Manuscript Details

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Title	PPEylation of proteins: synthesis, activity, and stability of myoglobin-polyphosphoesters conjugates.
Article type	Research paper

Abstract

Protein-polymer conjugates are used to treat several diseases. PEGylation, i.e. the modification with poly(ethylene glycol) (PEG) is the currently used strategy. However, due to its non-biodegradability, the design of effective and degradable conjugates is of both academic and industry potential. We present the preparation and studies of the activity and stability of novel biodegradable myoglobin-polyphosphoester conjugates. Poly(ethyl ethylene phosphate) (PEEP) is a water-soluble polyphosphoester, which had been reported to be biocompatible and biodegradable. PEEP is a promising candidate as a degradable substitute for the “gold standard” PEG, which can cause long-term effects, as it is not degradable. PEEPylated conjugates with a variable degree of polymer grafting were synthesized, characterized (with online triple detection size exclusion chromatography, mass spectrometry, and gel electrophoresis), and compared with PEGylated analogs. We highlight differences in how the structure, the number, and the length of the polymer influence the properties of the conjugates. Overall, the analyses conducted (including activity assay, calorimetry, and fluorimetry measurements) show that the covalent attachment of the polymer does not irrevocably affect the protein’s features under physiological conditions, suggesting the potential of this new class of polymers for the design of a new generation of fully degradable conjugates.

Keywords	protein-polymer conjugates; poly(phosphoester)s; n-DSF; n-DSC; PEGylation.
Manuscript category	Regular Paper

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Suggested reviewers	Michael Shaver, Dimitrios Fessas, Stefana Milioto, Fuwu Zhang, Concetta Giancola

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Data for: PPEylation of proteins: Synthesis, Activity, and Stability of Myoglobin-Polyphosphoester Conjugates

In this dataset are reported the NMR spectra and GPC curves in DMF of the polymer synthesized; moreover are reported the raw data of the GPC profiles in acqueous buffer of the conjugates (samples 1-7).

May, 6th , 2018

Dear Editor,

We would be grateful if you would consider the attached manuscript "PPE-ylation of proteins: studies of the activity and stability of novel conjugates myoglobin-poly(phosphoester)s" for publication in European Polymer Journal.

Protein-polymer conjugates are used to treat several diseases, therefore the design of novel efficient conjugates is very promising for academia and industry.

In the present work, we present the preparation and studies of activity and stability of novel PEEP-myoglobin conjugates. PEEP (poly(ethyl ethylene phosphate)) belongs to poly(phosphoester)s, a class of biocompatible and biodegradable polymers, promising candidate to substitute the "gold standard" poly(ethylene glycol) (PEG) in the biomedical field, which can cause long-term effects as it is not degradable.

PEEP-ylated conjugates with variable polymer chains were synthesized with high purity, characterized (with online triple detection size exclusion chromatography and gel electrophoresis) and compared with classical PEGylated analogs.

We performed a different kind of analyses on the conjugates, in order to better understand how the structure, the number and the length of the polymer influence protein's properties within the conjugates. In particular we studied the residual activity of the protein (measuring the peroxidase-like activity of the protein) , and its thermal stability, using *nano*-Differential Scanning Fluorimetry (n-DSF) and *nano*-Differential Scanning Calorimetry (n-DSC) techniques. The combined use of these two techniques is really innovative in bioconjugation field, and the comparison between them allows the study of the protein unfolding from two different points of views.

Overall, the analyses conducted show that the covalent attachment of the polymer does not irrevocably affect the protein's features under physiological conditions, suggesting the significant potentialities of this new class of polymers for the design of a new generation of conjugates classes.

We hope that this work will be of interest to readers of European Polymer Journal, as it fits with the new scopes of the journal in 2018, in particular with the field of the applied polymer chemistry for the development of new drugs.

I declare that the work presented in the manuscript is original and is not under consideration elsewhere.

Best regards,

Maria Rosaria Tinè

Maria Rosaria Tinè

Dear Editorial Office,

We thank the reviewers for the comments, related to our manuscript. Please find attached the revised version and the point-by-point response to the reviewers' comments.

We hope that with these changes the manuscript can be published in Eur. Polym. J.

Best regards,

Frederik Wurm

Reviewer 1

Protein polymer hybrids have found widespread use as therapeutics. In the realm of therapeutics the only commercialized protein polymer drugs utilize a PEG as the polymer component. Recent worrisome literature reports have demonstrated that PEG may be immunogenic and due to this the extend circulatory half-life typically achieved with PEGylayed proteins can be greatly diminished overtime. This report by Tine and coworkers highlights a novel water soluble polymer for protein conjugation- namely poly(ethyl ethylene phosphate). A series of myoglobin-PEEP biohybrids were prepared and the activity and stability of the PEEP biohybrids was compared to PEGylated myoglobin. It was found that PEEP-myoglobin conjugates retained their bioactivity and stability post polymer conjugation. The manuscript is well written and the results presented clearly.

As a reviewer I wonder if further block co-polymers could be prepared post bioconjugation if a alkyne polymerization initiator was used instead of phenyl polymerization initiator.

➔ *We thank the reviewer for this suggestion; the synthesis of functional block copolymers is planned for future experiments but would go beyond this manuscript.*

Reviewer 2

In this manuscript the authors describe a method for conjugating poly(ethyl ethylene phosphate) (PEEP) to myoglobin using NHS chemical methods.and assessing the residual protein activity. They show that the bioactivity decreasing with increasing number of grafted chains as expected. The focus of the paper is the assessment of bioactivity.

The methods for the synthesis of the monomers and polymer poly(ethyl ethylene phosphate) (PEEP) are reported in reference 21. No new chemistry is added in this manuscript.

In general, the characterization of the conjugates is limited. This reduces the enthusiasm for the paper. Mass spectra of the precursors and the conjugates or alternatively analysis of the SDS PAGE gels similar to those performed by Maynard et al should be included to demonstrate the successful conjugation and relative purity of the structures. S4 is a reasonable gel but there is no

explanation or assessment of the results. The fractions should be isolated and characterized further. As written, the data are not convincing.

➔ *The characterization of the conjugates is a multi-analysis approach (more different analysis have been performed to characterize them), and it is fully described in the dedicated paragraph.*
The SDS PAGEs reported in the Supporting information show that there is an upward shift of the molecular weight of the conjugates respect to the pure protein, and expected for a grafting-to-conjugation method, the conjugates may carry a variable number of polymer chains, resulting in several bands on the gel. SDS-PAGE (made with two different staining method, blue coomassie and silver staining) already proved the formation of polymer-protein conjugates. Moreover, we performed aqueous GPC with triple detection, a powerful technique that fits with the protein-polymer conjugate characterization, thanks to the three detectors.
As suggested by the reviewer, we have performed MALDI TOF mass spectra of three conjugates that prove successful bioconjugation.

Additional points
In each of the figures, there is no error analysis or statistical nomenclature. Are any of these data significant? A more rigorous analysis is needed throughout the paper.

➔ *In the original version, there were error bars of each measurement calculated and reported graphically in the manuscript, and numerically in the supporting information. Anyway, we have reported the error analysis more clear in both parts in the revision.*

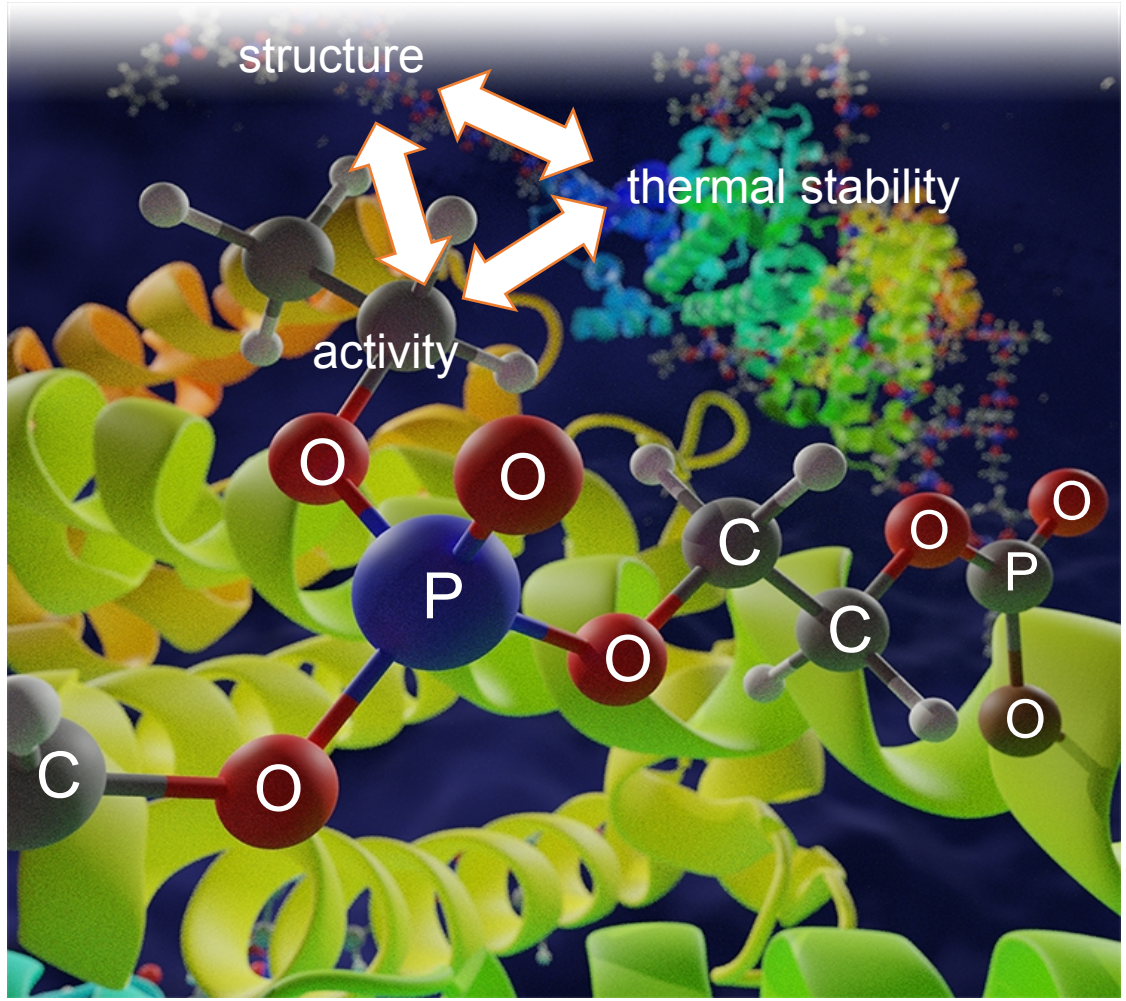
In the supporting information, the molecular mass distributions that are reported for conjugates 5 and 7 are somewhat absurd. If the molecular mass distributions are indeed this narrow, the authors should provide mass spectra to support these. SEC does not have 3 significant figures under any circumstances and must be corrected. That is true of all experimental sections were significant figures are applied liberally and without justification.

