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Unfreezing of molecular motions in protein-polymer conjugates: a calorimetric study

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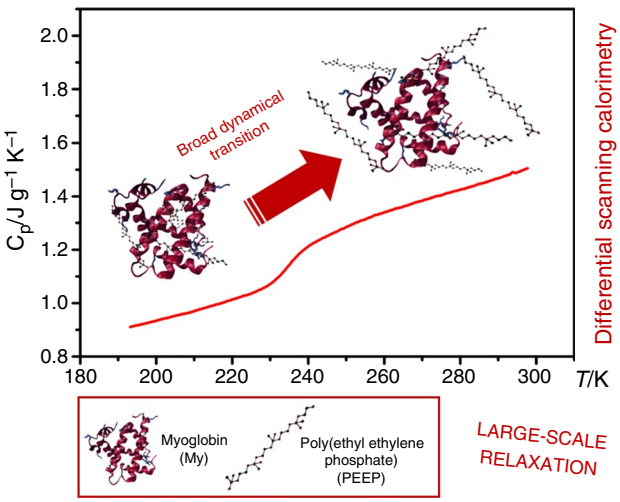
Abstract

Protein-polymer conjugates are a promising class of biohybrids. In this work, the dynamics of a set of biodegradable conjugates myoglobin-poly(ethyl ethylene phosphate) (My-PEEP) with variations in the number of attached polymers and their molar mass in the dry-state, have been investigated to understand the role of polymer on protein dynamics. We performed Differential Scanning Calorimetry measurements between 190 and 300 K, observing the large-scale dynamics arising from reorganization of conformational states, i.e. within the 100 s timescale. The application of an annealing time during the cooling scans was used to investigate the non-equilibrium glassy-state of the samples, observing the relaxation enthalpy at different annealing temperatures. This procedure permitted to extensively describe the transition broadness and the system relaxation kinetics in the glassy state. The samples show an experimental behaviour different from the theoretical predictions, suggesting the establishment of interactions among the protein and the polymer chains. The different behaviour of the conjugates and the physical mixture (composed of the protein and the polymer physically mixed) highlighted the importance of the covalent bond in defining the system dynamics.

Graphical abstract



Keywords Protein-polymer conjugates · Protein dynamics · Calorimetry · Polyphosphoesters · Physical ageing

Introduction

The covalent attachment of synthetic polymers to protein-based drugs, forming the so-called protein-polymer conjugates, is one of the most promising strategies employed to

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enhance the lifetime of protein-based drugs in the human body [1–3]. Poly(ethylene glycol) (PEG) is the most common water-soluble polymer used in the bioconjugation field, and it is covalently linked to proteins in 15 pharmaceutical formulations currently available on the market [4]. Despite their great success, in the last years, these drugs raised some concerns for their alleged long-term non-biodegradability [5, 6] and the formation of anti-PEG antibodies [7, 8], pushing the research of alternatives [5, 9–12]. In this frame, the synthesis of conjugates using different polymers and bioconjugation techniques were reported in the literature, focusing the attention on the sample preparation and their biochemical evaluation (either in vitro or in vivo) [4]. Nevertheless, at a fundamental level, it is crucial to understand their biophysical properties (i.e. structure, dynamics, etc.) to increase the knowledge at the basis to design new and improved protein-based drugs.

We used Differential Scanning Calorimetry (DSC) to study the unfreeze of molecular motions in a systematic set of protein-polymer conjugates in the dry form made by myoglobin and the hydrophilic and biodegradable polymer poly(ethyl ethylene phosphate) (PEEP). PEEP has been recently studied as a potential biodegradable alternative to PEG, belonging to the class of water-soluble polyphosphoesters (PPEs) [12]. Myoglobin is a small (17 kDa) and globular protein. It is often used as a model for haemoglobin because it has the same cofactor and similar tridimensional structure than the haemoglobin sub-units [13]. The evaluation of the thermal transition and structural relaxation of protein-based materials was extensively reported in the literature [14, 15]. Besides, the myoglobin dynamics at temperatures below 300 K, in the dry or hydrated form, was described in various papers [16–18]. In particular, the sudden increase of the atomic fluctuations at temperatures ranging between 180 and 220 K (called "protein dynamical transition", PDT) [19] was observed, in analogy with the general behaviour of all proteins. The transition was defined glass-like, as the protein goes from a rigid, glassy, native state, frozen into a distribution of conformational states, to a more dynamical flexible state [20]. Among the other works, Johari and co-workers studied by DSC the kinetic unfreezing of molecular motion in hydrated myoglobin [21], observing a broad transition extended from ca 150 K to the denaturation temperature, due to the presence of many segments or local structures with a different relaxation behaviour. They also observed the protein kinetic in the glassy state applying an annealing time during the cooling scan. On the other hand, no works on the conjugates' thermal analysis in the solid-state have been reported to date.

