lysozyme neutronic contrast.

When mixture is achieved a turbid solution that strongly scatters light is instantaneously obtained. If the excess charge is low (here [lysozyme] = 40g/L; [PSSNa] = 0.1Mol/L that corresponds to [+]/[-] = 0.3), solutions remains turbid with time. Complexes made with short chains length are fluid though they form gels with long chains. If the excess charge is large ([lysozyme] = 20g/L; [PSSNa] = 0.3Mol/L that corresponds to [+]/[-] = 0.05), samples progressively become clear with time. Samples that were originally gelled turn back liquid. The scale time of this clarification lies from some hours to a few days.

Figure 1 presents spectra obtained for a low excess of charge. For small chains (N =120), the spectrum of protein within the complexes (Fig 1.a, blue curve), is similar to the one of protein alone at large q (see form factor). But it shows a peak at $q \sim 0.2$ Å^{-1}. Its abscissa corresponds to protein-protein contact. At lower q the strong slope part can actually be divided in two regions : a q^{-4} law characteristic of scattering from the surface of a large dense object, followed at the lowest q by a $q^{-2.5}$ suggesting a fractal structure. Complexes look as compact objects made of several hundreds of

proteins arranged at larger scale in a kind of ramified clusters. Spectrum where polyions are

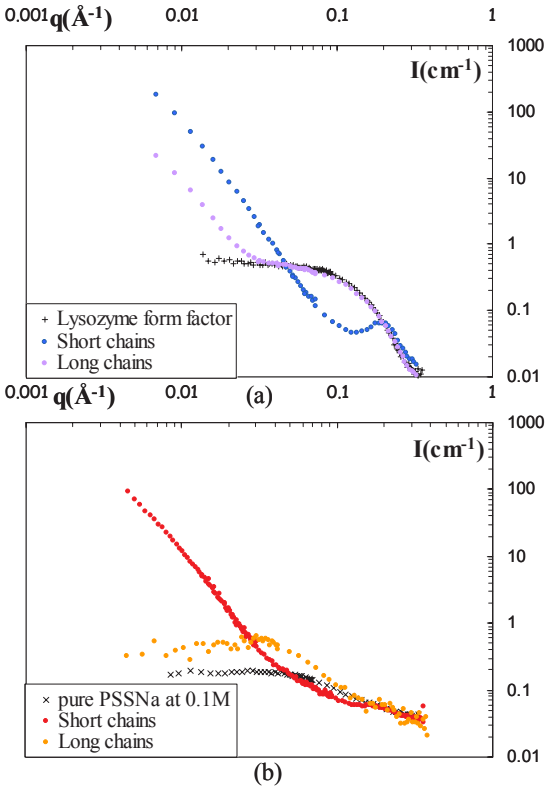

Figure 1. low charge excess ([lysozyme] = 40g/L; [PSSNa] = 0.1Mol/L) (a) Lysozyme signal (b) PSSNa signal

seen inside the complexes (Fig 1.b, red curve) is strikingly different from the one for pure polyions : the corresponding structure of the polyion solution has vanished. Conversely, it is quite similar to the scattering from protein: this indicates that lysozyme and protein are indeed embedded together inside the large dense complexes. Complexes are formed of dense 3-D objects made of several hundreds of proteins in which the polyelectrolyte plays the role of stickers between proteins. For long chains (N = 620), the protein signal (Fig 1.a, purple curve) is completely different as for short polyions: there is no more correlation peak at q ~ 0.2 $Å^{-1}$ thus no more contact. Dense aggregates have vanished, as confirmed by the low q law ($q^{-2.5}$) typical of fractal clusters, the latter are connected into a macroscopic gel. Looking now at the scattering from polyions (Fig 2.b, orange curve), we see that for long chains, it is close to the pure polyion one, with a similar maximum. The proteins are stuck on the polyions, which still form a chain network as in polyelectrolyte semi-dilute solution. However the maximum abscissa is lower here: this suggests a shrinking of the chains provoked by the proteins. We propose that part of the chain length is now looped around the protein. Complexes behave like charged polymeric networks in which proteins play the role of crosslinkers.