➔ *We observed that as correctly suggested by the referee, the molecular mass distribution calculated by GPC is not representative, as calculated from the light scattering data only. We decided to remove the values from the revised version. Concerning the figures reported, in the most of the cases we left the respective elugrams in the revision. In combination with SDS PAGE and mass spectra, the molecular weight characterization and the protein: polymer ratio was determined.*

The average number of attached chains should have error analysis. Is the standard deviation 50% or 100% of the mean?

➔ *We have reported in the manuscript the error bars.*

- Protein-polymer conjugation with biodegradable polymers;
- Multiple technique approach for the protein conjugate's characterization;
- Proteins still folded and active in physiological conditions after bioconjugation;
- Trend between the properties of the conjugates and the polymer molar mass.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PPEylation of proteins: Synthesis, Activity, and Stability of Myoglobin-Polyphosphoester Conjugates

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ABSTRACT

Protein-polymer conjugates are used to treat several diseases. PEGylation, i.e. the modification with poly(ethylene glycol) (PEG) is the currently used strategy. However, due to its non-biodegradability, the design of effective and degradable conjugates is of both academic and industry potential. We present the preparation and studies of the activity and stability of novel biodegradable myoglobin-polyphosphoester conjugates. Poly(ethyl ethylene phosphate) (PEEP) is a water-soluble polyphosphoester, which had been reported to be biocompatible and biodegradable. PEEP is a promising candidate as a degradable substitute for the “gold standard” PEG, which can cause long-term effects, as it is not degradable. PEEPylated conjugates with a variable degree of polymer grafting were synthesized, characterized (with online triple detection size exclusion chromatography, mass spectrometry, and gel electrophoresis), and compared with PEGylated analogs. We highlight differences in how the structure, the number, and the length of the polymer influence the properties of the conjugates. Overall, the analyses conducted (including activity assay, calorimetry, and fluorimetry measurements) show that the covalent attachment of the polymer does not irrevocably affect the protein’s features under physiological conditions, suggesting the potential of this new class of polymers for the design of a new generation of fully degradable conjugates.

Keywords: protein-polymer conjugates, polyphosphoesters, n-DSF, n-DSC, PEGylation.

INTRODUCTION

Proteins and peptides have high potential as therapeutic agents [1]. Several protein-based compounds, (such as interferon, insulin, erythropoietin, factor VIII and human growth hormone) are significantly involved in homeostasis regulation or in immunity processes and are produced industrially for use as potent drugs for the treatment of diseases. However, they often exhibit several drawbacks either outside the body (due to low solubility in water and short shelf-lives) or inside (due to rapid kidney clearance or susceptibility to destruction by proteolytic enzymes), with consequent non-negligible side effects for the patients [2]. One of the most effective strategies for decreasing their limitations and enhancing their pharmacological efficacy is an irreversible modification process called bioconjugation.

The bioconjugation of protein-based drugs is the covalent attachment of one or more polymer chains to the protein, with the formation of a protein-polymer conjugate [3]. The most common polymer used in bioconjugation is the well-known poly(ethylene glycol) (PEG), currently used in 13 protein-polymer conjugates approved by the U.S. Food and Drug Administration for therapeutic purposes, while several others are under clinical trials [4–6]. The benefits of PEG can be summarized as the formation of a polymer shield around the drug that protects it from degradation and opsonisation [7], which decreases the drug's immunogenicity [8] and at the same time increasing its solubility in aqueous media and its hydrodynamic radius (R_h), with a minor kidney clearance [9]. The overall effect is an improvement of the drug's pharmacokinetics, with an increase in its lifetime and a reduction in the necessary dosage frequency for the patients [10].

Despite these advantages, the use of PEGylated drugs has recently raised concerns, with some effects contradicting previous studies. Hypersensitivity reactions and anaphylactic shocks have been observed in some patients [11], along with the formation of PEG antibodies in patients with chronic diseases after systematic drug administration [12,13]. In model animals, the occurrence of renal tubular vacuolization [14] leads to the significant problem of PEG accumulation in the body after long-term treatment, due to its non-biodegradability [5]. Other drawbacks include the possibility of PEG degradation under heating, the possible presence of toxic side products resulting from its synthesis (e.g., 1,4 dioxane, ethylene oxide and formaldehyde) and the low chemical variability of the polymer structure [5]: its backbone cannot be easily modified. This limits the development of new strategies based on changes of the polymer properties (i.e., its architecture, solubility profile, charge, chemical function, etc.). Recent research has thus focused on the design of novel protein-polymer conjugates based on different types of polymers.

Here, we present the synthesis and characterization of novel protein-polymers conjugates, based on hydrophilic, biocompatible and biodegradable poly(ethyl ethylene phosphate) (PEEP), a promising substitute for PEG that belongs to the class of polyphosphoesters (PPEs). PPEs are biocompatible and biodegradable polymers, with an high chemical versatility (the presence of pentavalent phosphorus in the backbone allows the synthesis of polymers with a wide range of groups in the side chains and with an easy variation of the corresponding properties) and the possibility of a high synthesis control (low molar mass dispersity and high purity) [15,16]. They have already been investigated in biomedical applications (such as gene delivery [17,18], drug nanocarriers [19], and tissue engineering [20]) and appear to be possible substitutes for the gold standard poly(ethylene glycol) in the bioconjugation field. Steinbach et al. recently proposed and investigated their application in the bioconjugation field, and reported the synthesis and first characterization of bovine serum albumin (BSA) and catalase functionalized with poly(ethyl ethylene phosphate) (PEEP) [21]. In additional studies, BSA and maltose binding protein (MBP) was functionalized with structurally similar, but readily degradable poly(methyl ethylene phosphonate) [22,23]. Promising

results on protein folding and enzyme activities have thus been demonstrated. However, as the field of PPEylation is still new, we are continuing our research and report, for example, calorimetric techniques for a deeper evaluation of the conjugates' stability.

The protein chosen for the current study was myoglobin from the equine skeletal muscle. Myoglobin is a single-chain protein containing 153 amino acid residues and is present in almost all mammals, primarily in muscle tissues [24]. It was chosen for the following reasons: i) it has a simple primary and tertiary structure [24], ii) it contains numerous lysine residues (19) available for bioconjugation reactions and iii) deleterious changes in its tertiary structure can be easily detected with an easy activity assay, as described by Chilkoti and co-workers [25]. These features make it an excellent model protein that is perfect for the proof of concept and to study in detail the potentialities of "PPEylation" on new conjugates.

Conjugates with polymers of different lengths and with a different number of polymers attached were prepared and analyzed. Different techniques were used to detect the bioconjugation influence on the protein's residue activity and on its thermal behavior. The low influence the polymer has on the protein's features confirms the potential of poly(phosphoester)s in the bioconjugation field. This fundamental study is the basis for future works in this field (that are currently being explored in our group) and could pave the way for fully protein-polymer conjugates with clinical use.

EXPERIMENTAL SECTION

- **Materials**

Solvents were purchased from Sigma Aldrich (Germany) or Across Organics (Germany) and used as received unless otherwise specified. Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS) was purchased from Gibco/Life Technologies (Germany) and used as received for extensive analyses (Pierce assay, n-DSF, n-DSC, and activity assay). A 50 mM borate buffer was prepared from a solution of sodium tetraborate 50 mM (distilled water and $Na_2B_4O_7{}^*10H_2O$, Sigma Aldrich, Germany); the pH was adjusted with HCl 0.1 M until it reached pH = 8.825. 2-chloro-2-oxo-1,3,2-dioxaphospholane (COP), dry pyridine, N,N'-disuccinimidyl carbonate (N,N'-DSC), glacial acetic acid, myoglobin of the equine skeletal muscle (My) (purity: 99.5%) and bovine serum albumin (BSA) (purity: 99.5%) were purchased from Sigma Aldrich and used as received. Triethylamine (TEA) and 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) from Sigma Aldrich were dried, distilled and stored at 0°C, over molecular sieves (4 Å). 2-(benzyloxy)ethanol was purchased from ABCR distilled from sodium and stored at 0°C, over molecular sieves (4 Å). N-cyclohexyl-N'-(3,5-bis(trifluoromethyl)phenyl)thiourea (TU) was synthesized following a procedure previously reported in the literature [26]. Poly(ethylene glycol) monomethyl ether (M_n= 5 kDa) was purchased from Fluka and post-modified following the procedure reported by Zalipski [27]. Sephadex G-50 medium (Sigma Aldrich) was left to swell in water overnight before use, following the proportion Sephadex/water = 10mL/50mL; when the column was not in use it was stored in a solution of 5% EtOH at 4°C.

- **Instrumentation and characterization techniques**

For the analyses of PPEs, gel permeation chromatography (GPC) was performed in Dimethylformamide (DMF; containing 0.25 g/L of lithium bromide as an additive) using an Agilent 1100 Series as the integrated instrument, including a PSS GRAM columns (1000/1000/100 g), an UV detector (280 nm) and a RI (refractive index) detector at a flow rate of 1 mL/min at 60°C. Calibration was carried out using PS or PMMA

standards provided by the Polymer Standards Service. For the conjugate analyses, GPC was conducted using phosphate buffered saline (PBS, 100 mM phosphate, 50 mM sodium chloride, pH = 6.5) as the eluent. The conjugate samples were separated by a Superdex™ column (10/300 GL, 200 increase), with 100 µl injected at a flow rate of 0.5 mL min^{-1}. Elution profiles were detected with a UV-detector (280 nm, Agilent 1260), a multi-angle linear light scattering detector (Wyatt mini-DRAWN TREOS MALLS) and a differential refractive index detector (Agilent 1260), connected online in series, after the column. The software Astra 6.1.1 (in particular the method protein-polymer conjugates, based on principles described in the literature [28,29]) was used to analyze the elution profiles of the conjugates and to obtain their purity, estimated molecular weight and polydispersity index. The refractive index increment (dn/dc) of pure myoglobin in PBS, at 22°C and 632.8 nm, was established with an own-built off-line interferometer based on the principle of the Michelson Interferometer. ^{1}H, ^{13}C, and ^{31}P {H}-NMR spectra were acquired at 298.3 K with a Bruker AVANCE III 300, 500 or 700 MHz spectrometers. The spectra were calibrated against the solvent signal and analyzed using MestReNova 9.0.0 from Mestrelab Research S.L.

MALDI-TOF measurements were performed on an Ultraflex III TOF/TOF mass spectrometer from Bruker using sinapinic acid (3,5-Dimethoxy-4-hydroxycinnamic acid) as a matrix and a solution of Acetonitrile/TFA 0.1% (ratio 70/30) as a solvent. The samples (solved in water, with a concentration of 2 mg/mL) were put on the target with the matrix, with a ratio 1:1. The analysis was performed in a linear positive, with a mass range between 20 and 70 kDa.

The protein amount in the samples was quantified via the Pierce 660 Protein Assay from Thermo Scientific, following the microplate procedure instructions (absorption at 660 nm was measured with the Tecan infinite M1000 or with the absorbance measurement of a Jasco V-550 UV/VIS spectrometer). The solution's pH was measured with a TIM 900 Titration Manager pHmeter (Radiometer Analytical, Copenhagen, Denmark).

Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was conducted using the procedure of mixing 6 µL of the sample (concentration: 1 mg/mL previously determined by the Pierce assay) with 20 µL of DPBS, 4 µL of NuPAGE Sample Reducing agent and 10 µL of the NuPAGE LDS Sample Buffer. The electrophoresis was carried out in a NuPAGE 10% Bis-Tris Protein Gel, with a NuPAGE MED SDS Running Buffer at 100 V for 2h, using SeeBlue Plus2 Pre-Stained Standard (Invitrogen, USA) as a molecular marker. The gel was washed once with water and then stained for 24 h using SimplyBlue SafeStain (Novex, Thermo Fisher Scientific). The same procedure was repeated using 1 µL of the sample and 25 µL of DPBS. The gel was stained following the Basic Staining Protocol Silver Quest™, using the Silver Staining Kit (Silver Quest™, Thermo Fisher Scientific).

The activity assay was conducted following the procedure reported by Chilkoti and co-workers [25] with some modifications. At the end of the reaction, 200 µL of the sample was put in a 96 well plate, and the absorption at 409 nm was monitored with Tecan infinite M1000. The measure of a negative control (pure buffer) was used as a blank measurement. Errors were evaluated on the basis of at least three replicas. Further details are given in *Supplementary Material*, *Part 2*.

Nano-Differential Scanning Fluorimetry (n-DSF) analyses were conducted using Prometheus NT.48 from Nano-Temper technologies. The procedure was first to fill the instrument cell with the pure protein and the conjugate solutions in DPBS (concentration 1mg/mL of protein, previously determined with Pierce assay). A thermal scan was then conducted (from 20 to 95°C, rate 1°C/min) to detect the sample's emission fluorescence at 350 nm. Errors were evaluated on the basis of at least three replicas.

Nano-Differential Scanning Calorimetry (n-DSC) analyses were conducted using the differential scanning calorimeter N-DSC III (Calorimetry Science Corporation, model CS-6300). The procedure followed was first to filter (with a single-use, non-pyrogenic, sterile-EO hydrophilic syringe filter, with a pore size of 5 µm, from Minisart®, Sartorius, Germany) the solutions of the pure buffer (DPBS) and of the samples (pure protein, polymers or conjugates) and then to degas. The reference cell was then filled with DPBS, while the sample cell was filled with a sample solution in DPBS (concentration: 1mg/mL of a protein previously determined with the Pierce assay). These were submitted to a thermal scan from 25 to 90°C, at a rate of 1°C/min. Data were analyzed with the Nano-Analyze software. Errors were evaluated on the basis of at least three replicas.

- **Synthesis**

Synthesis of EEP: the synthesis was conducted following the procedure in the literature [30]. Dry TEA (35.51 g, 350.94 mmol, 1 eq, 0.049 mL) and dry ethanol (16.17 g, 350.94 mmol, 1 eq, 0.02 mL) were introduced with dry THF (50 mL) in a 500 mL three-necked flask under an argon atmosphere. The mixture was cooled to 0°C and a solution of COP (50.00 g, 350.94 mmol, 1 eq) in dry THF (70 mL) was added dropwise with a dropping funnel. After the complete addition, the solution was stirred at 0°C for two hours. During the reaction, triethylammonium chloride was precipitated. This was removed by filtration under the argon atmosphere and the filtrate was distilled under reduced pressure to obtain 25,065 g of pure product as a colorless oil (yield: 46.96%, T_{bp} = 96°C, 0.14 mbar). The product was stored at -28°C. ^{1}H-NMR (500 MHz, DMSO-d6): δ [ppm] 4.47-4.34 (m, 4H, O-C*H*$_2$-C*H*$_2$-O), 4.11-4.04 (m, 2H, O-C*H*$_2$-CH$_3$), 1.25 (t, 3H, O-CH$_2$-C*H*$_3$). ^{13}C{H}-NMR (125 MHz, DMSO-d_6): δ [ppm] 66.35 (-O-CH$_2$-CH$_2$-O-), 64.18 (-O-CH$_2$-CH$_3$), 15.96 (-O-CH$_2$-*C*H$_3$). ^{31}P{H}-NMR (202 MHz, DMSO-d_6): δ [ppm] 16.83.

Synthesis of PEEP-SC (a-b): The polymerization was carried out in 25 and 50 mL Schlenk tubes, flamed-dried under reduced pressure and purged with argon twice before use. The monomer EEP and the catalyst TU were introduced into a tube with dry DCM. In the other two tubes, stock solutions of DBU and of 2-(benzyloxy)ethanol in dry DCM were prepared. All the solutions were cooled down to 0°C. The 2-(benzyloxy)ethanol solution was then added to the monomer. The polymerization was started by the addition of 0.5 mL of DBU stock solution to the monomer/initiator/TU mixture, obtaining a solution in which the monomer concentration was ca. 4 mol/l. The reaction was quenched after 90 min by the addition of a N,N'-DSC solution in 10-15 mL of dry acetonitrile while the mixture was under the argon flow. The reaction was left to stand for 20 min. The product was purified by precipitation into cold diethyl ether and centrifugation (15 min, 4000 rpm, 0°C). The residue was again dissolved in a few mL of dry DCM, filtered and finally dried *in vacuo* to obtain a colorless oil. The specific reaction details are reported in *Supplementary Material*, *Part 1*.

Synthesis of My-PEEP (1-5) and My-PEG (6-7): the myoglobin was dissolved in a few mL of borate buffer (50 mM, pH = 8.825). The NHS-activated polymer was divided into three parts and each was dissolved in a few mL of distilled water (as little as possible) and added dropwise to the protein solution, waiting 45 min between the additions of each portion. The reaction was left to stand at room temperature for 4 h. The product was dialyzed in water (MWCO = 50,000 kDa) for two days. It was then lyophilized to obtain the red conjugate and analyzed by online triple detection size exclusion chromatography. When necessary, additional purification was conducted using gel permeation chromatography over Sephadex G-50, with water as the eluent. Only the fractions with a purity ≥ 95% were unified and lyophilized, yielding the product as a red solid. The details of the specific reactions are given in *Supplementary Material*, *Part 1*. The

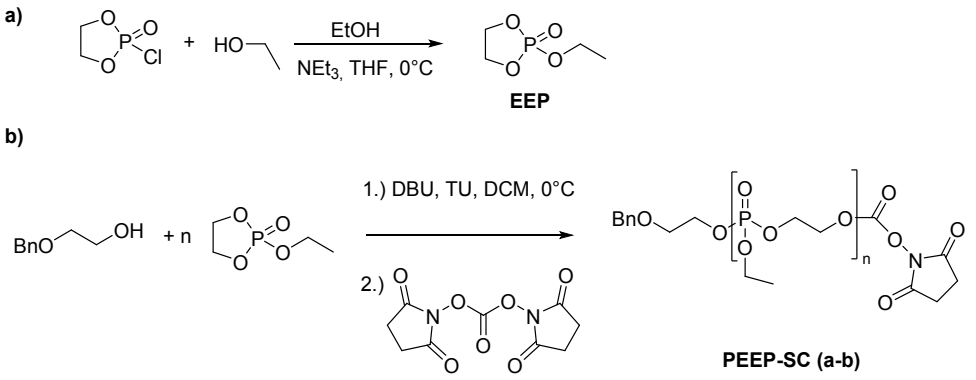
same procedure (omitting the polymer addition) was repeated on a myoglobin sample, to test the influence of the method on the protein's features.

RESULTS AND DISCUSSION

The focus of this study is the synthesis, purification, and characterization of new fully degradable protein-polymer conjugates, to find effective alternatives to drug modifications with PEG and to understand how polymer attachment influences the protein structure and properties within the conjugates.

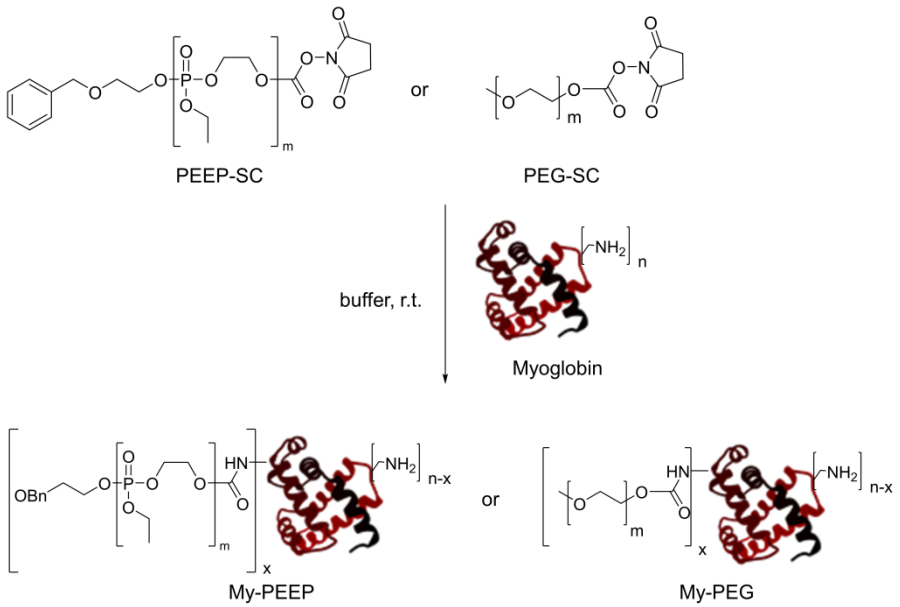
We synthesized conjugates with a variable number of polymer chains and polymers with a different degree of polymerization, to enable us to examine how the number of chains and the molecular weight influence the properties of the conjugates. We thus prepared five different conjugates using PEEP-SC (a) of ca. 6 kDa and PEEP-SC (b) of ca. 11 kDa, and different molar ratios between proteins and polymers. Two additional PEGylated conjugates (with a different molar ratio of protein and polymer used in the synthesis) were prepared and used for comparison, as a gold reference standard. The protein/polymer molar ratios used in the synthesis, the molecular weight (M_n), and the purity of the conjugates synthesized are reported in *Table 1*.

Synthesis of the conjugates. The monomer 2-ethoxy-2-oxo-1,3,2-dioxaphospholane (EEP) was synthesized according to the literature [31]. It was then used in the polymerization reaction to obtain the polymer poly(ethyl ethylene phosphate) (PEEP), which in our previous studies we characterized as water-soluble, non-toxic and degradable under physiological conditions [21]. PEEP of two different weights (ca. 6 and 11 kDa) were prepared by an organocatalyzed anionic ring opening polymerization, using 2-benzyloxy(ethanol) as the initiator, cyclohexyl-N'-(3,5-bis(trifluoromethyl)phenyl)thiourea (TU) as the co-catalyst, 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) as the catalyst and N,N'-disuccinimidyl carbonate (N-N'-DSC) as the terminator (*Scheme 1*). The choice of catalyst follows the work of Lecomte and co-workers [32], who explained that the mixture DBU/TU activates the nucleophilic and electrophilic sites of the growing chain at the same time, giving a narrow molecular weight distribution to the final polymer and where undesired transesterification side reactions are minimized. The use of N-N'-DSC as the terminator enabled the functionalization of the polymer end to be obtained. This was found to form a succinimidyl-carbonate (SC) ester, a well-known and reliable group that has been used in the literature for mPEG-OH [33] and poly(oxazoline)s [34] activation. The successful introduction of the succinimidyl carbonate group, the molecular weight and the polydispersity index of the polymers were verified by ^{1}H and 31P NMR spectroscopy and GPC/SEC in DMF. The results are reported in *Supplementary Material*, *Figure S1-3*.



