Insight into protein-polymer conjugate relaxation dynamics: the importance of polymer grafting

D. Russo,[a,b] *C.Pelosi*[c], *F. R. Wurm,*[d] *B. Frick,*[e] *J. Ollivier,*[e] *and J. Teixeira*[f]

a) Consiglio Nazionale delle Ricerche & Istituto Officina dei Materiali c/o Institut Laue Langevin, 38042 Grenoble, France

b) Australian Nuclear Science and Technology Organisation, New Illawarra Road, Lucas Heights NSW 2234, Australia

c) Dipartimento di Chimica e Chimica Industriale, Università di Pisa, Via Moruzzi, 56124, Pisa, Italy.

d) Max-Planck-Institut für Polymerforschung, Ackermannweg 10, 55128 Mainz, Germany

e) Institut Laue Langevin, 38042 Grenoble, France

f) Laboratoire Léon Brillouin (CEA/CNRS), CEA Saclay, 91191 Gif-sur-Yvette Cedex, France

Corresponding author: * E-mail:. russo@ill.fr

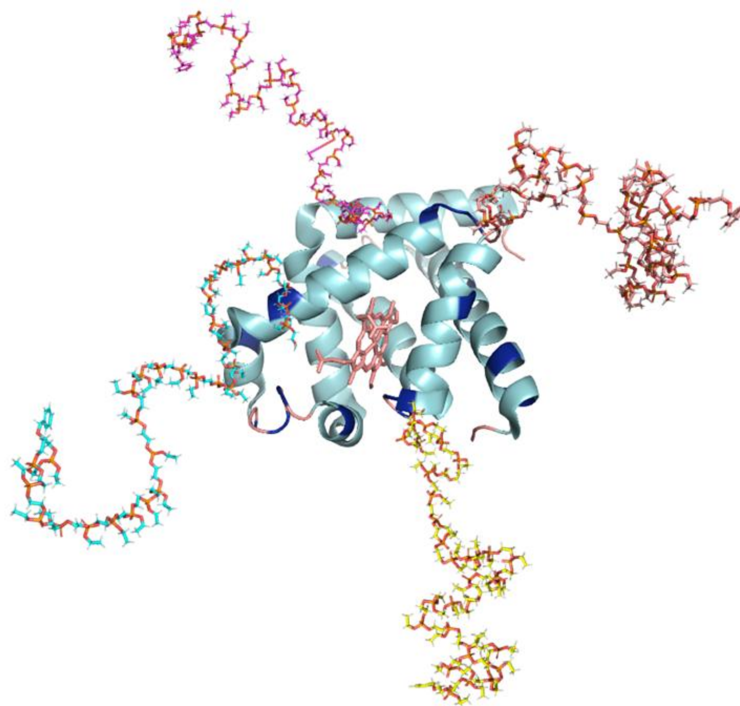
Abstract

The bio and chemical physics of protein-polymer conjugates are related to parameters that characterize each component. With this work, we intend to feature the dynamical properties of Myoglobin (Mb)-poly(ethyl ethylene phosphate)(PEEP) protein-polymer conjugate, in the *ps* and *ns* time scales, in order to understand the role of protein and of polymer size in the protein-conjugate dynamics. We performed elastic and quasi-elastic neutron scattering on completely hydrogenated samples with variable number of polymer chains covalently attached to the protein. The role of the polymer length in the protein solvation and internal dynamics are investigated using two polymers of different molecular weights to form each conjugate. We confirmed that flexibility of the complex increases with the number of grafted polymer chains and that a sharp dynamical transition appears when either grafting density or polymer molecular weight is high. We proved how the protein size is crucial for the polymer structural organization

and interaction on the protein surface and we established that the glass properties of the polymer change upon conjugation. The resulting general picture is a better insight into the polymer coating equivalence and of the role of water on the protein surface.

KEYWORDS: *Properties of protein-polymer conjugates, protein dynamics, neutron scattering, hydration water, dynamical transition*

Table of Content



Myoglobin PEEP conjugate representation. The role of the polymer length in the protein solvation and internal dynamics is investigated. The dynamics and flexibility of the whoel conjugate ncreases with the number of grafted polymer chains and a sharp dynamical transition appears when either grafting density or polymer molecular weight is high.

INTRODUCTION

Protein–polymer conjugates have been developed in order to combine the complementary advantages of each component. Polymer grafting to proteins impacts the stability, solubility, activity, and immunogenicity of the protein. For these reasons, they are widely taken as hybrid materials and drugs in medicine, bio- and nanotechnology.[1,2,3,4] A convenient handling and processing of synthetic polymers allows to change their chemical structure, functionality, degradability, chain length, etc. according to the desired application and requested physical properties. Therefore, the rational design links the chemical properties of a particular polymer to its ultimate effect on the biological molecule.[5] New strategies and designs in protein conjugation techniques led to outstanding alternatives of the pioneered PEGylation, i.e. the covalent attachment of poly(ethylene glycol). In particular, polyphosphoesters (PPEs), a chemical versatile class of polyesters that degrades hydrolytically and enzymatically, represent a promising novel and biocompatible alternative with a maximum of synthetic flexibility. In recent years, several research groups and our lab used PPEs[6] as hydrophilic and degradable polymers[7,8,9] for the preparation of various nanocarriers or protein conjugates and their biocompatibility was studied in detail.[10,11,12,13,14,1] Especially water-soluble and degradable poly(ethylene alkylene)phosphates and – phosphonates are interesting candidates for such conjugates as they are water-soluble and can be prepared by a living ring-opening polymerization with high control over molar mass and dispersity.[15] Further, copolymers can be prepared that alter hydrophilicity or install additional chemical functionality, [10]which cannot be achieved by conventional PEGylation strategies. As the hydrophilicity for PPEs can be adjusted similar to PEG, they exhibit similar protein-adsorption patterns from human blood plasma comparable to PEG (on polymeric nanocarriers). [10] Taken together, PPEs are a very interesting and versatile polymer class to be used in protein-polymer conjugates.