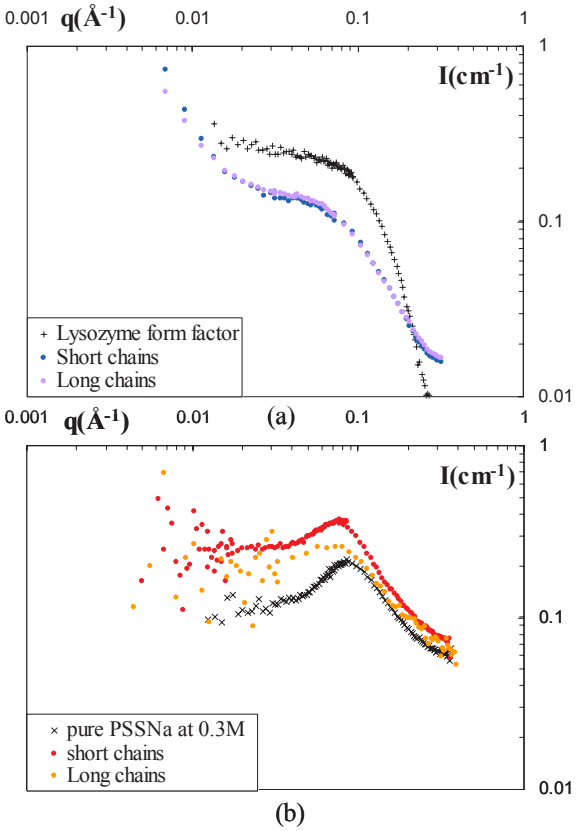

Figure 2 : low charge excess ([lysozyme] = 20g/L; [PSSNa] = 0.3Mol/L) (a) Lysozyme signal (b) PSSNa signal

Figure 2 present spectra obtained for a large excess of charge. All samples have turned clear. The chain length is no longer important. The most striking is the scattering of protein at large q (Fig 2.a), which varies as $q^{-1.7}$ instead of q^{-4}. This is typical of excluded volume chain, and is often observed for unfolded protein. Indeed, FTIR spectroscopy confirm that the α-helix 1654cm^{-1} peak is now absent (it is shifted towards 1645cm^{-1}). Looking at polyelectrolyte (Fig 2.b), we again see a "solution-like" organization, with a maximum shifted to low q compared to pure polyion solutions as in Fig. 1b. This can here also be due to some shrinking of the chain. A second striking fact is the location of this peak. It is located at the same q as the shoulder shown in the protein signal (Fig.2a) The two chainlike objects are now organized in a costructure. For a strong excess of charge, we realize the best situation for the PSSNa to interact with the lysozyme. It can bring its hydrophobic regions in contact with the protein hydrophobic ones and then unfold it.

Inner structure of complexes: what we can see without deuteration, the pectin example.

In this second part lysozyme is mixed with pectin, a polysaccharide with a hydrophilic backbone and

a large stiffness. Its charge density can be changed through a chemical composition of the backbone : each monomer can bear either a methyl group either a dissociable carboxylic group that provide a negative charge for pH > 3. In all experiment described pectin chains are in dilute regime. In order to vary the strength of electrostatic interactions, we vary either the pH to vary lysozyme charges at constant pectin charge (it has a global net charge Z = + 15 at pH 3.5, Z = + 10 at pH 5 and Z = + 8 at pH 7), either the degree of methylation (DM) to vary pectin charged at constant pH.