Scheme 1: a) Synthesis of the monomer EEP; b) Organocatalyzed ring-opening polymerization of EEP and termination with DSC to protein-reactive PEEP-SC.

The bioconjugation reaction (*Scheme 2*) was carried out for 4 h in aqueous borate buffer (50 mM, pH = 8.8), using different quantities of PEEP and polymers of different degrees of polymerization, to obtain a library of conjugates (*Table 1*). The conjugates were purified by dialysis against water (MWCO = 50 kDa), and when required by an additional gel permeation chromatography over Sephadex G-50. PEGylated conjugates (to be used for comparison) were synthesized and purified following the same procedure, starting from the commercially available poly(ethylene glycol monomethyl ether) with M_n = 5.0 kDa.



Scheme 2: Bioconjugation of protein-reactive PEEP and PEG to myoglobin.

Characterization of the conjugates. A comparative analysis using data obtained from the different methods was used to verify the successful and effective bioconjugation of PEEP to myoglobin and to determine the molecular weight of the conjugates. The first analysis performed was an SDS-PAGE (*Supplementary Material*, *Figure S4*). The upward shift of the molecular weight (in accordance with the polymer ratio used in the bioconjugation reaction) and the absence of a residual protein band in the samples confirm the conjugate's formation and the samples' purity [35]. As expected for a grafting-to-conjugation method, the

conjugates may carry a variable number of polymer chains, resulting in several bands on the gel. SDS-PAGE already proved the formation of polymer-protein conjugates. In addition, we performed aqueous GPC with triple detection for further characterizations. The instrument uses UV and RI detectors to gather information on the purity, molecular weight distribution and relative molecular weight of the samples. In combination with the light scattering detector (specifically, multi-angle laser light scattering or MALLS), this enables the absolute molecular weight of the samples to be determined [28,29]. We calibrated the RI detector using the same value of dn/dc for all the signals, which is the value calculated for the pure protein in PBS with an off-line interferometer (dn/dc = 0.1793 mL/g). The calculation of the myoglobin dn/dc in PBS and the elution profiles of the samples are reported in *Supplementary Material*, *Figures S5-6*. In addition, MALDI-ToF mass spectrometry was conducted exemplarily on three conjugates, proving the covalent attachment of the polymer chains to myoglobin (*Supplementary Material*, *Figure S7*). Sample 2 was detected in MALDI with 37,000 Da, which is close to the value obtained from GPC (see *Table 1*). Sample 3 was adjusted to a 1:1: ratio (PEEP: myoglobin) and MALDI ToF proves the formation of mainly a single product with ca 25,000 Da (and a minor product with 31,000 Da).

The features of the samples synthesized in this work are summarized in *Table 1*. We prepared seven conjugates (compounds 1-7), performing the bioconjugation reactions with three different polymers (PEEP-SC (a) of ca. 6.4 kDa, PEEP-SC (b) of ca. 11.25 kDa and PEG-SC of c.a. 5 kDa), using a different experimental ratio of protein/polymer (*Table 1)* for each conjugate. Myoglobin has 19 lysine residues available for the bioconjugation with the NHS-terminated PEEP [25], and preliminary trials have proven that the bioconjugation reaction is not quantitative, so we opted to use 1:5 or 1:10 as the protein/polymer ratio, with an additional 1:1 conjugate synthesized with the longer polymer (the only one with a weight quite different to that of the protein, to permit acceptable purification). The analysis of the conjugates with GPC provided us with an estimation of their molecular weight, from which it is possible to calculate the average number of polymer chains attached to each protein by subtracting the protein weight and then dividing for the polymer weight. *Table 1* also shows that the final fractions collected for each conjugate have a high purity (all above 95%).

Table 1: Conjugates' features, concerning experimental information (the type of polymer and the molar ratio used for the synthesis), and other information derived from the GPC analysis (as the M_n, the calculated average number of polymer chains attached to each protein and the purity of the synthesized conjugates).

Sample	Polymer[a]	([My]/[polymer])[b]	M_n (kDa)[c]	polymer chains attached[d]	Purity (%)[e,f]
1	PEEP-SC (a)	1:5	41.0	3.8	98
2	PEEP-SC (a)	1:10	41.2	4.5	96
3	PEEP-SC (b)	1:1	26.4	1.2	99
4	PEEP-SC (b)	1:5	46.0	3.3	99
5	PEEP-SC (b)	1:10	62.0	5.0	98
6	PEG-SC	1:5	47.5	3.3	99
7	PEG-SC	1:10	64.0	5.2	98

[a]:polymer used for bioconjugation: PEEP-SC (a) of ca. 6 kDa, PEEP-SC (b) of ca. 11 kDa, PEG-SC of ca. 5 kDa; [b]: experimental molar ratio protein/polymer used in the synthesis; [c]: numeral average molecular weight determined by triple detection SEC analysis in phosphate buffer; [d]: average number of polymer chain attached, reported with an error bar of ± 2 polymers; [e]: calculated as the ratio between the area of the peak of the conjugate and the sum of the areas of all the peaks registered in the chromatogram.

Activity and conformational studies. As a control reaction (to detect if and how the conjugation and the purification processes have affected the protein properties), we subjected the myoglobin to the bioconjugation procedure (omitting the polymer addition) and to the purification with GPC over Sephadex G-50. The activity assay and the calorimetry experiments conducted on the samples (the first fraction recovered after the dialysis, and the second after the purification with GPC over Sephadex G-50) showed no significant differences from the values obtained for the pure protein measured before the bioconjugation treatment. These trials confirmed that the process does not significantly affect the protein features, therefore any change found in the protein features of the conjugates will be due to the covalent attachment of the polymer on the protein (or from their interactions), but not from a partial denaturation of the protein induced by the procedure. The results obtained are reported in *Supplementary Material*, *Table S1.*

One of the first properties that must be detected on a new protein-polymer conjugate is the residual enzymatic activity, to evaluate the effect of covalent PPE-ylation. High activity retention is important for the final applications of the conjugates (their use as drugs) because it results in lower drug dosages for patients. Polymer attachment to enzymes is known to change their activity, and in most cases results in a significant activity reduction (e.g., the PEGylated drug Pegasys®, which retains ca. 7% of native protein activity [36]). Nevertheless, polymer attachment often improves the pharmacokinetics, and therefore the activity loss is accepted in many formulations.

Deleterious changes in the tertiary structure of the myoglobin of equine skeletal muscle can be easily detected with an easy activity assay [25]. We conducted an activity assay on the samples following the procedure of Chilkoti and co-workers [25], which consisted of observing the myoglobin peroxidase-like activity and monitoring the absorbance at 409 nm of the samples that were previously subjected to a specific treatment. The procedure is described in detail in *Supplementary Material*, *Part 2*. The results obtained are reported in *Figure 1* and in *Supplementary Material*, *Table S2*.

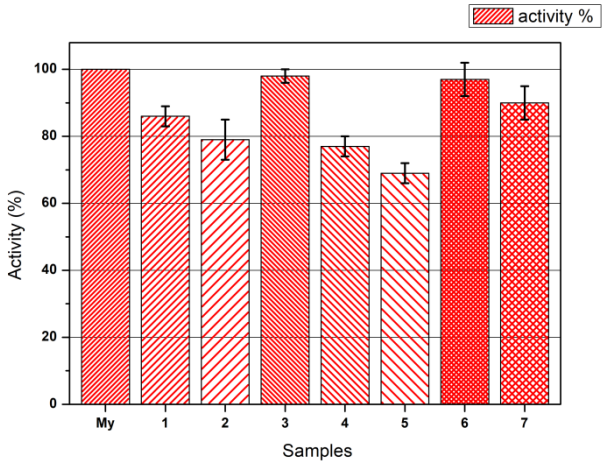


Figure 1: Enzyme activity of myoglobin (My) and the of the polymer-conjugates (samples 1-7), further details cf. Table S2.

The graph in *Figure 1* shows that the bioconjugation process is always followed by a loss in protein activity, and this is more remarkable when the number of polymer chains attached to the protein and their degree of polymerization is increased. The loss of activity can be associated with both a change in a protein's tertiary structure and the reduced active-site accessibility caused by polymer obstruction. However, it is important to note that at least 70% of the activity is retained in all the conjugates, which is a positive result in conjugate characterization. The values obtained are in fact higher than those reported for similar conjugates [21,22].

The potential of new conjugates is dependent on their stability. We, therefore, determined the thermal stability of the samples by *nano*-Differential Scanning Fluorimetry (n-DSF) and *nano*-Differential Scanning Calorimetry (n-DSC). The combination of these techniques is particularly innovative in the field of bioconjugation, and the comparison between them allows the study of the protein from two different points of view.

n-DSF concerns the measurement of emission fluorescence at a fixed wavelength (350 or 330 nm) during a thermal scan of the sample. The fluorescence intensity and the wavelength of maximum fluorescence emission strongly depend on the close surroundings of tyrosine or tryptophan residues in the protein [37], and therefore the measured value is linked to changes in the protein structure. n-DSC is a similar technique that measures the energy needed to compensate the temperature differences between a reference cell and a sample cell during a thermal scan at a constant rate. The energy is then opportunely converted in heat capacity, and the profile obtained can give important information on the thermodynamics of the studied process.

In this study, solutions of pure protein and of conjugates with the same protein concentration inside (1 mg/mL, previously determined by Pierce assay) were detected during a thermal scan (from 20 to 95°C, rate

1°C/min) by n-DSF. The onset temperature (T_{onset}) (calculated as the intercept between baseline before the peak and the curve's tangent at the inflection point) and the melting temperature (T_m) (corresponding to the inflection point in the fluorescence intensity and the maximum in the derivative graph) were measured, and compared with those obtained by n-DSC. The sample concentration, heating rate, and temperature range were the same. The pH measurements of the sample solutions before and after the scan showed unremarkable differences, confirming the effective buffering action during the heating scan. The results obtained with the two different techniques (and their error bars, calculated in a confidence interval of 95%) are reported in *Figure 2* and *Supplementary Material*, *Table S3*.