We expanded the previous studies by looking at the large-scale myoglobin relaxation dynamics when it is covalently attached to a polymer, so in a myoglobin-PEEP set of conjugates. Differential Scanning Calorimetry was employed as a pivotal technique to obtain information on the large-scale molecular motion unfreezing induced by temperature between 200 and 300 K. We used a set of conjugates formed by myoglobin attached to the polymer PEEP with two different molar masses (6.4 and 12 kDa), with 3, 5 or 7 polymer chains attached to the protein. The effect of the covalent bond was also questioned by the parallel evaluation of the sample obtained by physically mixing the protein and the polymer (physical mixture), without any chemical linkage. Besides, the application of an annealing time during the cooling scan was performed for the first time on this kind of system, revealing similarities among myoglobin-PEEP conjugates and hydrated myoglobin, both possessing a broad-relaxation time range. Overall, the experimental data permitted a biophysical interpretation of the system in the dry state, at the basis of any future conjugates' application, e.g., for a critical evaluation of the drug storage.

Materials and methods

Synthesis and characterization of the conjugates

The conjugates Myoglobin-poly(ethyl ethylene phosphate) (My-PEEP) were synthesised and characterized as previously described [22]. Briefly, the polymer PEEP of 6.4 kDa or 12 kDa were prepared by anionic ring opening polymerization using the monomer 2-ethoxy-2-oxo-1,3,2-dioxaphospholane, the initiator 2-(benzyloxy)ethanol, the cocatalyst N-cyclohexyl-N-(3,5-bis(trifluoromethyl)phenyl)thiourea, the catalyst 1,8-diazobicyclo[5.4.0]undec-7-ene and the quencher N,N-disuccinimidyl carbonate. Then the protein myoglobin of equine skeletal muscle (Sigma Aldrich) was covalently attached to the polymers, using different molar ratios protein/polymer. The reaction was conducted in borate buffer (50 mM, pH = 8.8) for 4 h at room temperature, the product was dialyzed in water (MWCO = 50,000 kDa), lyophilized, and analyzed by online triple detection size exclusion chromatography. When necessary, additional purification was conducted by gel permeation chromatography over Sephadex G-50, with water as the eluent. Only the fractions with a purity $\geq 95\%$ were used in the present study. The set of conjugates used for the analysis reported in this paper are the following: My, i.e. unmodified myoglobin; PEEP (6.4) and PEEP (12), i.e. the polymers PEEP with molar masses respectively of 6.4 kDa or 12 kDa; Conj 3p (6.4), Conj 5p (6.4), Conj 7p (6.4), i.e. the conjugates synthesized using myoglobin and respectively 3, 5, or 7 PEEP (6.4); Conj 3p (12 kDa) that is the conjugates synthesized with myoglobin and 3 PEEP (12); Mix My-3p (6.4) that is the physical mixture of myoglobin with 3 equivalents of PEEP (6.4). The composition of the samples is reported in the *Supporting Information, Table S1*.

The physical mixture Mix-My 3p (6.4) has been prepared by dissolving the protein and the polymer in a molar ratio of 3/1 in water. When both the components were completely dissolved, the solution was lyophilised and stored in the freezer at − 20 °C.

Sample preparation for calorimetric measurements

My, all the conjugates and Mix-My 3p (6.4) were weighed (between 10 and 20 mg) in an aluminium pan, closed manually with a plat lid and perforated with a needle. PEEP (6.4) and PEEP (12) were weighed into a sealed aluminium pan, subsequently perforated with a needle. All the samples were stored in a desiccator with P_2O_4 until a constant mass measurement was achieved. The maximum dryness was reached after ten days.