As we have stressed in previous publications,[7,16,17,18] a fundamental knowledge of molecular processes that modulate biological functions in polymer-conjugated proteins is necessary both to improve the design and to increase potential applications. The structure-function-dynamics relationship and biophysical properties need to be revealed to depict the polymer impact in the protein folding and activity. In other words, it is necessary to find the

common parameters that govern the stability of polymer-protein conjugates. For example, the way polymers can structurally organize themselves around a protein and influence its structural stability and how polymer dynamics contribute to protein activity remain fundamentally open questions. The degree to which polymers interact with the protein surface is governed by parameters such as grafting density, molecular weight, physicochemical properties of polymers more external conditions such as solvent, pH, ionic strength, and temperature.[19,20,]

In the past few years, we performed a complete study of Bovine Serum Albumin (BSA) and Maltose Binding Protein (MBP) to examine the general ability of polymers to mimic the effects of water in the protein hydration shell.[7,21] Polymers at the protein interface are dynamically active and can participate in non-covalent interactions such as electrostatic, van der Waals and hydrogen bonds formed with the protein or between themselves. We were able to prove that polymer interactions on the protein surface play an important role, enhancing the magnitude of protein dynamics and demonstrated that polymer modification has the same effect on the protein dynamics as hydration water. Further, we were able to show that polymers on the protein surface adsorb all water molecules when hydrated. Concerning the structure, we have observed that conjugation does not affect the secondary structure but has a role in its stability and that a globular shape of the whole complex tertiary structure is preferred. A gradual change of the polymer conformation as a function of grafting density, from a compact to open Gaussian chain toward a star-like was also evidenced. [18]

Among the large variety of applications, Cell-free hemoglobin (Hb)-based oxygen carriers is one of the more interesting. The cell-free Hb has long been proposed as a potential blood substitute but the clinical use remains hard due to problems related to inefficiency and toxicity, while, in contrast, PEGylated Hb greatly improved its performance.[22] In this work, keeping in mind biotechnological applications, we focus our attention on the dynamics of conjugates of Myoglobin (Mb) protein in the dry state. The impact of size and secondary structure of the protein, as well as the size and mass of the polymer chains, were evaluated and compared to results of our previous work.

EXPERIMENTAL SECTION

Conjugates: Myoglobin (Mb) of equine skeletal muscle (Sigma Aldrich) and Bovine Serum Albumin (BSA, Sigma Aldrich) proteins conjugated with poly(ethyl ethylene phosphate) (PEEP) polymer were synthesized with 5, 10, 20 polymer chains. PEEP polymer chains of about 5-6.4 KDa and 12 kDa, where grafted following the synthesis and purification process described by Wurm and co-workers [9]. The bio-conjugation reaction using different molar ratios Mb-polymer brought to the formation of conjugates with 4, 7 and 10 polymer chains attached to each protein molecule, for both 6 kDa and 12 kDa PEEP polymer (Table S2). For the conjugates BSA-PEEP we obtained conjugates with 4, 7, and 20 polymer chains attached to each protein molecule. However for clarity, in this work, we will adopt the following notation for the conjugates: Mb-*xp* and BSA-*xp*, where *x* is the number of polymer in the synthesis ratios (5, 10, 20) rather than the final one attached to the protein surface.

Sample preparation for neutron scattering: All the samples were dissolved in D_2O, lyophilized, kept under secondary vacuum for several days and stored, in powder form, into a dryer under vacuum in presence of P_2O_4, until measurements. Before the measurements, the dry samples were inserted in a sealed standard flat aluminum holder 0.2 mm thick. A total mass of 100-200 mg per conjugate was loaded for the measurements.

Neutron Scattering Experiments: Elastic neutron scattering experiments on Mb-PEEP conjugates were performed at the high flux backscattering spectrometer IN16B[23,24,25,26] at the Institute Laue-Langevin (ILL, Grenoble, France) using the standard energy resolution 0.9 μeV. Elastic and inelastic fixed windows scans were recorded in a temperature range between 10 and 300 K using a heating rate of 1.5 K/min. A quasi-elastic measurement at room temperature (300 K) was also performed. The collected signal was measured in a Q range extending from 0.1 to 1.8 Å $^{-1}$

Quasi-elastic neutron scattering measurements, in the ps time scale, were performed using the high flux time-of-flight (t.o.f.) spectrometer IN5[27,28] at ILL. Data were collected for both BSA and Mb-PEEP conjugates at 300 and 200 K. The experiment was carried out using an incident neutron wavelength λ = 5 Å^{-1} corresponding to a full-width energy resolution 100 μeV, and a wave vector range from 0.2 to 2.2 Å^{-1}. Preliminary data were collected on Pelican t.o.f.

spectrometer[29,30,31] (Bragg Institut, ANSTO, Australia) and recorded with an energy resolution 65 μeV, in a *Q* range from 0.2 to 1.8 Å $^{-1}$, at 300 and 200 K

All spectra were corrected for detector efficiency, subtracted of the background and normalized following the standards procedures. QENS data were analyzed with a delta function and a Lorentzian. Given the complexity of the systems, the experiments probe average dynamics.

RESULTS AND DISCUSSION

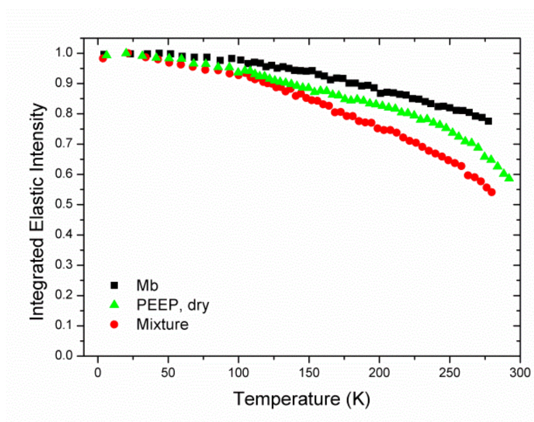
In this work, we studied the dynamical properties of Mb-PEEP protein-polymer conjugates, in the *ps* and *ns* time scale, and try to unravel the role of the protein and of the polymer size in their dynamic behavior. We have performed elastic and quasi-elastic measurements to evaluate the dynamics in the *ps* and *ns* time scale. We compared the *elastic* Mb-PEEP neutron measurements with the previously published results on BSA-PEEP dynamics as affected by polymer coating and interactions. We measured the average relaxation time of BSA and Mb conjugates in the *ps* time scale. This observation provides information about the relation between polymer coating and water on the protein surface.