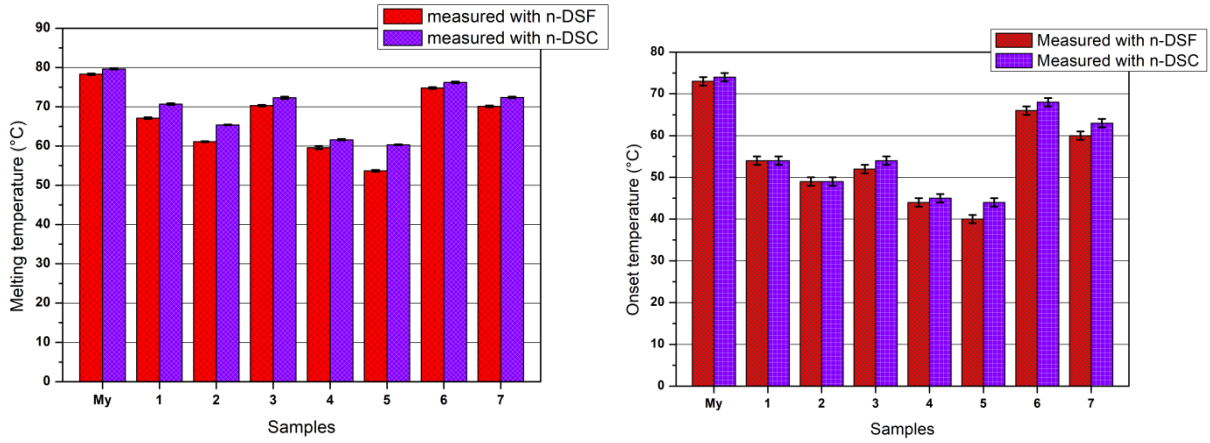


Figure 2: a) The melting temperatures (T_m) of myoglobin (My) and of the conjugates (1-7) obtained by n-DSF and n-DSC; b) the onset temperatures (T_{onset}) of myoglobin (My) and of the conjugates (1-7) obtained by n-DSF and n-DSC. The error bar is reported graphically as the confidence interval of the value (within 95% of probability) and it is specified in the Supplementary Materials, Table S3.

The first observation that can be made from the graphs is that the values obtained with n-DSF are slightly lower than those obtained by n-DSC, but they follow the same trend so they can be considered not significantly different. We also observed that the bioconjugation process causes a reduction of T_m and T_{onset} values, which become more remarkable when increasing the number of polymer chains attached. The polymer that has less influence on T_m and T_{onset} values is PEG (values in good agreement with the literature [38]), followed by PEEP (a) (with a shorter chain) and then PEEP (b) (with a longer chain). A reduction in the unfolding temperature results in the conjugation destabilizing the protein, but it is important to note that the unfolding process begins at temperatures that cannot be found in the body, and therefore at the administration temperature the drug is completely folded.

The trend observed in the variations of the onset and melting temperatures exactly reproduces the variations of activity previously described. All the properties studied present a reduction (with respect to the pure protein) when increasing the number of polymer chains attached to the protein and the degree of polymerization of the polymer. We can thus hypothesize that the loss of activity is connected to a reduction in the active site accessibility (longer polymers bring a major activity reduction) and possibly to a partial denaturation of the protein active structure, which is linked to the reduction of the melting temperature observed using the two calorimetric techniques. Nevertheless, the reduction of the properties present in the PPEylated conjugates is acceptable in all cases, because the activity is always greater than 70% and the unfolding process always begins at a temperature greater than that present in the body.

CONCLUSIONS

We have reported the synthesis, the purification, the characterization and the first evaluation of myoglobin-poly (ethyl ethylene phosphate) protein-polymer conjugates. We prepared different conjugates with high purity using an optimized bioconjugation strategy and studied their thermal stability and residual enzyme activity, which are fundamental properties for investigating their potential as therapeutic agents. Despite the reduction in the values observed with respect to pure and PEGylated protein, PPEylated conjugates show significant thermal stability in physiological conditions (no unfolding occurs at the drug administration temperature, i.e., body temperature) and relatively high activity retention, which was above 70% in all cases. We also observed that the protein's features within the conjugates can be modulated by changing the degree of polymerization and the number of polymer chains attached to the protein. These results, together with the polymers' biodegradability under physiological conditions, underline PPEs as promising candidates for the stabilization of clinically important proteins.

ACKNOWLEDGMENTS

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DATA AVAILABILITY

The raw data will be made available upon request.

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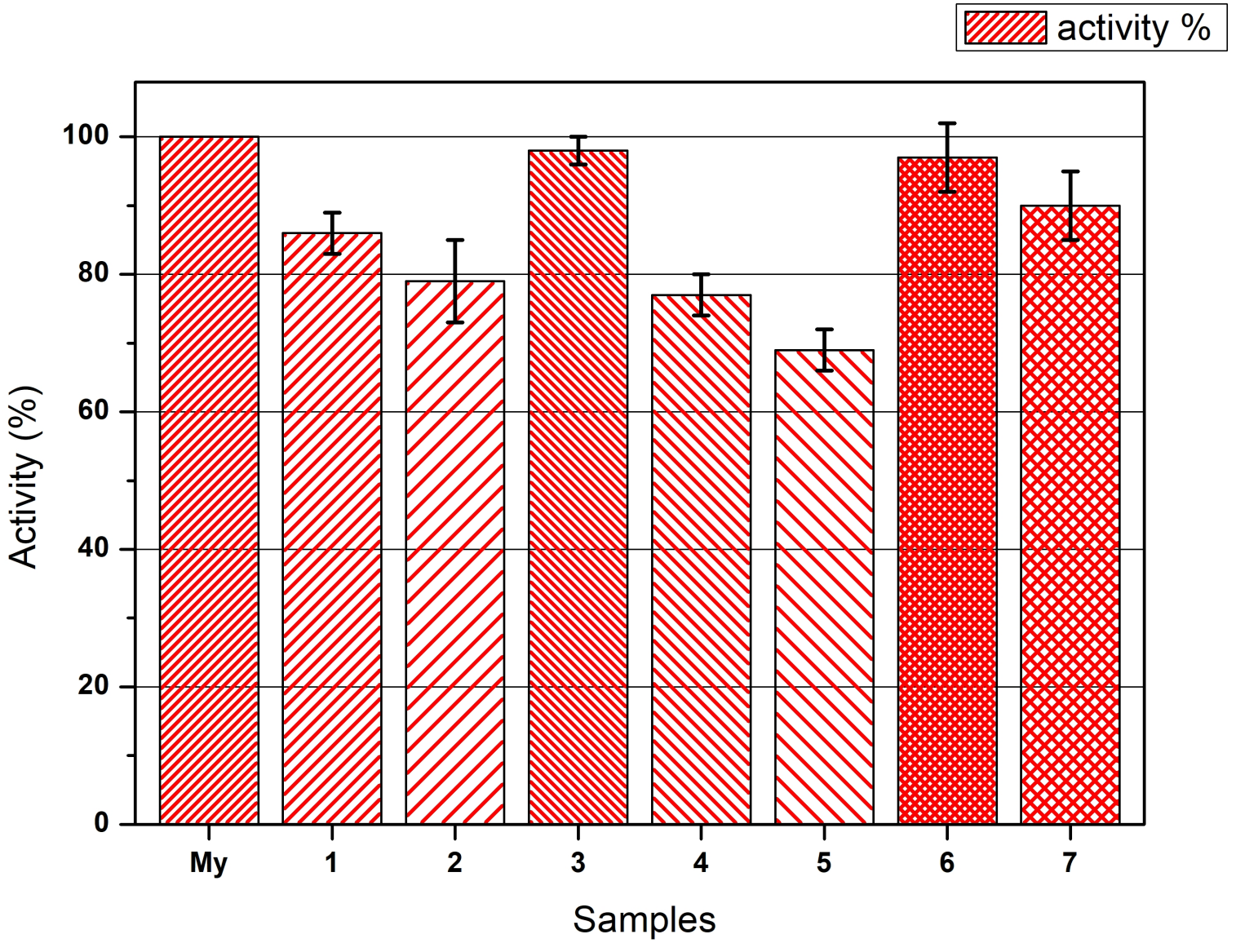
FIGURE CAPTIONS

Figure 1: Enzyme activity of myoglobin (My) and the of the polymer-conjugates (samples 1-7), further details cf. Table S2.

Figure 2: a) The melting temperatures (T_m) of myoglobin (My) and of the conjugates (1-7) obtained by n-DSF and n-DSC; b) the onset temperatures (T_{onset}) of myoglobin (My) and of the conjugates (1-7) obtained by n-DSF and n-DSC. The error bar is reported graphically as the confidence interval of the value (within 95% of probability) and it is specified in the Supplementary Materials, Table S3.

