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Counterions Release from Electrostatic Complexes of Polyelectrolytes and Proteins of Opposite Charge: A Direct Measurement

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There has been recently a large interest[1] in the understanding of the mechanisms governing the complexation of polyelectrolytes and proteins of opposite charges because of the growing potential of applications (proteins fractionation,[2] controlled drug release,[3] biosensors[4]). This is also linked to the emergence of new polyelectrolyte architectures such as spherical brushes or multilayers. While an obvious and confirmed driving force of complexation is electrostatic attraction, the role of the counterions (c.i.) is a key point. Being many and of small mass, free c.i. have a high-translational entropy: for example, they increase strikingly the osmotic pressure of polyions solutions. If they are dispersed into the solvent after complexation, the entropy gain is important and could balance the loss of conformational entropy of the polymer. To estimate this entropy, one has to take into account the "condensation" phenomenon: if the distance a between two charges is shorter than the Bjerrum length above which thermal agitation is higher than electrostatic attraction, l_B, a large fraction $(1- a/l_B)$ of c.i. is trapped close to the polyion;[5] condensation is relevant also for spherical objects[6] such as globular proteins. During complexation, the charge neutralization of oppositely charged species can lead to a release of the condensed c.i. that regain as much translational entropy as the free ones.[7,8] Such effects could differentiate strongly charged synthetic polyelectrolyte, where condensation is important, from less charged chains like polysaccharides, where condensation is negligible. In numerical simulations,[9] condensation and release of free c.i. is observed. But experiments gave until now only indirect indications: calorimetric measurements during complexation (proteins/polycation[10] or polyanion/polycation[11]) evidence an endothermic entropically driven contribution. Protein penetration within a polyelectrolyte brush[12] is also indirectly attributed to c.i. release.

The present paper reports the first direct structural observation on proteins/polyelectrolyte complexes with an unambiguous 'yes−no' SANS experiment. It is performed on four samples where we measure the scattering of the c.i only with a specific "c.i." labeling. The protein is lysozyme, positively charged at low pH, and the polyion is polystyrene sulfonate (PSS), with one negative charge per unit. We take advantage in SANS experiments of the fact that hydrogenated PSS chains and lysozyme have exactly the same neutron length density. Thus the scattering from both species can be switched off simultaneously using a solvent at the same neutron length density (a 57% H_2O/43% D_2O mixture) to measure only the remaining scattered signal of the counterions within the complexes.

Our choice of samples is based on already known well-defined structures, observed by SANS[13] in complexes of lysozyme and short PSS chains with Na^+ c.i. in dilute regime.[14] When the ratio of negative to positive effective charges brought by components, $[-]/[+]_{intro}$, is close to 1, the system is made of dense globular primary complexes (radius $\approx$ 10 nm) organized at a larger scale in a fractal way, similar to the structures observed on polyanion/polycation complexes.[15] Using deuterated PSS chains, we could switch off either the protein scattering or the *d*-PSS scattering. For such globular structure, comparison of data for these two contrasts gives accurately the inner composition of the globules. Let us summarize three important points: (i) Whatever $[-]/[+]_{intro}$, globules have a neutral dense core, that is, $[-]/[+]_{inner} \approx 1$ ($[-]_{intro}$ takes into account all units of the polyion, so core neutrality suggests the release of condensed c.i.). (ii) The core is surrounded by a polymer corona when PSS is in excess (hairy core). (iii) This gives four typical structures (Figure 1): for $[-]/[+]_{intro} < 1$, naked cores plus free protein; for $[-]/[+]_{intro} = 1$, naked cores; for $[-]/[+]_{intro} > 1$, hairy cores; for $[-]/[+]_{intro} > 2$, hairy cores plus free polyions.