Elastic neutron scattering experiment.

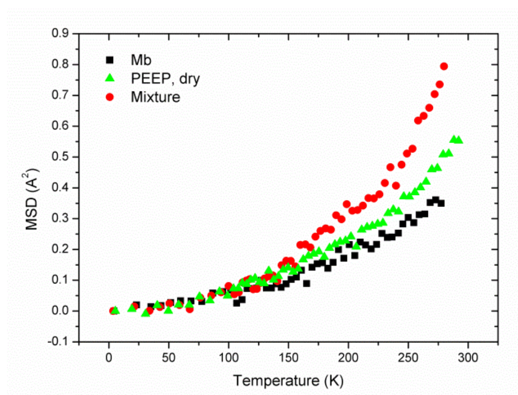
The intensity probed in an elastic neutron scattering experiment arises from atoms, which are *immobile* on the timescale of the instrumental resolution. Elastic scans give access to the mean square displacements and eventual transition temperatures.[32,33] The intensity probed during a fixed window inelastic scan arises from atoms that are *mobile* within the instrumental resolution and gives information about the relaxation dynamics at the chosen fixed energy.[34]

Elastic scans. We performed elastic and inelastic fixed windows scans as a function of the temperature on the Mb-PEEP dry conjugated following the same procedure used for the BSA-PEEP conjugates.[17] In the beginning, it was tempting to compare the integrated elastic intensity and the mean square displacement (MSD) of native Mb, PEEP and Mb-polymer mixture (Figure 1a, Figure 1b) in their dry preparation in order to validate previously published results. However, we verified that results obtained for the mixture are not simple linear

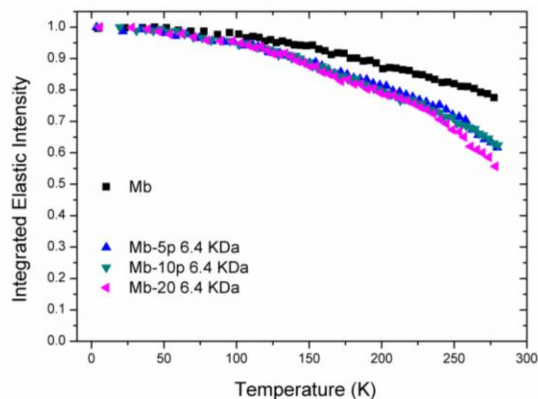
combinations of results obtained with the components, PEEP polymer and Mb native protein (Figure S1) and that the scattered intensity due to Mb protein is dominant (Table S6 in S.I). The behavior of the elastic intensities and the inferred MSD show that mobility increased in the mixtures compared to the native Mb. As observed for BSA, the net effect of interactions between the two components is the enhancement of the global dynamics of the complexes, including thus the protein. For all profiles we observed the well-known transition of the methyl group at 130 K.[35,36,37] In the protein-polymer mixture, we observed a change of the slope and an important increase of the amplitude fluctuation at ca. 220 K, before the glass transition temperature of the pure polymer (230 K). Likely, the evolution of the polymer mobility just below T_g is enough to boost the protein dynamics. The same phenomenon was already observed for BSA-conjugates,[17] which however show less flexibility (Figure S2a in S.I.). The protein size plays a role in the grafting density while secondary structure plays a role in the intrinsic dynamics of the proteins, which can be more or less flexible. These results support the hypothesis that polymer coating, similarly to hydration water, impacts the dynamics of the protein. Non-covalent bonded polymers can interact either with the protein or with polymer chains and their high mobility enhances the dynamics of the protein mainly through hydrogen bonds.



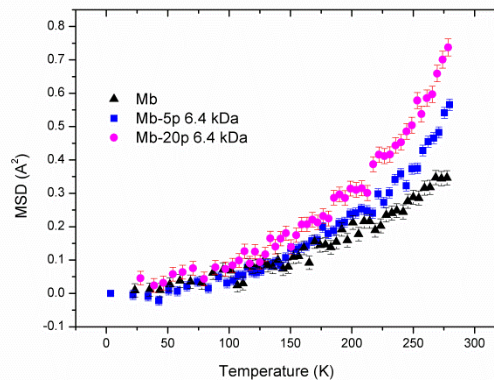
a)



b)



c)



d)

Figure 1. *Temperature dependence of the integrated elastic intensity (a,c) and mean square displacement (c,d) of protein, polymer, mixture and conjugated dry samples.(a,b) Mb protein, PEEP polymer, and Mb-polymer mixture; (c,d) comparison between all Mb-xp conjugated and the native Mb. The MSD of Mb-10p is not represented, for the clarity of the figure, since is very close to 5p. Discontinuities of slopes reveal a change in the dynamical process.*

The impact of non-covalent polymer interactions versus covalently attached polymer chains to the protein surface was investigated performing complementary measurements on Mb-PEEP conjugates with a variable number of polymer chains attached and different degrees of polymerization (S.I., section 2). From a *structural point* of view, the radius of gyration of the investigated samples increases with the number of polymers. A core-shell model describes the structure in all cases very well: the thickness of the shell increases while the radius of gyration of the protein remains constant, suggesting a lack of unfolding due to conjugation [SANS *data not yet published. Russo et al*].