Differential scanning calorimetry

Differential Scanning Calorimetry was performed with a Perkin Elmer DSC 8500 calorimeter. We performed a cooling-heating scan at 10 K min^{-1} between 190 and 300 K, followed by an additional cooling-heating scan at 20 K min^{-1} in the same temperature range. Between each ramp, the temperature was kept constant for one minute to evaluate the presence of the hysteresis and the long-term drift of the instrument. The measurements were performed using a reference pan; the baseline was corrected for the instrumental asymmetry and the long-term drift. Data were analysed using OriginPro8 SR4 (version 8, Origin Lab Corporation). The measured heat flow was divided by the scan rate and the sample mass (measured directly after the thermal scan) to obtain the intrinsic specific heat capacity of the sample (C_p). Besides, the curves were multiplied by a correction factor, obtained with a polystyrene standard sample. The curves obtained with this procedure are reported in the Supporting information, Fig. S1. The onset temperature (T_{onset}) was calculated as intersection between the extrapolated baseline and tangent passing by the first inflection point. Theoretical curves were obtained as a linear combination of the polymer and protein heat capacities, weighted for the quantity of each component expressed in grams of components divided for total grams of sample. More in detail, the protein and the polymer heat capacities were multiplied, respectively, for the ratio percentages (in mass) of protein and polymer contained in the samples (reported in Table S1). The resulting curves were summed to obtain the theoretical curves.

We further evaluated the samples' relaxation kinetics. We performed repeated cooling-heating cycles at a constant scan rate of 20 K min^{-1}, keeping the temperature constant for one minute between each ramp, to evaluate the hysteresis' presence and the long-term drift of the instrument. About 20 min of annealing time at specific temperatures were introduced during the cooling scans and we observed the changes induced in the subsequent heating scan. The measurements were performed using a reference pan and the baseline was corrected for the instrumental asymmetry and the long-term drift. Data were analysed using OriginPro8 SR4 (version 8, Origin Lab Corporation). The measured heat flow was divided by the scan rate and the polymer mass (referred to the percentage of polymer in the samples reported in Table S1), to observe the polymer behaviour within the samples. The heating curve obtained after annealing was subtracted from the heating curve obtained after annealing, to observe the relaxation induced in the glassy state. We calculated for each temperature the peak area to obtain the enthalpy ($\Delta^{\circ}H_{relax}$.) of relaxation process.

Results and discussion

We analysed the dynamical transition of a systematic library of biodegradable protein-polymer conjugates in the dry form (Fig. 1). We performed Differential Scanning Calorimetry (DSC) measurements between 190 and 300 K focusing on the large-scale dynamics arising from reorganization of conformational states, i.e. within 100 s time scale. Calorimetry is a technique naturally addressed to detect conformational heat-induced changes in biological or synthetic materials such as proteins and polymers [23, 24]. The extreme sensibility of this technique permits obtaining information on the thermodynamics of the processes and the characteristic temperatures with a precision of ± 1 K, difficult to achieve with other techniques.

The curves obtained by DSC analysis are reported in Fig. 2. The thermal profile of the native protein (Fig. 2a) was in accordance with data reported in the literature for dry proteins [16], showing only the harmonic vibrational motions given by thermal energy, with the lack of any PDT. The glass transition was visible in the polymers PEEP (6.4) and PEEP (12) as an endothermic heat step in the specific heat capacity (C_p), with the onset temperature in accordance with similar data reported in the literature [11]. The transition showed the presence of an overshoot peak, a physical phenomenon occurring often during a heating scan, due to the temporary loss of thermodynamic equilibrium during the transition, compensated with a rapid energetic regain. Differently, the conjugates exhibited a transition occurring in a broad temperature range. The enlargement of the transition and the loss of the overshoot peak suggested the onset of motions with a wide range of activation temperatures, corresponding to different relaxation times. The transition's initial slope (Table 1) increased when increasing the number of polymers attached to the protein (Conj 3p (6.4) < Conj 5p (6.4) < Conj 7p (6.4)) and the polymer length (Conj 3p (6.4) < Conj 3p (12)), suggesting a gradual recovery of the