TABLES

Table 1: Conjugates' features, concerning experimental information (the type of polymer and the molar ratio used for the synthesis), and other information derived from the GPC analysis (as the M_n, the calculated average number of polymer chains attached to each protein and the purity of the synthesized conjugates).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Activity (%)
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80
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40
20
0
My
1
2
3
4
5
6
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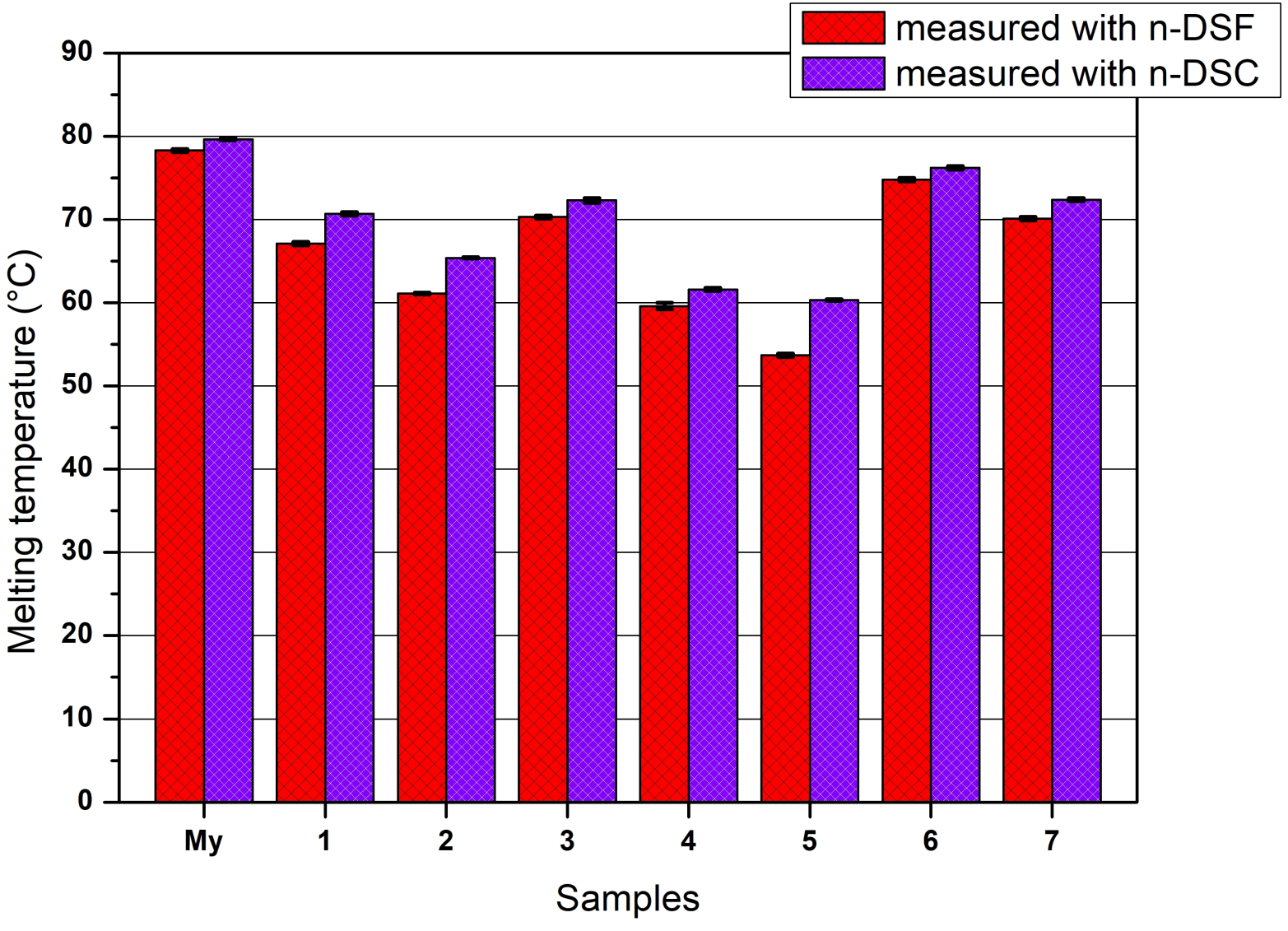
Samples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measured with n-DSC
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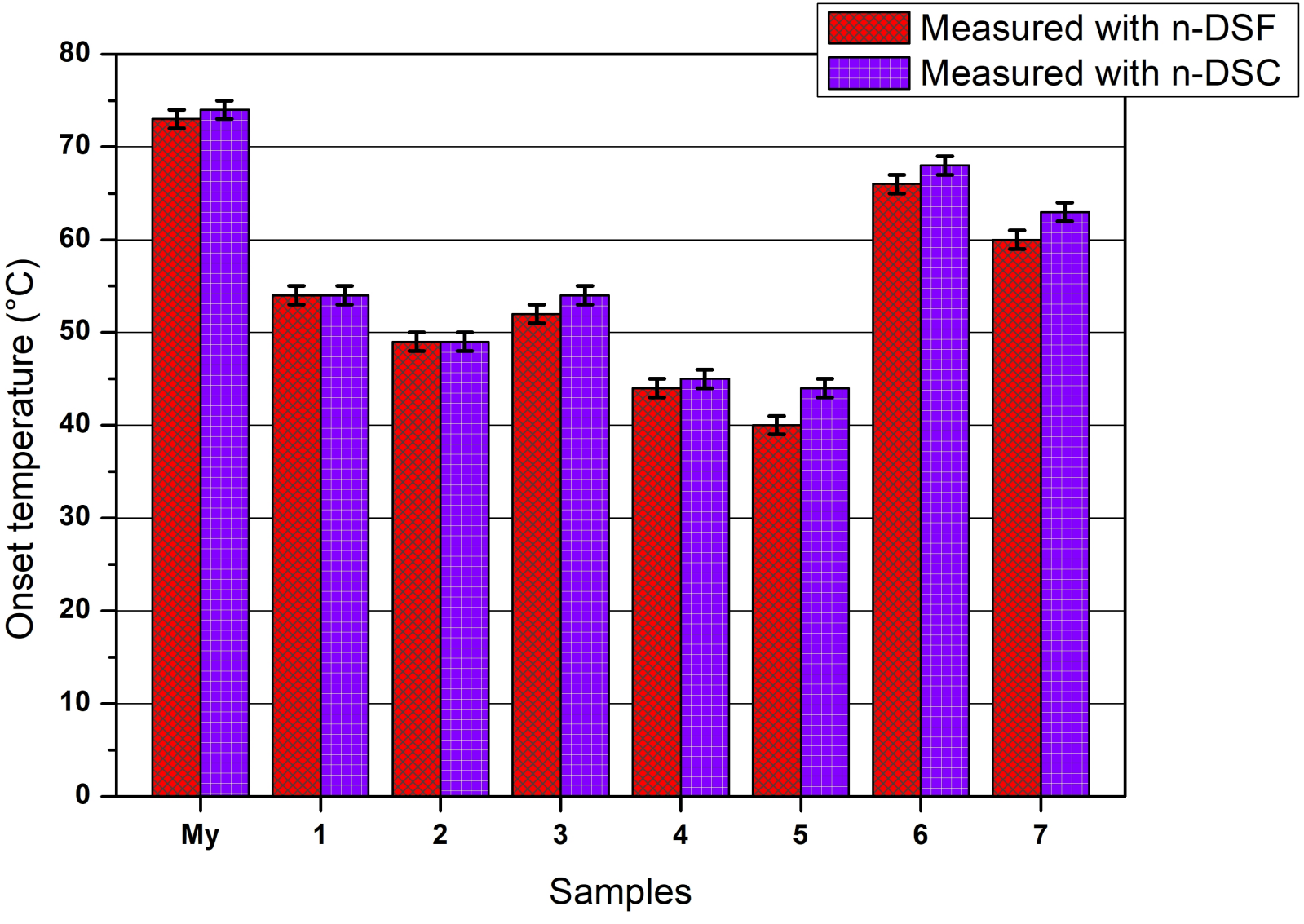
Samples


Measured with n-DSF
Measured with n-DSC
Onset temperature (°C)
0
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2
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4
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6
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Samples

SUPPLEMENTARY MATERIAL

PPEylation of proteins: Synthesis, Activity and Stability of Myoglobin-Polyphosphoester Conjugates.

Chiara Pelosi, Celia Duce, Daniela Russo, Maria Rosaria Tiné and Frederik R. Wurm.

TABLE OF CONTENTS

1. PART 1: SYNTHETIC PROCEDURES

Synthesis of PEEP-SC (a): The reaction was performed following the general procedure, using the reported quantities: EEP (4.96 g, 32.59 mmol, 32 eq); TU (1.89 g, 5.09 mmol, 5 eq); DBU (775.24 mg, 5.09 mmol, 5 eq); 2-(benzyloxy)ethanol (155 mg, 1.02 mmol, 1 eq); DSC (1.28 g, 5.1 mmol, 5 eq), affording 7.085 g of product as a colourless oil (Yield 89.41 %, functionalization: 100 %). M_n (NMR): 6400 g/mol (41 repeated units). Đ (GPC in DMF): 1.15. ^{1}H-NMR (DMSO-d6, 300 MHz): δ [ppm] 10.55 (s, 3H, NO-H byproduct), 7.39-7.28 (m, 5H, Ar), 4.57 (s, 2H, PEEP-O-CH_2-CH_2-O-C(=O)-O-NHS), 4.52 (s, 2 H, Ar-CH_2-O), 4.19-3.99 (m, 244 H, O-CH_2-CH_2-O and O-CH_2-CH_3), 3.60 (m, 2H, Ar-CH_2-O-CH_2-CH_2-OP), 2.81 (s, 4H, -C(=O)-CH_2CH_2-C(=O)), 2.65 (s, 14 H, -C(=O)-CH_2CH_2-C(=O) byproduct), 1.27 (dt, 123 H, O-CH_2-CH_3). ^{31}P {H}-NMR (DMSO-d6, 202 MHz): δ [ppm] -1.16, -1.24.

Synthesis of PEEP-SC (b): The reaction was performed following the general procedure, using the reported quantities: EEP (5.12 g, 33.60 mmol, 64 eq); TU (973.3 mg, 2.63 mmol, 5 eq); DBU (400.13 mg, 2.63 mmol, 5 eq); 2-(benzyloxy)ethanol (80 mg, 52.56 µmol, 1 eq); DSC (571 mg, 2.63 mmol, 5 eq), affording 5.42 g of product as a colourless oil (Yield 87.93 %, activation: 100 %). M_n (NMR): 11250 g/mol (72 repeated units); Đ (GPC): 1.10. ^{1}H-NMR (DMSO-d6, 300 MHz): δ [ppm] 10.54 (s, 3H, NO-H byproduct), 7.38-7.28 (m, 5H, Ar), 4.55 (s, 2H, PEEP-O-CH_2-CH_2-O-C(=O)-O-NHS), 4.51 (s, 2 H, Ar-CH_2-O), 4.18-3.98 (m, 428 H, O-CH_2-CH_2-O and O-CH_2-CH_3), 3.62 (m, 2H, Ar-CH_2-O-CH_2-CH_2-OP), 2.80 (s, 4H, -C(=O)-CH_2CH_2-C(=O)), 2.6 (s, 14 H, -C(=O)-CH_2CH_2-C(=O) byproduct), 1.26 (dt, 217 H, O-CH_2-CH_3). ^{31}P {H}-NMR (DMSO, 300 MHz): δ [ppm] -0.97, -1.12, -1.24.

Synthesis of My-PEEP (1): The reaction was performed following the general procedure, using the reported quantities: myoglobin (170 mg, 10 µmol, 1 eq), polymer ($PEEP_{41}$: 326 mg, 50 µmol, 5 eq), yielding 250 mg of product as a red dust (yield: 50.4%). No Sephadex purification was performed for this conjugate. Purity: 100 %; Đ = 1.031; M_n: 41.000 kDa; average number of attached chains: 3.8.

Synthesis of My-PEEP (2): the reaction was performed following the general procedure, using the reported quantities: myoglobin (110 mg, 6.5 µmol, 1 eq), polymer ($PEEP_{41}$: 413 mg, 65 µmol, 10 eq), yielding 90 mg of product as a red dust (yield: 17%). Purity 96.1%; Đ= 1.104; M_n: 41.154 average number of attached chains: 4.5.

Synthesis of My-PEEP (3): the reaction was performed following the general procedure, using the reported quantities: myoglobin (510 mg, 30 µmol, 1 eq), polymer ($PEEP_{72}$: 338 mg, 30 µmol, 1 eq), yielding 80 mg of product as a red dust (yield: 9.4%). Purity 98.5 %; Đ= 1.037; M_n: 26450; average number of attached chains: 1.2.

Synthesis of My-PEEP (4): the reaction was performed following the general procedure, using the reported quantities: myoglobin (204 mg, 12 µmol, 1 eq), polymer ($PEEP_{72}$: 675 mg, 60 µmol, 5 eq), yielding 125 mg of product as a red dust (yield:14.2%). Purity 98.72 %; Đ= 1.038; M_n: 46.010; average number of attached chains: 3.3.

Synthesis of My-PEEP (5): the reaction was performed following the general procedure, using the reported quantities: myoglobin (136 mg, 8 µmol, 1 eq), polymer ($PEEP_{72}$: 900 mg, 80 µmol, 10 eq), yielding 145 mg of product as a red dust (yield: 14.3 %). Purity 98 %; Đ= 1.020; M_n: 62.040 average number of attached chains: 5.0.