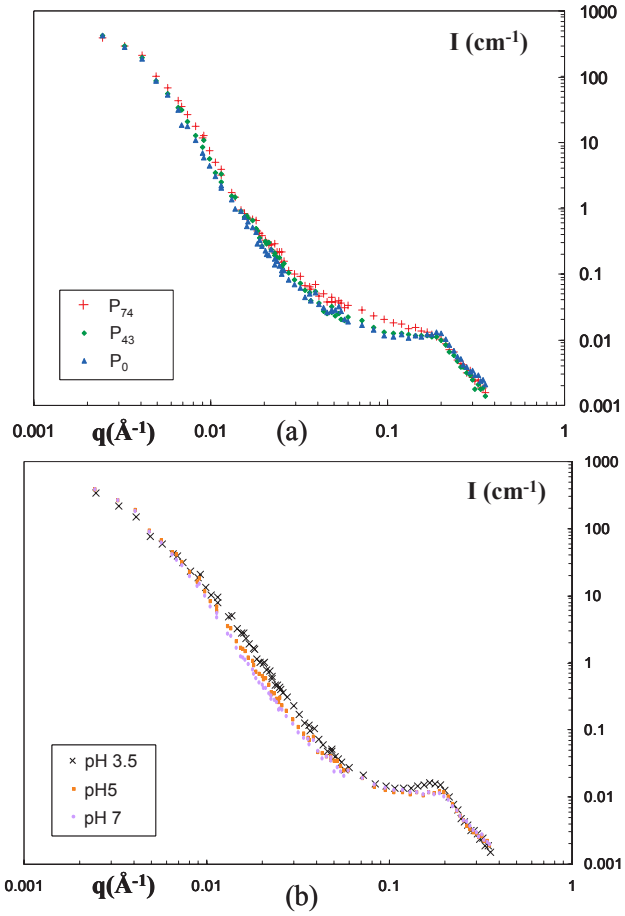

Figure 3. (a) Pectin charge variation signal (b) Lysozyme charge variation

Samples are realized with the same method as lysozyme/PSSNa mixtures. Here we use a mixture of KH_2PO_4 and $Na_2HPO_42H_2O$ with a global ionic strength of 2.5 10^{-2} mol/L. pH is adjusted with NaOH. All samples have been realized in pure D_2O with $\Phi_{lysozyme} \sim \Phi_{pectin} = 0.003$. Whatever the conditions, a turbid solution that strongly scatters light is instantaneously obtained. If left at rest, some samples demix between a turbid liquid phase and precipitate on a scale time of a week. The complexes size slowly grows with time, up to final sedimentation. Mixtures of complexes are thus out of equilibrium on such a time scale and the kinetics of their evolution is a two-steps process: (i) a first fast complexation of lysozyme and pectin followed by (ii) a slow aggregation. All SANS measurements have been performed after two days on macroscopically homogeneous solutions.

Figure 3 presents all SANS spectra obtained by varying pH and DM. They all present the same features as spectrum measured on lysozyme/PSSNa mixtures for short chains and low excess charges. I(q) decreases as q^{-4} at large q due to the globular form factor of the lysozyme. There is a correlation peak that corresponds to protein-protein contact around 0.2 $Å^{-1}$. All spectra present a correlation peak that corresponds to protein-protein contact. At low q, the intensity scatters again as q^{-4}. As one probes scales much larger than the lysozyme scale, this q^{-4} behaviour, typical of scattering form factor of a 3-D shaped object, arises from the form factor of the 3-D shaped complexes. At very small q, the q^{-4} scattering law vanishes and the intensity present a shoulder.

In order to go further and to get the size and inner volume fraction of the complexes, we plot $I(q)q^4$ as a function of q (figure 4). Two plateaus appear. For each q range where I(q) scatters as q^{-4}, the Porod law can be applied. $I(q)q^4/2\pi$ should present a plateau that equals $\Delta\rho^2 S/V$ where S/V is the total specific area of the objects probed at the scale of the q range. At large q, it provides the specific surface of lysozyme within the complexes. At low q , the plot of $I(q)q^4 / 2\pi$ presents two features : a peak followed by a plateau (figure 4). The peak position is linked to the complex size and the plateau value to its specific surface. Assuming spherical complex, the peak arises at the maximum of $P_{comp}(q)q^4$ where $P_{comp}(q)$ is the form factor of a spherical complex of radius R_{comp}. The maximum enables to get R_{comp}.

$$P_{comp}(q) = \left(3 \frac{(\sin qR_{comp} - qR_{comp} \cos qR_{comp})}{(qR_{comp})^3} \right)^2 \qquad (1)$$

From the plateau value of $Iq^4/2\pi$ the complexes contrast $\Delta\rho_{comp}^2$ can be deduced by assuming complexes as perfect monodisperses spheres. The Porod radius R_p of complexes (where the specific area of complexes is 3/ R_p) equals its real radius R_{comp}. If Φ_{comp} is the volume fraction occupied by the complexes, we get :

$\Delta\rho_{comp}^2 = Iq^4 R_{comp} / ((1-\Phi_{comp})\Phi_{comp} 2\pi 3)$

The contrast $\Delta\rho_{comp}^2$ can be written as a function of the volume fraction of lysozyme Φ_{lyso} and of pectin Φ_{pectin} and their respective neutron length densities ρ_{lyso} and ρ_{pectin} and we obtain the volume fraction of water in complexes Φ_{D20_comp}:

$$\Phi_{D2O\_comp} = \frac{1}{1+\frac{Iq^4 R_{comp}}{2\pi 3}} - (\Phi_{lyso} + \Phi_{pectin}) \qquad (2)$$
$$(\Phi_{lyso}(\rho_{D2O} - \rho_{lyso}) + \Phi_{pectin}(\rho_{D2O} - \rho_{pectin}))^2$$