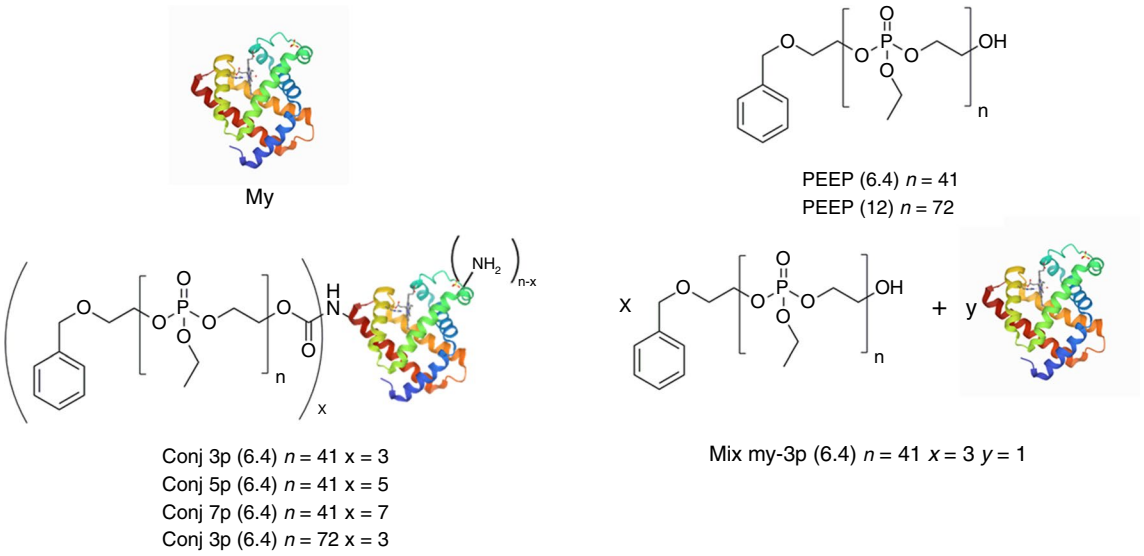


Fig. 1 Schematic representation of the library of biodegradable protein-polymer conjugates analysed in this work. Myoglobin image was taken from, PBD source, with the PDB ID: 1WLA (myoglobin, horse heart, recombinant wild-type), taken from the literature [25]

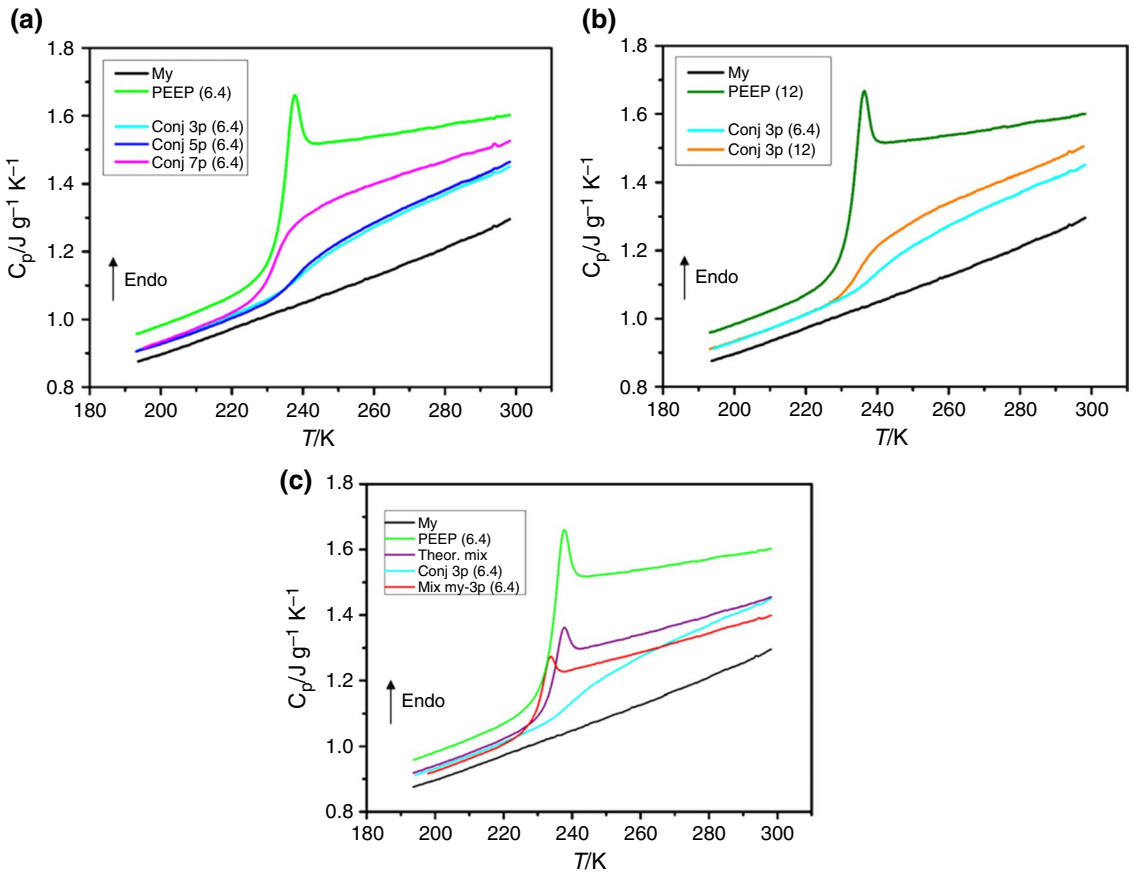


Fig. 2 curves obtained by a heating scan at 10 K min^{-1} for the samples: **a** My, PEEP (6.4), Conj 3p (6.4), Conj 5p (6.4), Conj 7p (6.4); **b** My, PEEP (12), Conj 3p (6.4), Conj 3p (12); **c** My, PEEP (6.4), Mix My-3p (6.4), Conj 3p (6.4) and the theoretical curves calculated as a linear combination of the two components