Figures 1c and 1d represent the integrated intensity and MSD of Mb-PEEP conjugates for 6.4 kDa polymer. The data prove that the global mobility increased with the number of grafted polymer chains with a first discontinuity of the slopes at ca. 130K and another another at 220 +/- 5 K (Figure S3) representing a major change in the whole dynamical process. As previously speculated for BSA-PEEP,[17] interactions between the phosphate groups of the polymer and the polar groups on the protein surface might enhance protein dynamics. In addition, a remarkable

outcome, such as the lack of polymer glass transition upon low-density conjugation was observed using partially deuterated conjugates.[7] Mb-*5p* and Mb-*10p* exhibited equivalent dynamics. The dominant protein scattering contribution demonstrates the effect of the enhancement on the protein dynamics compared to the native Mb and the lack of a well-defined dynamical transition as a consequence of the modified polymer glass transition properties (in particular in the integrated intensity that includes the whole Q range). The polymer behaves as a plasticizer of the dry protein. Given the absence of a well-pronounced dynamical transition at this polymer ratio, an analogy with a low hydration water scenario is more appropriate. In fact, comparing the MSD of Mb hydrated powder our data behaves such as the Mb hydrated with 20 % of water.[38]

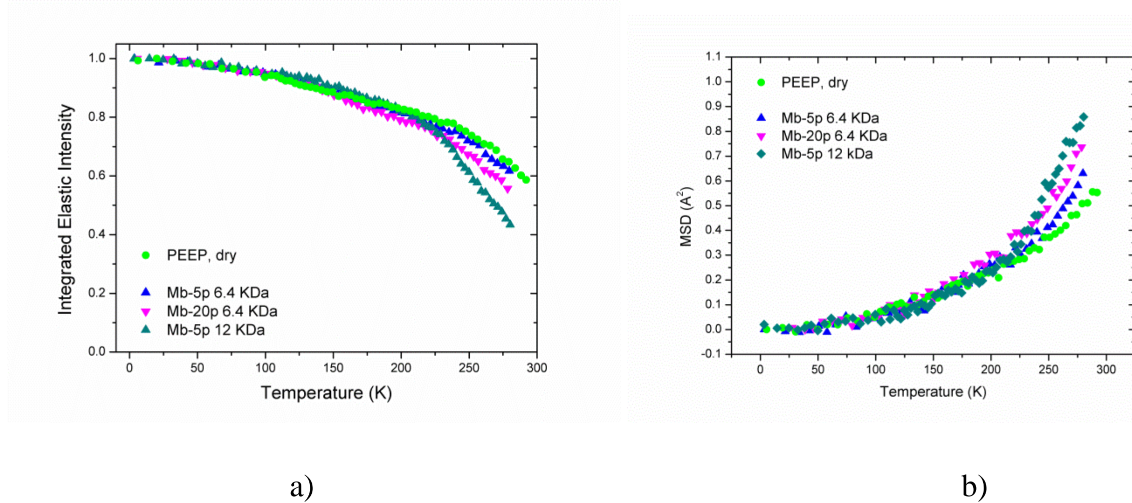
More importantly, in agreement with our previous investigation on BSA-PEEP, we observed that, at equivalent polymer content, mixtures (*3p*) and Mb-*5p* have a different dynamical profile (Figure S4), showing that the protein-polymer interaction and polymer distribution on the protein surface play an important role and are independent of the protein size. The arrangement of the polymer chains on the protein surface is different in the mixture and in the conjugates, and the presence of constraint points between them reduces the dynamical impact.

The behavior of Mb-*20p* is clearly different since it shows a more pronounced transition at about 225 K compared to *5p* and *10p* conjugates. Given the high grafting density, the ratio of the *incoherent* cross-section (scattering power) was slightly higher for PEEP in the case Mb-*20p*. It could be tempting to associate the observed enhancement to the polymer glass transition and attribute the downshift of about 10 K of the T_g to a conjugation effect (eventually also for the *5p* and *10p*). However, even if we are aware of the fact that the polymer changes its glass transition properties upon conjugation, we remind that the same behavior was observed for the BSA-*20p* at high grafting density, although in this case, the protein scattering power was dominant (Figure S2b in the S.I.). The Mb-*20p* profile provided a signature of the reciprocal and complex influence of both components in the dynamical profile. In fact, a more realistic scenario could be an important change in the polymer dynamics due to a large number of grafted chains, which reflects in a more efficient increase of the protein and conjugate dynamics. When the number of polymer chains increases, the geometrical constraints and chain-chain interactions allow the polymer to explore conformations in the free space between conjugates and attempt to recover

the glass transition. In this context we highlight the resemblance of the means square displacement of the mixture and Mb-20p (Figure S4). The number of the polymers is different in the two samples (as a result the relative scattering power) but the interactions experienced from the polymers are likely very similar. Therefore we believe that amplitude fluctuations (or elastic intensity) arise from the whole system (polymer and protein) and the transition temperature is mostly imposed by the protein dynamics as observed in BSA-*20p*. We provide the transition temperatures evaluated from the integrated elastic intensities in the Supplementary Information, Figure S3 and Table 8. We remarked that only PEEP, the Mb-20p and Mb-5p 12 kDa have well-defined intensity transitions; while it was more challenging to define the T_{onset} values for the other samples. The effect of the polymer grafting density on the protein surface is to increase the amplitude of the anharmonic mean square displacement and to increase the temperature of the dynamical transition. Since it was observed that the onset temperature is hydration independent[38] we are tempted to think that the increase of the temperature transition arises on the one side, from a confinement effect of the polymer on the protein surface and on the oher side, from the evolvutiojn of the polymer-polymer interaction properties.