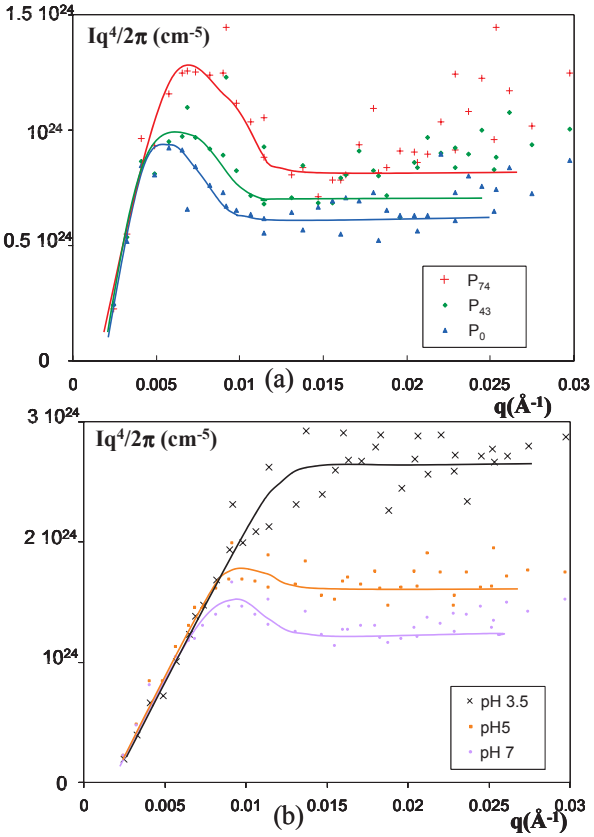

Figure 4 : $I(q)q^4$ versus q (low q) (a) Pectin charge variation signal (b) Lysozyme charge variation. Lines are guides for the eye.

As complexes are dense spheres, the intensity scattered by the complexes writes :

$I(q)\ (cm^{-1}) = \Phi V_{comp} \Delta\rho_{comp}^2 P_{comp}(q) S_{comp}(q)$ (3)

where n is the object density (cm^{-3}) , $\Delta\rho_{comp}^2$ the contrast (cm^{-4}), V_{comp} the complexes volume (cm^3), $P_{comp}(q)$ the form factor and $S_{comp}(q)$ the structure factor. $S_{comp}(q)$ can thus be valued from I(q). $P_{comp}(q)$ is calculated according to equation 1. $S_{comp}(q)$ is linked to the interactions between complexes. Whatever the system, it is superior to 1 and strongly increases when S(q) tends to 0, indicating then a high compressibility in the complexes solution. There are thus **effectivesattractions** between the complexes. The interactions strength can be valued through the compressibility. The latter evaluation is achieved by the measure of the correlation length of the density fluctuations in the system, Ξ, fitting to S(q) $\sim 1/(1 + q^2\Xi^2)$ at low q. Table 1 gives values ranging from 450Å to 150Å.

Sample	Φ_{comp}	R_{comp} (Å)	Ξ (Å)
pH 3.5	~ 0.35	~ 230	450
pH 5	0.25	280	310
pH 7	0.21	310	320
P_0	0.17	480	175
P_{43}	0.17	420	230
P_{74}	0.175	400	160

Table 1. Structural parameters of complexes

These results can then be used to understand the formation of complexes. We can see the complex as a network of pectin chains cross-linked by lysozyme. The latter controls the number of cross-links ν in the network. Like in a charged polymer gel, the swelling trend due to repulsion between charged units is limited by the network elastic modulus, proportional to ν. The inner volume fraction of complexes, i.e. gel swelling appears controlled by lysozyme, hence by ν, and not by pectin electrostatic repulsion. Pectin charge density plays nevertheless a huge role on the final size of complexes and on their effective interactions. This can be due to the fact that increase of pectin charge density: *(i)* increases complex radius by increasing electrostatic interactions with positive charges at the nucleation stage *(ii)* decreases attraction between larger complexes by increasing the electrostatic repulsive part of the interaction potential in a later maturation stage.

Reference

[1] C.Tribet, Surfactant Science Series "Physical chemistry of polyelectrolytes", T. Radeva Ed., M. Dekker, 99, 2000 chap 19, 687.