Table 1 Onset temperature (T_{onset}) of the transition exhibited by the samples

Sample	T_{onset} / K*	*Tangent slope***
Conj 3p (6.4)	230	0.009
Conj 5p (6.4)	229	0.011
Conj 7p (6.4)	227	0.026
Mix My-3p (6.4)	229	0.052
PEEP (6.4)	233	0.115
Conj 3p (12)	229	0.017
PEEP (12)	232	0.103

*Onset temperature of the transition, calculated as intersection between the extrapolated baseline and tangent passing by the first inflection point. Data reported with an error bar of ± 1 K; **Slope of the line tangent to the curve passing from the first inflection point. Data reported with an error bar of ± 0.001

polymer properties in bulk when increasing the number of the polymer chains or their length.

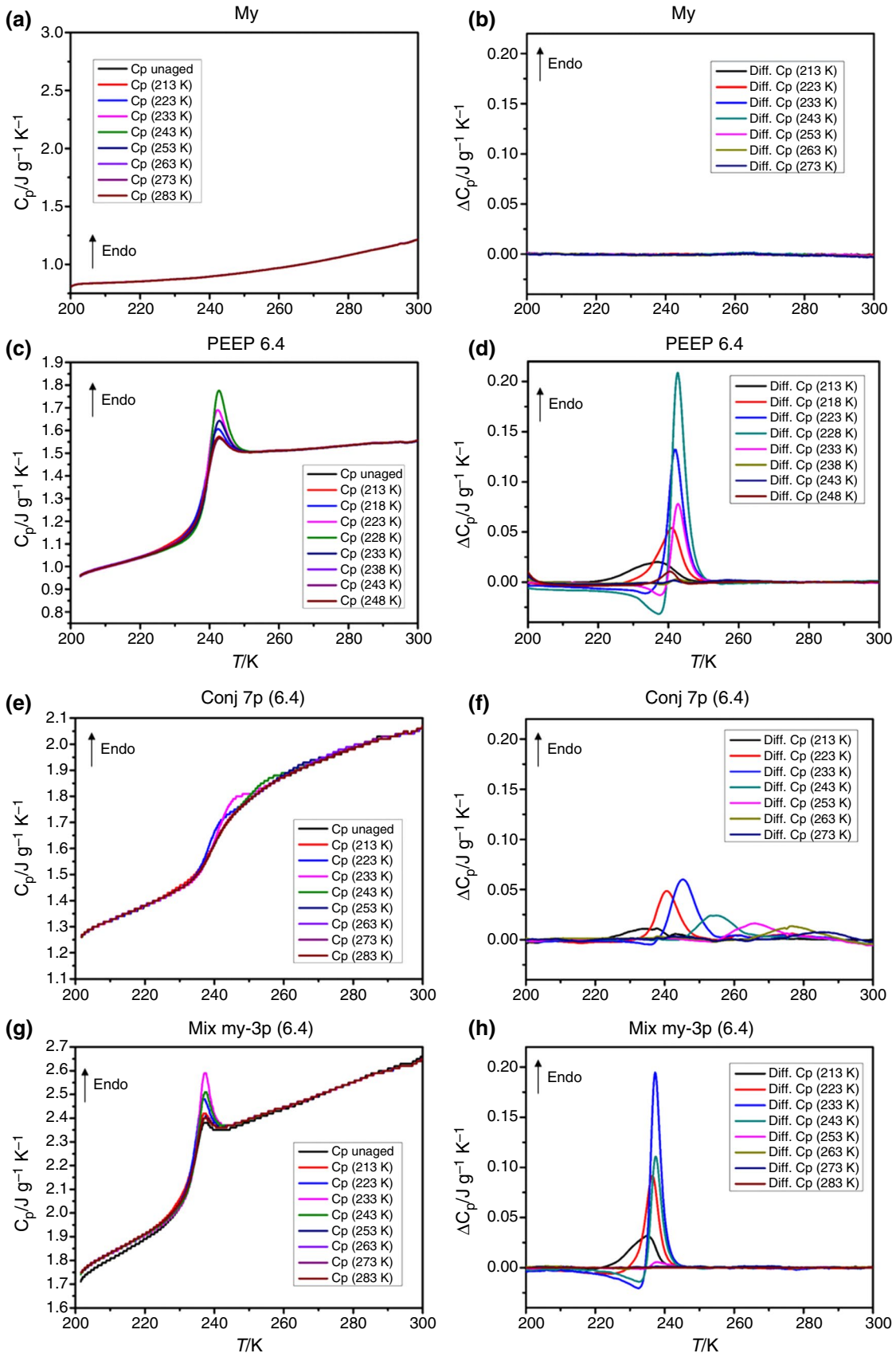
We calculated the theoretical curves as a linear combination of the two components (Figs. 2c and S2), as explained more in detail in the Material and Method section. The differences observed among the experimental and the theoretical curves suggested the establishment of interactions between protein and polymer, e.g. among the phosphate groups in PEEP and the polar amino acids on the protein surface. We hypothesized that the polymer, which forms interphase with the protein, gradually gained the degree of freedom, contributing to the formation of a broad signal. The alleged interactions of the hydrophilic polymer with the protein surface, similar to those depicted for similar samples in aqueous solution [26, 27], allowed the view of the polymer as a protein protector, e.g. during the protein storage. Besides, it is important to mention that also the boost of protein motions might contribute to the observed signals, especially at high temperatures. This last hypothesis was supported by previous studies performed on similar samples by neutron scattering experiments [28, 29] (which allowed the evaluation of samples dynamics on a faster timescale), and by UV–Vis spectroscopy [26]. This last technique, based on the observation of the protein Soret band (a strong signal originated from the protein heme group sensible to the chemical surrounding [30]), permitted us to assess that the bioconjugation procedure itself did not induce any alteration in the protein structure [22], while the polymer presence slightly altered the protein tertiary structure, without changing its secondary structure [26]. The observed changes in tertiary structure reinforced the possibility of increased protein mobility within the samples, which contributed to the differences observed among the experimental and theoretical curves recorded. Further evaluations of the conjugates structure were beyond the scope of this work.