Synthesis of My-PEG (6): the reaction was performed following the general procedure, using the reported quantities: myoglobin (129 mg, 7.6 µmol, 1 eq), polymer (PEG_{5000}: 195 mg, 38 µmol, 5 eq), yielding 51 mg of product as a red dust (yield:15.7 %). Purity 99 %; Đ= 1.051; M_n: 47.490; average number of attached chains:3.3.

Synthesis of My-PEG (7): the reaction was performed following the general procedure, using the reported quantities: myoglobin (76 mg, 4.5 µmol, 1 eq), polymer (PEG_{5000}: 231 mg, 45 µmol, 10 eq), yielding 71 mg of product as a red dust (yield: 23.6 %). Purity 97.5 %; Đ= 1.008; M_n: 63.970; average number of attached chains:5.2.

2. PART 2: POLYMER ANALYSIS

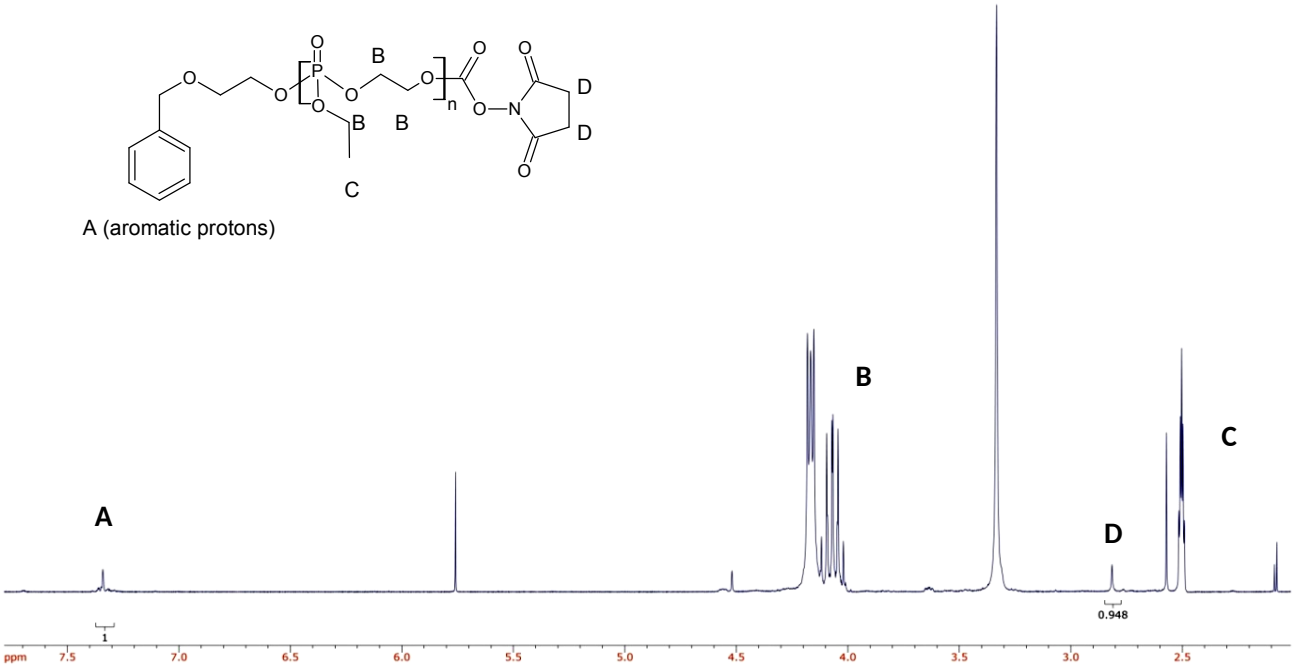

Figure S1: Representative ^1H NMR of PEEP-SC (DMSO, 300 MHz, 298 K).

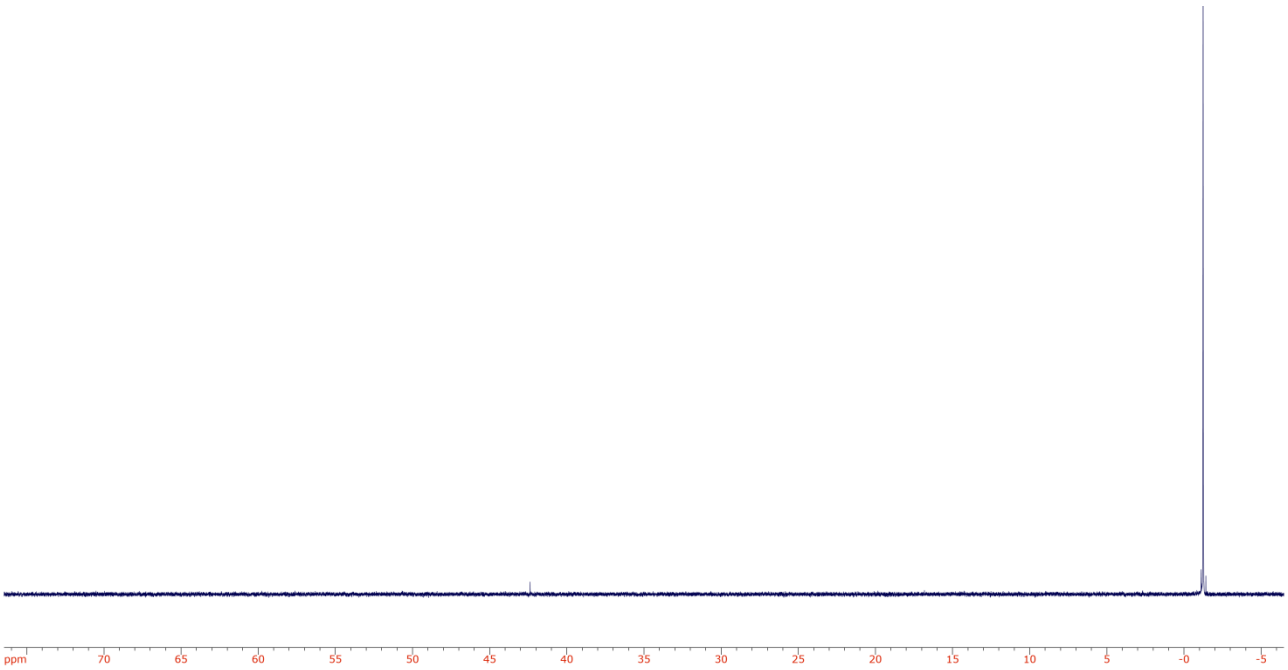

Figure S2: Representative ^{31}P NMR of PEEP-SC (DMSO, 300 MHz, 298 K).

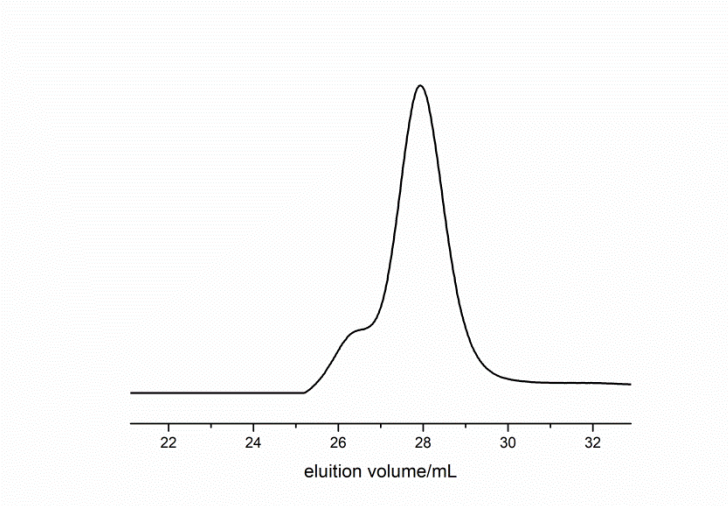

Figure S3: Representative GPC analysis of PEEP-SC.

3. PART 3: CONJUGATES ANALYSIS

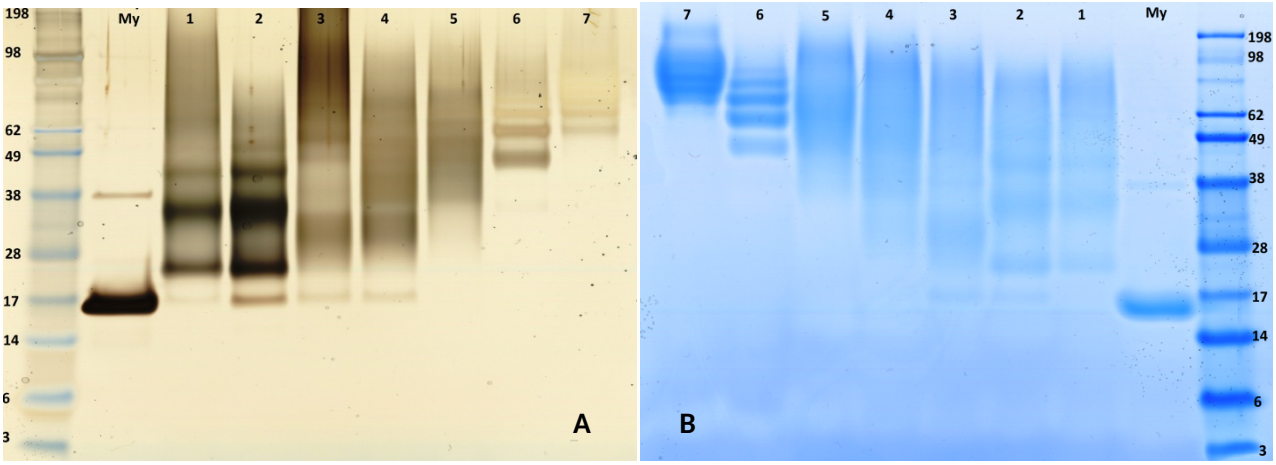

Figure S4. A: SDS-PAGE of myoglobin and conjugates stained with a silver staining. From the left to the right: ladder, My, sample 1, 2, 3, 4, 5, 6, 7. B: SDS-PAGE of myoglobin and conjugates stained with a blue Coomassie staining. From the left to the right: sample 7, 6, 5, 4, 3, 2, 1, My, ladder.

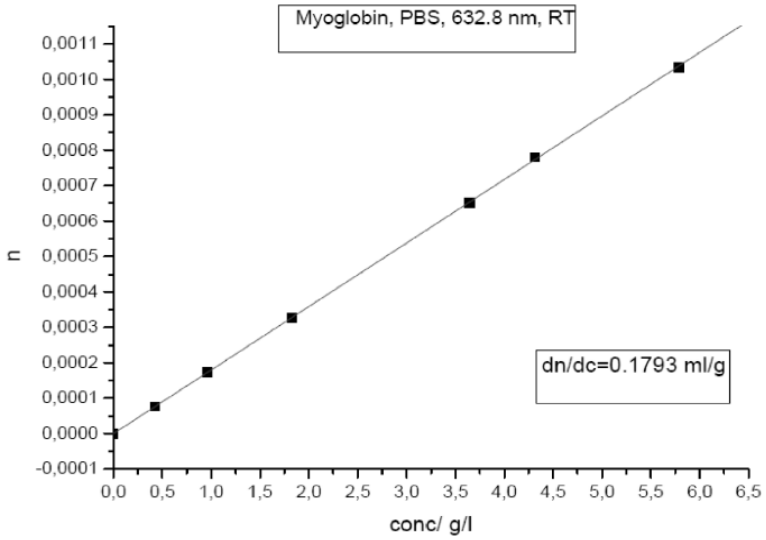

Figure S5: Off-line measurement of myoglobin d_n/d_c.

