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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.

1. 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].

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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 coworkers [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.

2. Experimental section

2.1. 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 = 10 mL/50 mL; when the column was not in use it was stored in a solution of 5% EtOH at 4 °C.

2.2. 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