The physical mixture of myoglobin and PEEP showed a different behaviour, i.e. the transition occurred in a defined temperature range, without the loss of the overshoot peak (Fig. 1c). Nevertheless, also in this case, the experimental curve differed from the theoretical curve calculated as a linear combination of the two components, exhibiting a smaller heat capacity after the transition and a different transition slope (see Table 1). This behaviour suggested, also in this case, the establishment of interactions between protein and polymer.

Table 1 shows the transition onset temperatures (T_{onset}) of the samples studied, and the slope of the line tangent to the curve, passing by the first inflection point. We observed that the process started for all samples between 227 and 233 K. The conjugates and the physical mixture presented a slight decrease of T_{onset} with respect to the polymer, with a reducing trend with increasing the number of polymer chains attached to the protein. The broadness of the transition did not permit us to identify with certainty the end of the transition in the temperature range studied. Therefore, at this stage, we were unable to calculate additional parameters to define the process (e.g. transition temperature or heat capacity drop). For comparison purposes only, we report in the Supporting Information the values of ΔC_p calculated in the approximation that the transition was ended at 300 K in all the samples (see Table S2).

To deeply investigate the thermal behaviour of our samples, we studied their spontaneous relaxation in the glassy state, by introducing an annealing time in the thermal scans, thus inducing the process commonly known as "physical ageing". When a sample in the glass form is annealed at temperatures below the glass transition (in other terms, when a sample is subjected to physical ageing), all the dynamical movements with a relaxation time faster than the annealing time become kinetically active. The sample undergoes to a relaxation process from a non-equilibrium state toward a thermodynamic equilibrium, with enthalpy and entropy release, following a non-exponential and nonlinear kinetics [31]. The energy lost during the annealing time is regained during the subsequent heating scan, and it is usually visible as an endothermic peak, with a shape that resembles the overshoot peak commonly observed in many glasses, starting around 20 K above the ageing temperature. The application of this methodology is particularly useful to analyse complex systems (e.g. interpenetrating network polymer [31] or hydrates proteins [21]), which present a broad transition induced by a broad relaxation time distribution. Besides, physical ageing of proteins at different temperatures, can mimic the storage conditions [32], thus it gives important information on the stability and conservation performances of the material.