It is well-known that there is a dynamical transition of the protein when the hydration level is around 40%.[39,40,41] In our case, that polymer solvation, we observed that 10 or more grafted polymers were necessary for the transition. Still, we think that not only the number but also the length of the polymer can play a role in the whole dynamics.[7] To check this point, we measured the elastic scattering of the Mb-*5p* conjugated with a polymer of about 12 kDa (in this conjugate, the scattering power of protein and polymer are equivalent, as shown in S. I.) Figures 2a and 2b show a surprising well defined dynamical transition, more pronounced than that observed with Mb-*5p* sample for the 6.4 kDa polymer. The dynamical process started at ca. 210 K, and the marked loss of the elastic intensity is a signature that the length of the polymer drastically enhances the whole dynamics. This result is supported by previously published investigation of Maltose Binding protein (43 kDa) conjugated with 5 and 10 kDa polymers (PMeP).[7] In that work, using partially deuterated samples, it was shown that the increase of the polymer molecular weight considerably affects the protein dynamics in the conjugate. It was also shown a lack of dynamical transition, given the low grafting density (3-4 polymers) and the larger size of MBP.

Instead, comparison with Mb-*20p* simply suggests that the length of the polymer is more efficient than the number of polymer chains to affect the protein dynamics in a core-shell way. From the structural point of view, the core shell model remains valid.



a) b)

Figure 2. *Temperature dependence of the (a) Integrated elastic intensity (b) and mean square displacement of PEEP polymer and 5p (6 kDa and 12 kDa) 20p conjugated dry samples. Mb-5p 12 kDa shows a MSD with larger fluctuation than the Mb-20p and Mb5p with 6 kDa polymer.*

Fixed window inelastic scan. Figure 3 depicts the integrated inelastic intensity scattered with energy transfer ω=2 μeV, by the conjugates, the mixture, and the Mb native protein. Measurements are performed within the fixed energy window $\Delta\omega$=0.9 μeV. Fixed windows inelastic scans allow a better understanding and offer a different point of view from elastic Intensity and MSD temperature dependence. The behavior of dry PEEP polymer is reported in reference.[17] All conjugates and mixtures showed a relaxation process at ca. 200 K, which corresponds to the bump characterizing Mb dry. It confirms that attachment of polymer on the protein surface promotes and enhances the intrinsic protein dynamics. PEEP relaxation process observed at 150 K [17] can be related to what is discussed above, while for Mb-*20p* and Mb-mixture the kink in the INS amplitude above 230 K is possibly related to the higher polymer mobility at the glass transition.

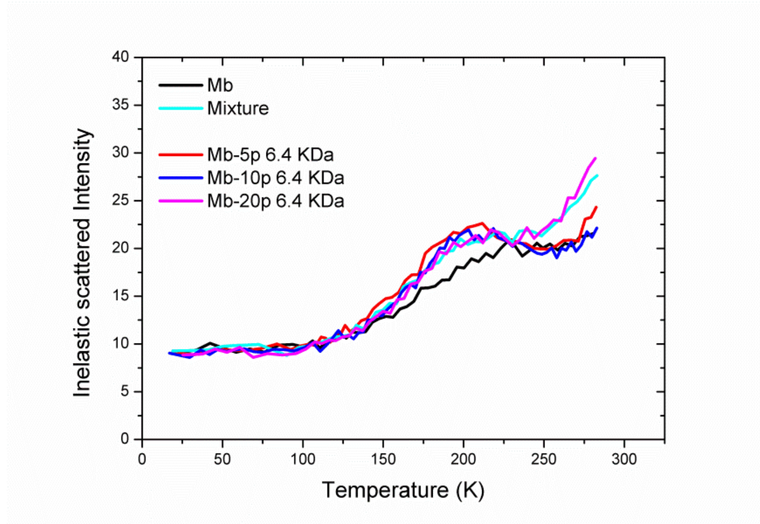


Figure 3. *Temperature dependence of inelastic scattering intensity of all dry samples at ω=2 μeV, integrated over Q*

The emerging dynamical panorama depends on the structural and dynamical parameters of each component of the conjugates. Unfortunately, the lack of partially deuterated samples does not allow measurements to discriminate each component. Therefore, we naturally address our attention to complementary measurements for a complete picture of the specific dynamical processes of conjugates.

Quasi-elastic neutron scattering experiment.

Quasi-elastic neutron scattering (QENS) measures small energy exchanges between neutrons and atoms of the sample, essentially hydrogen atoms. It gives information about local diffusive motions expressed by relaxation times. *Q* dependence of relaxation times provides information about geometry associated to such motions. To complete the dynamical profile of the Mb-PEEP conjugates, we performed QENS experiments at two distinct time scales: *ns* (at the spectrometer IN16B) and *ps* (IN5 and PELICAN).

Data generated by IN16B were fitted with one delta function, representing immobile atoms within the instrumental resolution, and one Lorentzian that describes the global diffusive

dynamics at the *ns* time scale. The native protein required only a delta function. Figure 4 reports the half-width at half maximum (HWHM) of the fitted Lorentzians as a function of Q^2 for all conjugates. They do not depend on Q, meaning that probed motions are local, likely restricted either to rotations or very local diffusive jumps between neighboring sites. The time scale of these motions is of the order of 700 ps.

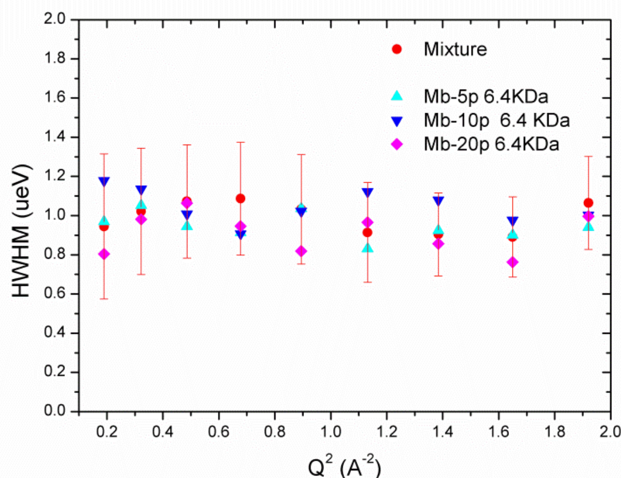


Figure 4: *HWHM of the fitted Lorentzian functions plotted versus Q^2 for Mb-PEEP conjugated and the mixture in their dry state. The error bar reported applies to all samples.*

The dynamics of the samples in the picoseconds time-scale were investigated at IN5 and PELICAN (results in S.I.) time of flight spectrometers. Again, data are fitted with a delta function and a Lorentzian. Figure S7 shows an example of the collected data.