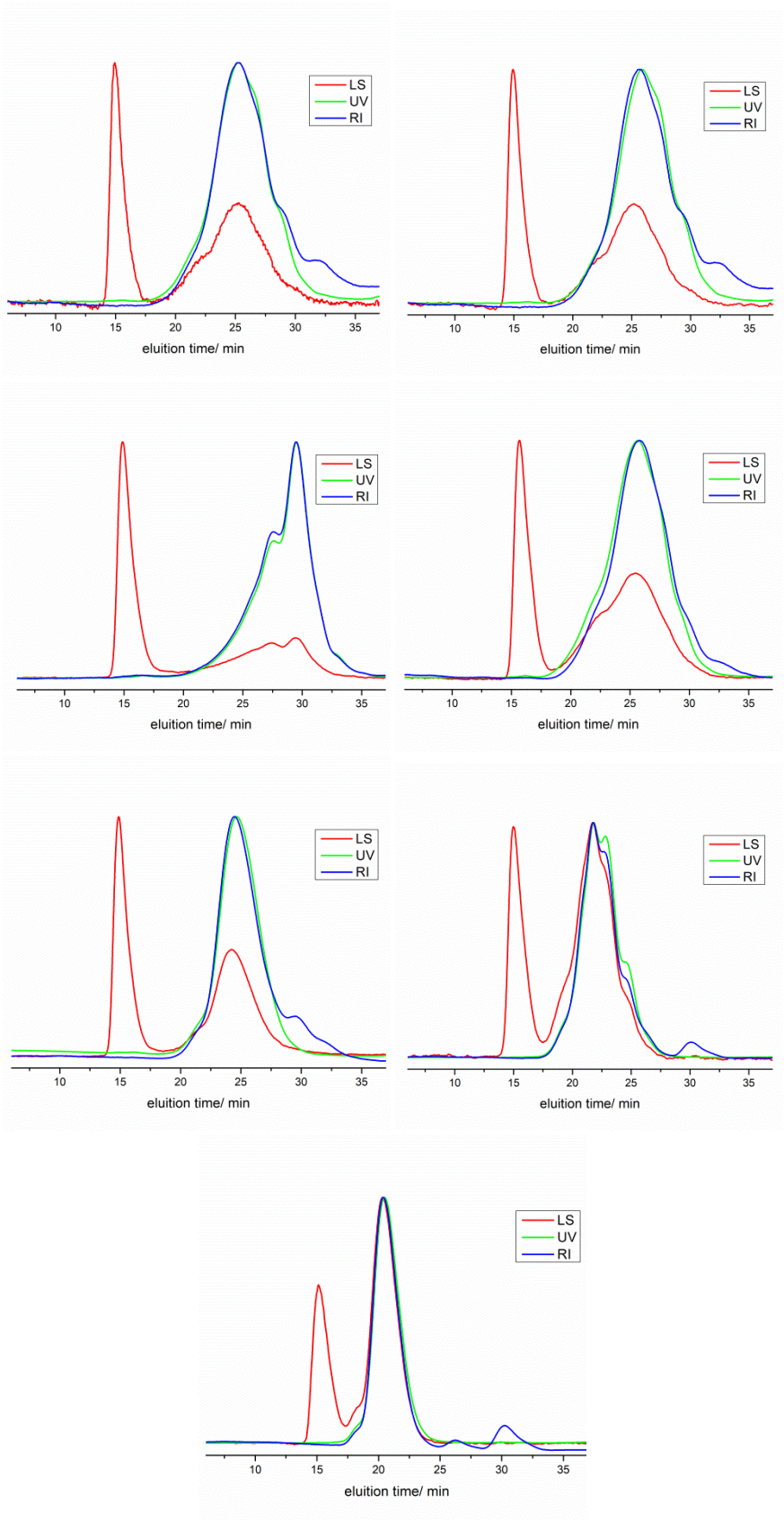

Figure S6: Triple detection SEC of samples 1, 2, 3, 4, 5, 6, 7 (from left to right, from the top to the bottom) after purification. The peak at 15' visible with the light scattering detector is typical of the instrument, the others peaks are characteristic of the conjugates.

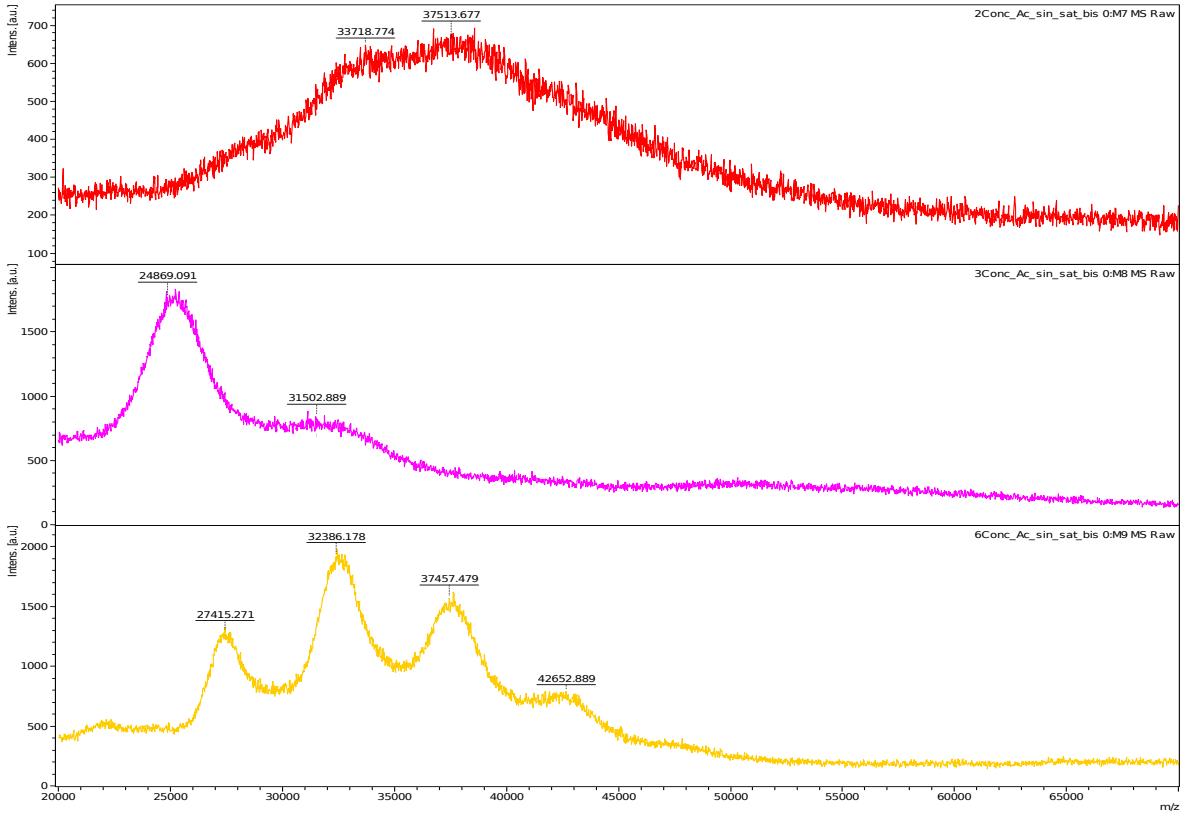

Figure S7: Representative MALDI-ToF mass spectra of My-PEEP (2) (red line), My-PEEP (3) (purple line) and My-PEG (6) (yellow line), proving successful conjugation.

Table S1: Values for the influence of the procedure of the protein, measured as activity, melting temperature from n-DSC on the following samples: My: myoglobin without any treatment; MD: myoglobin after the bioconjugation reaction without the polymer addition and the first dialysis; MS: myoglobin after the bioconjugation reaction without the polymer, the dialysis and the purification with GPC over Sephadex G-50. The error bar is reported as the confidence interval at 95% of probability.

	My	MD	MS
Activity	0.18 ± 0.03	0. 17 ± 0.03	0.15 ± 0.02
T_m (n-DSC)	79.5± 0.1	79.3± 0.2	79.3± 0.2

Experimental procedure for the activity assay: Each sample solution (My and samples 1-7 at concentration 1 µM of protein, previously determined by Pierce assay) was treated with hydrogen peroxide (5 µL, 40 mM). After 5 min of incubation at 20°C, an excess of catalase from bovine liver (2 mg/mL, 5 µL) was added to destroy residue hydrogen peroxide and 2,2' azino-di-(3-ethyl)benothiazoline-6-sulfonic-acid (ABTS) (5 µL, 10 mM) was added. The amount of $ABTS^{+\cdot}$ (the oxidation product), directly linked to protein activity, was quantified by absorbance measurements at 409 nm (200 µL of the sample were put in a 96 well plate, monitoring the absorption at 409 nm with Tecan infinite M1000). The blank measurements (of pure buffer) was subtracted to all the absorbance values. The final value is expressed as the percentage of residue activity, calculated as the ratio between the activity of conjugates and pure protein.

Table S2: Percentage of activity of myoglobin (My) and the conjugates (1-7). The error bar is reported as the confidence interval at 95% of probability.

	My	1	2	3	4	5	6	7
activity %	100.0 ± 0.1	86 ± 3	79 ± 6	98 ± 2	77 ± 3	69 ± 3	97 ± 5	90 ± 5

Table S3: Onset and melting temperature of myoglobin (My) and the conjugates (1-7) obtained with n-DSF and n-DSC. The error bar is reported as the confidence interval at 95% of probability.

	My	1	2	3	4	5	6	7
T_m (n-DSF)	78.28 ± 0.03	67.1 ± 0.2	61.1 ± 0.1	70.3 ± 0.2	59.6 ± 0.4	53.7 ± 0.2	74.8 ± 0.1	70.1 ± 0.2
T_m (n-DSC)	79.5 ± 0.1	70.7 ± 0.2	65.4 ± 0.1	72.3 ± 0.3	61.6 ± 0.2	60.3 ± 0.1	76.2 ± 0.1	72.4 ± 0.2
T_{onset} (n-DSF)	73 ± 1	54 ± 1	49 ± 1	52 ± 1	44 ± 1	40 ± 1	66 ± 1	60 ± 1
T_{onset} (n-DSC)	74 ± 1	54 ± 1	49 ± 1	54 ± 1	45 ± 1	44 ± 1	68 ± 1	63 ± 1