Such possibility to tune from "naked" to "hairy" cores permits us to compare the scattered intensity of samples where all c.i. should be released ("naked" cores), with the one where some c.i. are still present ("hairy" cores where c.i. are condensed on the dangling polyions of the shell). For a strong conclusion the second should have a high-scattered intensity, while the first should be much lower. This is indeed possible because the scattering of the c.i condensed on the small species that may coexist with globules (free proteins or free small PSS chains) is very low.[16]

Experimental success relies on the efficiency of the "c.i. labeling", counterions have to be visible when lysozyme and hydrogenated PSS chains are masked by the 57% H_2O/43% D_2O solvent. The c.i. scattering length density must thus be very different from the one of this solvent. For this we use deuterated tetramethylammonium c.i., $(CD_3)_4N^+$ (noted *d*-TMA), which contains 12 nonlabile deuterium atoms (conversely Na^+ has, accounting for solvatation, a very poor contrast in this solvent[17]).

We first checked that replacing Na by TMA does not change the structure of the complexes. We performed SANS measurements on samples made with *d*-PSS-*h*-TMA and $[-]/[+]_{intro}$: 0.65, 1, 1.66, and 3.33 that correspond to the four cases depicted in Figure 1. We used thus the same concentrations as in ref 13 to characterize *d*-PSSNa/lysozyme complexes. The synthesis of *d*-PSS-*h*-TMA chains, the samples realization, and the details concerning SANS measurements are given in the Supporting Information (SI). Results for $[-]/[+]_{intro} = 1.66$ is shown in Figure 2 ($[-]/[+]_{intro} = 3.33$ results are in SI). PSS and lysozyme signals present the same features, indicating that the two species are spatially organized in the same way in the system.[13] We observe a correlation peak at 0.2 $Å^{-1}$ corresponding to the contact distance between two proteins, a q^{-4} decay at intermediate q due to surface scattering of the globules (which look dense at the corresponding larger scale), and an upturn toward a $q^{-2.1}$ decay at low q due to the fractal organization of the globules at even larger scale. The upturn onset is at lower q for the PSS signal because globules with a shell look larger in PSS contrast. Thus features are all the same for samples made with *h*-TMA^+ c.i. and for samples made with Na^+.[13] The only small difference is the size of the primary complexes which is slightly larger with TMA counterions. The mean radius of the

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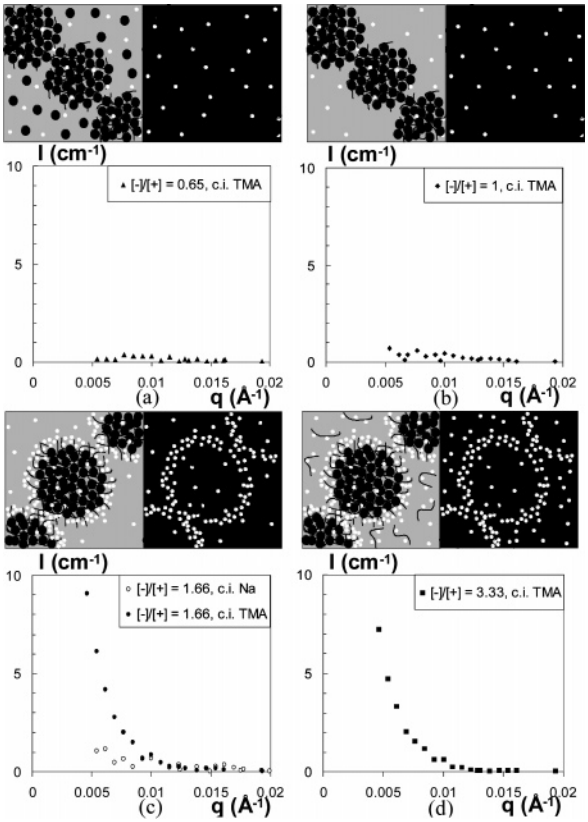

Figure 1. Structures of globules as a function of $[-]/[+]_{intro}$: (a,b) "naked cores" ($[-]/[+]_{intro} = 0.65$ and $[-]/[+]_{intro} = 1$); (c,d) "hairy cores" ($[-]/[+]_{intro} = 1.66$ and $[-]/[+]_{intro} = 3.33$). For each charge ratio, we present a drawing of the system where both of lysozyme and *h*-PSS are in black and TMA^+ counterions are in white, either in 100% H_2O solvent (gray, in top in the left) or in a 57% H_2O/43% D_2O solvent (black, top in the right) and the corresponding scattering of TMA^+ in the 57% H_2O/43% D_2O solvent (in bottom).