Figure 5a shows the HWHM of the fitted Lorentzians plotted against Q^2, at room temperature, for native Mb, PEEP polymer, protein-polymer mixture, and all Mb-polymer conjugates. Comparison with the results in Figure 4 shows two important features: 1) There is an enhancement of the dynamics upon conjugation. The same applies to the mixture compared to native Mb; 2) HWHM is slightly dependent on Q, corresponding to the presence of diffusive motions. HWHM associated with the native Mb does not depend on Q in agreement with what was observed.[42] The evolution of the Mb dynamics is reported in Figure 5b, where the correlation time (calculated from the HWHM at Q=0) drops from 9 ps for the native Mb to 5 ps for the Mb-*5p* conjugate down to *4* ps for the mixture and the PEEP. The decay toward the polymer relaxation values indicates a dominant *polymer like* dynamics at the ps time scale.

Therefore, we conclude that the observed fast dynamics are due to diffusive motions of CH_x groups attached to the polymer and protein chain. We verify that the elastic contribution is dominant compared to the quasi-elastic fraction (Figure S8) and, from the analysis of the Elastic Incoherent Scattering Function (S.I.) we observe that the number of *immobile protons* decreases as a function of polymer density although remaining dominant (Figure S9c). The geometry of the motions shows a general homogeneity of explored space (Figure S9c)

Preliminary experiments were performed at the PELICAN t.o.f. spectrometer and the analysis obtained from the signal summed over all Q values are reported in S. I. A new and important result was obtained at 200 K, when both protein and conjugates are completely "frozen" while the bulk polymer shows a quasi-elastic signal (Figure S10), confirming the assumption that polymers linked to the protein do change their properties.

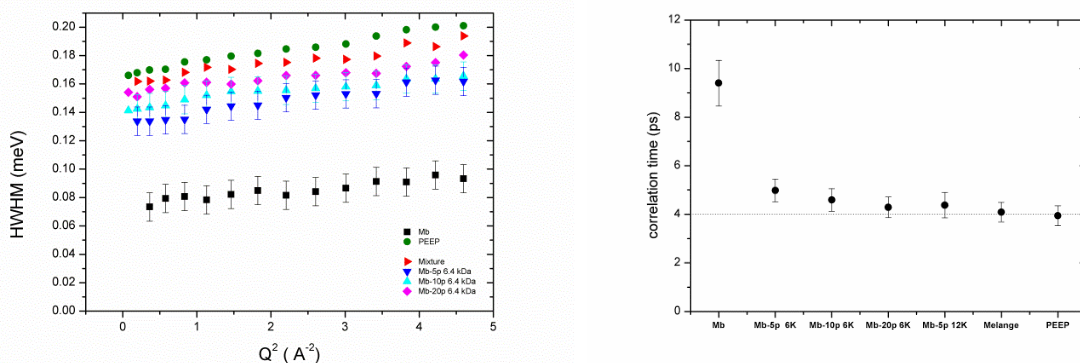


Figure 5: *a) HWHM of the fitted Lorentzian functions plotted versus Q^2 for Mb protein, PEEP polymer, the mixture and all conjugated. The error bar reported applies to all samples. b) Correlation time inferred from the intercept of the HWHM at Q=0. The behavior is a trend toward the polymer relaxation time.*

To better understand the role of the size and protein secondary structure on the conjugates we also compare the measurement of the fast dynamic of BSA-PEEP with that of Mb-PEEP (Figure S11, S12). Similarly to previously discussed Mb results, BSA shows as well a high percentage of immobile protons and confined motions. Analysis of the mean square displacement of BSA and its conjugates present a longer correlation time (13-6 ps) that smoothly decreases toward the polymer values (4ps), in contrast with the sudden drop observed in Mb conjugates. This result

proves that the protein size is crucial for the polymer structural organization and interaction on the protein surface. In fact, the BSA being larger than the Mb, the same number of grafted polymer chains will not be distributed in the same way and the local crowding will be less important. In both cases, the correlation time in the mixture is equivalent showing how the side grafting has still a non negligible impact in the polymer-polymer interaction.

Conclusion

Protein-polymer conjugates are a new class of bio-hybrids materials. Despite their great potential for biomedical and technological applications, the majority of the works present in literature are focused on the synthesis of new conjugates and their biochemical evaluation. Very few studies on their biophysical properties, focusing on the structure-dynamics-function relationship, have been reported.

Here, we have considered the evolution of protein-polymer conjugates internal dynamics as a function of polymer grafting density and polymer chain lengths. The ultimate goal of this work is the study of the impact on dynamics of a polymer coating compared to that of hydration water. For this purpose, we investigated only dry samples. Single-particle dynamics experiments reveal a consistent dynamical picture where the general effect of polymer-protein interaction interaction promotes and enhances the protein dynamics. However, the puzzle is not trivial and we understood that the number of attached polymer chains, the size of the polymer and of the protein together with the kind of interaction, have a role and different impact in triggering the protein dynamical transition. We find indication that non-covalent protein-polymer interaction does not change the polymer glass transition properties, explaining its pronounced mean square amplitude. We confirmed that polymers under conjugation (with covalent bonds) change the glass transition properties but still have a macroscopic evolution able to influence the whole conjugate dynamics. Increasing polymer chain length reduces the effect of grafting. The impact is similar to a higher grafting density.. The impact of the protein size is clear when comparing the Mb to the BSA protein. At low polymer grafting density, the protein dynamical transition is observed in Myoglobin protein, while not for the BSA. The mean square fluctuation of the BSA reveals the presence of a dynamical transition only when the number of attached polymers is

high. Observation of fast ps dynamics of conjugates provided interesting information about homogeneous *confined* polymer like motions.