We performed repeated heating/cooling scans in all the samples, applying 20 min of ageing time during the cooling


(a)
My
Cp unaged
Cp (213 K)
Cp (223 K)
Cp (233 K)
Cp (243 K)
Cp (253 K)
Cp (263 K)
Cp (273 K)
Cp (283 K)
Endo
Cp/J g−1 K−1
(b)
My
Endo
Diff. Cp (213 K)
Diff. Cp (223 K)
Diff. Cp (233 K)
Diff. Cp (243 K)
Diff. Cp (253 K)
Diff. Cp (263 K)
Diff. Cp (273 K)
ΔCp/J g−1 K−1
(c)
PEEP 6.4
Endo
Cp unaged
Cp (213 K)
Cp (218 K)
Cp (223 K)
Cp (228 K)
Cp (233 K)
Cp (238 K)
Cp (243 K)
Cp (248 K)
Cp/J g−1 K−1
T/K
(d)
PEEP 6.4
Endo
Diff. Cp (213 K)
Diff. Cp (218 K)
Diff. Cp (223 K)
Diff. Cp (228 K)
Diff. Cp (233 K)
Diff. Cp (238 K)
Diff. Cp (243 K)
Diff. Cp (248 K)
ΔCp/J g−1 K−1
T/K
(e)
Conj 7p (6.4)
Endo
Cp unaged
Cp (213 K)
Cp (223 K)
Cp (233 K)
Cp (243 K)
Cp (253 K)
Cp (263 K)
Cp (273 K)
Cp (283 K)
Cp/J g−1 K−1
(f)
Conj 7p (6.4)
Endo
Diff. Cp (213 K)
Diff. Cp (223 K)
Diff. Cp (233 K)
Diff. Cp (243 K)
Diff. Cp (253 K)
Diff. Cp (263 K)
Diff. Cp (273 K)
ΔCp/J g−1 K−1
(g)
Mix my-3p (6.4)
Endo
Cp unaged
Cp (213 K)
Cp (223 K)
Cp (233 K)
Cp (243 K)
Cp (253 K)
Cp (263 K)
Cp (273 K)
Cp (283 K)
Cp/J g−1 K−1
T/K
(h)
Mix my-3p (6.4)
Endo
Diff. Cp (213 K)
Diff. Cp (223 K)
Diff. Cp (233 K)
Diff. Cp (243 K)
Diff. Cp (253 K)
Diff. Cp (263 K)
Diff. Cp (273 K)
Diff. Cp (283 K)
ΔCp/J g−1 K−1
T/K

◂**Fig. 3** Heating scans performed at 20 K min^{-1} obtained after 20 min of annealing performed in the previous cooling scan for the samples **a** My; **c** PEEP (6.4); **e** Conj. 7p (6.4); **g**) Mix My-3p (6.4) and the correspondent curve obtained subtracting the unaged curves (curves b, d, f, h). The heat capacity (C_p) was normalised for the amount of polymer present in the samples. The curves obtained for the other samples are reported in Fig. S3. The annealing temperature (induced in the previous cooling scan) is indicated in brackets

scans at growing temperatures, looking at the effect on the following heating scan. The thermal profile adopted to investigate the samples is reported in Figure S3. We reported the data normalised for the quantity of polymer contained in the sample (percentages reported in Table S1), assuming the polymer as the component that mostly contribute to the transition. The results obtained for My, PEEP (6.4), Conj 7p (6.4), and Mix My-3p (6.4) are reported in Fig. 3, while the results obtained for the other samples are reported in S.I. Fig. S4.

We observed that the heat capacities (C_p) of the polymer, the conjugates, and the mixture the presence of an additional endothermic peak in the heating scan with respect to the unaged curve, related to the energy lost by the system in the glass-state during the annealing time. On the contrary, myoglobin alone did not present any relevant effect with the ageing time, confirming the absence of any transition in the temperature range observed. The experimental curves of myoglobin confirmed the effective removal of superficial water from the protein during the sample preparation (see Experimental part), as a similar behaviour was previously reported in the literature for dry myoglobin with a hydration level below 0.1 w/w [21].