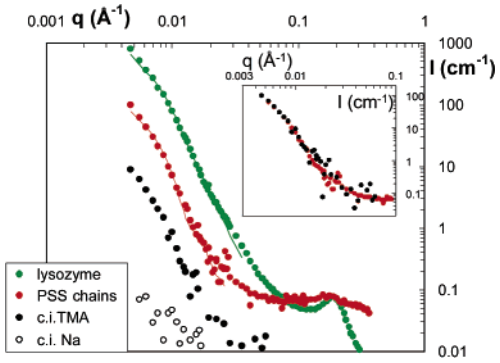

Figure 2. Comparison of lysozyme scattering (*d*-PSS in 100% D_2O solvent), PSS scattering (*d*-PSS in 57% H_2O/43% D_2O solvent), c.i. *d*-TMA^+ (57% H_2O/43% D_2O solvent with *h*-PSS chains) and c.i. Na^+ (57% H_2O/43% D_2O solvent with *h*-PSS chains) in hairy globules made at $[-]/[+]_{intro} = 1.66$. The full lines correspond to the fits (see SI). The inset compares the PSS scattering with the *d*-TMA^+ scattering renormalized with a factor 11 in the low-*q* regime (see text).

core R_{core} is here 190 Å (see SI) and the shell layer is ~50 Å though we obtained $R_{core} \approx 150$ Å (shell ≈ 40 Å) with Na.[13]

Then the specific signal of TMA c.i. was measured for the four cases in the 57% H_2O/43% D_2O solvent at the same concentrations (Figure 1), but replacing *d*-PSS*h*-TMA by *h*-PSS*d*-TMA (see SI). At first sight, samples containing "naked" globules ($[-]/[+]_{intro} =$ 1 and 0.65) *do not scatter* at low *q*, while samples with "hairy" globules ($[-]/[+]_{intro} = 1.66$ and $[-]/[+]_{intro} = 3.33$) *do scatter*. A control sample with Na^+ c.i. has been measured for $[-]/[+]_{intro} =$ 1.66. It is compared in Figure 1 and Figure 2 with the *d*-TMA and does not show any scattering at low *q*. This confirms that the low *q* scattering from hairy globules comes only from the *d*-TMA c.i.

To check whether this scattering comes from the decoration of the shell by the c.i., we compare it to the scattering of PSS chains. Applying a renormalization factor *F* of the order of 10 (11 for $[-]/[+]_{intro} = 1.66$, insert of Figure 2, and 12 for $[-]/[+]_{intro} =$ 3.33, see SI), the c.i. signal fits quite well the PSS signal at low *q*. One should expect the shape of the curves to be different since corona (for c.i.) and sphere (for PSS) have different form factor; however, the differences should lie at larger *q*-range ($q > 0.02$ Å^{-1}), where the c.i. signal is too noisy to be significant. A quick calculation gives an order of the magnitude of the normalization factor *F*. It should be proportional to the squared ratio of the *d*-TMA and *d*-PSS contrasts (~1, the neutron density length of *d*-TMA and *d*-PSS are close) times the square ratio of their volume fraction ($(1 - f)V_{c.i.}/V_{PSS}$).[2] Using $1 - f = 1 - (a/l_B) \approx {}^2/_3$), and our estimate of $V_{c.i.}/V_{PSS}$, the calculated *F* is not very far from the experimental value of about 10. This confirms that the PSS shell is decorated by counterions.

In summary, we have shown by a specific labeling experiment that in a system where proteins and polyelectrolyte chains strongly interact to form dense globules, the inner charge stoichiometry of the globules is accompanied by a complete release of the counterions from the core of the globules. When the complexes are surrounded by a shell of dangling chains, the only significant scattering signal from c.i. comes from the ones trapped in the shell.

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Supporting Information Available: *d*-PSS*h*-TMA and *h*-PSS*d*-TMA synthesis, samples realization, experimental details concerning SANS experiments and SANS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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