An important outcome compares to the effect of hydration water on the protein dynamics and activity is that protein-polymer solvation has a variety of control parameters allowing a higher degree of investigation. It is established that hydration water promotes the protein dynamics and once a first hydration shell is attaint the protein dynamics transition occurs. Hydration water rotation and diffusion dynamics need to be enabled at the water glass transition. With our work we established that also polymer solvation enhances protein flexibility and dynamics putting in evidence that the process can be induced not only by the *density* (conjugates) of the polymer on the protein surface but also by its *chain length* and the *local interactions* (mixture vs. conjugates). To control the threshold of the protein dynamics and activity it is possible to control the polymer T_g properties varying the grafting density (glass transition properties modified by the conjugation) or simply choosing a polymer with a different Tg. In addition polymer solvation act as a confinement medium and its effect is single out in the fast dynamics profile. In the solid-state conformation the polymer solvation allows a storage whiteout changing the intrinsic enhanced dynamical properties which are not straightforward in hydrated protein. Finally, protein-polymer conjugates can be also hydrated, and the properties of the polymer (hydrophilic and hydrophobic sites) will play an important role.[17]

Associated Content

Supporting Information: Sample preparation, sample composition, Neutron scattering cross-section calculation, Elastic Intensity and MSD Comparison between BSA and Mb protein conjugated, Determination of the Transition temperature, DSC.

Acknowledgment

C.P. is grateful to the Institut Laue Langevin (ILL) for the financial support during her permanence as Visiting Scientist in order to learn procedures for IN16B and IN5 neutron scattering data analysis. D.R. thanks Dr. Dehong Yu, and Dr. Richard Mole, for their assistance

during the experiment at the PELICAN t.o.f. spectrometer at the Bragg Institute (Australian Centre for Neutron Scattering) .

References

1. I. Cobo, M. Li, B. S. Sumerlin and S. Perrier, Smart hybrid materials by conjugation of responsive polymers to biomacromolecules, *Nat. Mater.*, , DOI:10.1038/nmat4106.
2. A. N. Zelikin, C. Ehrhardt and A. M. Healy, Materials and methods for delivery of biological drugs, *Nat. Chem.*, 2016, **8**, 997–1007.
3. Y. Hou and H. Lu, Protein PEPylation: A New Paradigm of Protein–Polymer Conjugation, *Bioconjug. Chem.*, 2019, **30**, 1604–1616.
4. E. P. DeBenedictis, E. Hamed and S. Keten, Mechanical Reinforcement of Proteins with Polymer Conjugation, *ACS Nano*, 2016, **10**, 2259–2267.
5. A. J. Russell, S. L. Baker, C. M. Colina, C. A. Figg, J. L. Kaar, K. Matyjaszewski, A. Simakova and B. S. Sumerlin, Next generation protein-polymer conjugates, *AIChE J.*, 2018, **64**, 3230–3245.
6. K. N. Bauer, H. T. Tee, M. M. Velencoso and F. R. Wurm, Main-chain poly(phosphoester)s: History, syntheses, degradation, bio-and flame-retardant applications, *Prog. Polym. Sci.*, 2017, **73**, 61–122.
7. D. Russo, M. Plazanet, J. Teixeira, M. Moulin, M. Härtlein, F. R. Wurm and T. Steinbach, Investigation into the Relaxation Dynamics of Polymer–Protein Conjugates Reveals Surprising Role of Polymer Solvation on Inherent Protein Flexibility, *Biomacromolecules*, 2016, **17**, 141–147.
8. T. Steinbach, G. Becker, A. Spiegel, T. Figueiredo, D. Russo and F. R. Wurm, Reversible Bioconjugation: Biodegradable Poly(phosphate)-Protein Conjugates, *Macromol. Biosci.*, , DOI:10.1002/mabi.201600377.
9. T. Steinbach and F. R. Wurm, Degradable Polyphosphoester-Protein Conjugates: "PPEylation" of Proteins, *Biomacromolecules*, 2016, **17**, 3338–3346.
10. J. Simon, T. Wolf, K. Klein, K. Landfester, F. R. Wurm and V. Mailänder, Hydrophilicity Regulates the Stealth Properties of Polyphosphoester-Coated Nanocarriers, *Angew. Chemie Int. Ed.*, 2018, **57**, 5548–5553.
11. F. Zhang, J. A. Smolen, S. Zhang, R. Li, P. N. Shah, S. Cho, H. Wang, J. E. Raymond, C. L. Cannon and K. L. Wooley, Degradable polyphosphoester-based silver-loaded nanoparticles as therapeutics for bacterial lung infections, *Nanoscale*, 2015, **7**, 2265–2270.
12. S. M. Ryan, G. Mantovani, X. Wang, D. M. Haddleton and D. J. Brayden, Advances in PEGylation of important biotech molecules: delivery aspects, *Expert Opin. Drug Deliv.*, , DOI:10.1517/17425247.5.4.371.
13. J. Wang, H. Q. Mao and K. W. Leong, *J. Am. Chem. Soc.*, 2001.

14 J. Müller, K. N. Bauer, D. Prozeller, J. Simon, V. Mailänder, F. R. Wurm, S. Winzen and K. Landfester, Coating nanoparticles with tunable surfactants facilitates control over the protein corona, *Biomaterials*, , DOI:10.1016/j.biomaterials.2016.11.015.

15 T. Steinbach, S. Ritz and F. R. Wurm, Water-Soluble Poly(phosphonate)s via Living Ring-Opening Polymerization, *ACS Macro Lett.*, 2014, **3**, 244–248.

16 C. Pelosi, C. Duce, D. Russo, M. R. Tiné and F. R. Wurm, PPEylation of proteins: Synthesis, Activity, and Stability of Myoglobin-Polyphosphoester Conjugates, *Eur. Polym. J.*

17 D. Russo, A. De Angelis, A. Paciaroni, B. Frick, N. de Sousa, F. R. Wurm and J. Teixeira, Protein–Polymer Dynamics as Affected by Polymer Coating and Interactions, *Langmuir*, 2019, **35**, 2674–2679.