The effect of physical ageing was highlighted by subtracting the unaged curve (i.e. the curves obtained without the application of any annealing time) from the curves obtained after ageing (Fig. 3b, d, f). The area of the peaks obtained with this operation reflected the relaxation enthalpy in the annealing experimental conditions. The peaks had smaller areas at lower ageing temperatures, as the samples were more kinetically frozen. The area increased with increasing the ageing temperature until it reached a maximum, then it decreased again, and finally disappeared. The disappearance of the peak means that, at the correspondent ageing temperature, the sample is no longer in the glassy state anymore, but it is in a thermodynamic-equilibrium liquid state, and therefore the ageing time do not involve any relaxation. This treatment is useful to understand the transition enlargement and to correctly compare samples with a broad-relaxation time range. Looking at our samples, (on the right side of the panel), we observed that the polymer PEEP and the physical mixture presented a rapid peak grow, with the disappearance

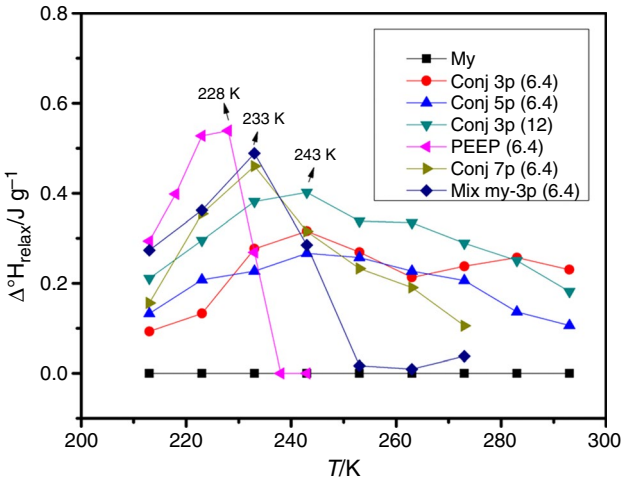


Fig. 4 Relaxation enthalpy ($\Delta^\circ H_{relax}$.) of the samples after 20 min of annealing time performed at different temperatures

of any annealing effect at temperatures above 238 and 253 K respectively. The conjugates, instead, presented a broader transition, involving a minor amount of energy for a broader temperature range.

The relaxation enthalpy ($\Delta^\circ H_{relax}$.), calculated as the area of the heat capacity variation at each annealing temperature, is reported in Fig. 4.

The $\Delta^\circ H_{relax}$. data reported in Fig. 4 clearly highlight the differences among the samples. The polymer exhibited a rapid increase of the relaxation enthalpy, with the maximum at 228 K, and a rapid decrease, with the sample in the liquid state (with a corresponding $\Delta^\circ H_{relax}$.=0) above 238 K. The physical mixture had the same relaxation enthalpy than the polymer at 213 K (ca 0.28 J g^{-1}), but it needed higher temperatures to reach the maximum (233 K) and the sample liquid state (above 253 K). On the other hand, all the conjugates presented a broader transition whit decreasing the number of polymers attached to the protein, and the samples did not reach the complete liquid state even at room temperature. The effect of the polymer on myoglobin was comparable to the one induced on the protein by the presence of hydration water, already described the literature by Johari and co-workers [21]. The similarity observed corroborated the hypothesis of the establishment of interactions protein-polymer, suggesting a solvent-like effect of the polymer on the protein.

Conclusions

We described an extensive thermodynamic characterization of the large-scale relaxation dynamics of biodegradable protein-polymer conjugates. The conjugates exhibited a broad transition from the glassy to the liquid state, which implied the presence of a large number of local structures in the samples, with different relaxation times. Besides, we investigated the conjugates transition by applying subsequent annealing times during the cooling scan at different temperatures. This procedure, applied for the first time on this kind of systems, permitted to fully characterize the samples glassy-state, defining the broadness of the transition and the kinetic of the system in the non-equilibrium glassy state. Overall, the most important results obtained include: (i) the differences among the theoretical and the experimental curves, which suggested the establishment of interactions protein-polymer in the conjugates; (ii) the broadness of the conjugates transition (comparable to the effect induced on the protein by the presence of hydration water) which suggests a solvent-like effect of the polymer on the protein; (iii) the peculiar behaviour of the physical mixture (with intermediate features among the conjugates and the polymer), which highlight the importance of the covalent bonds in the induction of interactions protein-polymer. We intended this study as a starting point to reach full knowledge of the conjugates' biophysical properties, useful to orientate future applications in the solid state, e.g. to allow a better comprehension of the phenomena occurring to the protein during the storage.

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Author contribution CP: Conceptualization, Investigation, Data curation, Methodology, Roles/writing–original draft; ET: Conceptualization, Investigation, Data curation, Methodology, Supervision Writing—review & editing; FRW: Supervision, Writing—review & editing; MRT: Conceptualization, Supervision, Writing—review & editing.

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Availability of data and material Raw data will be made available upon request.

Declarations

Conflict of interests The authors declare no conflicts of interests.

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