18 D. Russo, A. de Angelis, C. J. Garvey, F. R. Wurm, M.-S. Appavou and S. Prevost, Effect of Polymer Chain Density on Protein–Polymer Conjugate Conformation, *Biomacromolecules*, 2019, **20**, 1944–1955.

19 H. Murata, C. S. Cummings, R. R. Koepsel and A. J. Russell, Polymer-based protein engineering can rationally tune enzyme activity, pH-dependence, and stability, *Biomacromolecules*, , DOI:10.1021/bm4002816.

20 M. Lucius, R. Falatach, C. McGlone, K. Makaroff, A. Danielson, C. Williams, J. C. Nix, D. Konkolewicz, R. C. Page and J. A. Berberich, Investigating the Impact of Polymer Functional Groups on the Stability and Activity of Lysozyme–Polymer Conjugates, *Biomacromolecules*, 2016, **17**, 1123–1134.

21 D. Russo, A. De Angelis, A. Paciaroni, B. Frick, N. De Sousa, F. R. Wurm and J. Teixeira, Protein−Polymer Dynamics as A ff ected by Polymer Coating and Interactions, *Langmuir*, , DOI:10.1021/acs.langmuir.8b03636.

22 C. Le Cœur, S. Combet, G. Carrot, P. Busch, J. Teixeira and S. Longeville, Conformation of the Poly(ethylene Glycol) Chains in DiPEGylated Hemoglobin Specifically Probed by SANS: Correlation with PEG Length and in Vivo Efficiency, *Langmuir*, 2015, **31**, 8402–8410.

23 B. Frick, E. Mamontov, L. Van Eijck and T. Seydel, *Zeitschrift fur Phys. Chemie*, 2010.

24 M. Hennig, B. Frick and T. Seydel, Optimum velocity of a phase-space transformer for cold-neutron backscattering spectroscopy, *J. Appl. Crystallogr.*, 2011, **44**, 467–472.

25 M. Appel, B. Frick and A. Magerl, First results with the neutron backscattering and TOF spectrometer option BATS on IN16B, *Phys. B Condens. Matter*, 2019, **562**, 6–8.

26 M. Appel, B. Frick and A. Magerl, A flexible high speed pulse chopper system for an inverted neutron time-of-flight option on backscattering spectrometers, *Sci. Rep.*, 2018, **8**, 13580.

27 J. Ollivier and H. Mutka, IN5 Cold Neutron Time-of-Flight Spectrometer, Prepared to

Tackle Single Crystal Spectroscopy, *J. Phys. Soc. Japan*, 2011, **80**, SB003.

28 O. Jacques, H. Mutka and L. Didier, *Neutron News*, 2010.

29 D. Yu, R. Mole, T. Noakes, S. Kennedy and R. Robinson, Pelican — a Time of Flight Cold Neutron Polarization Analysis Spectrometer at OPAL, *J. Phys. Soc. Japan*, , DOI:10.7566/jpsjs.82sa.sa027.

30 D. Yu, R. A. Mole and G. J. Kearley, in *EPJ Web of Conferences*, 2015.

31 R. A. Mole and D. Yu, PELICAN: The cold-neutron time-of-flight-spectrometer with polarisation analysis at ANSTO, *Neutron News*, 2016, **27**, 33–34.

32 D. Russo, M. D. Lambreva, C. A. Simionesco, P. Sebban and G. Rea, Dynamics Properties of Photosynthetic Microorganisms Probed by Incoherent Neutron Scattering, *Biophys. J.*, 2019, **116**, 1759–1768.

33 D. Russo, J. Teixeira and J. Ollivier, The impact of hydration water on the dynamics of side chains of hydrophobic peptides: From dry powder to highly concentrated solutions, *J. Chem. Phys.*, 2009, **130**, 235101.

34 B. Frick, J. Combet and L. Van Eijck, New possibilities with inelastic fixed window scans and linear motor Doppler drives on high resolution neutron backscattering spectrometers, *Nucl. Instruments Methods Phys. Res. Sect. A Accel. Spectrometers, Detect. Assoc. Equip.*, , DOI:10.1016/j.nima.2011.11.090.

35 D. Russo, J. Ollivier and J. Teixeira, Water hydrogen bond analysis on hydrophilic and hydrophobic biomolecule sites, *Phys. Chem. Chem. Phys.*, 2008, **10**, 4968.

36 D. Russo, J. R. D. Copley, J. Ollivier and J. Teixeira, On the behaviour of water hydrogen bonds at biomolecular sites: Dependences on temperature and on network dimensionality, *J. Mol. Struct.*, 2010, **972**, 81–86.

37 L. Hong, N. Smolin, B. Lindner, A. P. Sokolov and J. C. Smith, Three Classes of Motion in the Dynamic Neutron-Scattering Susceptibility of a Globular Protein, *Phys. Rev. Lett.*, 2011, **107**, 148102.

38 M. Fomina, G. Schirò and A. Cupane, Hydration dependence of myoglobin dynamics studied with elastic neutron scattering, differential scanning calorimetry and broadband dielectric spectroscopy, *Biophys. Chem.*, 2014, **185**, 25–31.

39 W. Doster, S. Cusack and W. Petry, Dynamical transition of myoglobin revealed by inelastic neutron scattering, *Nature*, 1989, **337**, 754–756.

40 A. Paciaroni, S. Cinelli, E. Cornicchi, A. De Francesco and G. Onori, Fast fluctuations in protein powders: The role of hydration, *Chem. Phys. Lett.*, 2005, **410**, 400–403.

41 A. L. Tournier and J. C. Smith, Principal components of the protein dynamical transition, *Phys. Rev. Lett.*, , DOI:10.1103/PhysRevLett.91.208106.

42 J. Pérez, J. M. Zanotti and D. Durand, Evolution of the internal dynamics of two globular

proteins from dry powder to solution, *Biophys. J.*, , DOI:10.1016/S0006-3495(99)76903-1.