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Research paper

Tryptophan-mPEGs: Novel excipients that stabilize salmon calcitonin against aggregation by non-covalent PEGylation

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ABSTRACT

Protein aggregation, which is triggered by various factors, is still one of the most prevalent problems encountered during all stages of protein formulation development. In this publication, we present novel excipients, tryptophan-mPEGs (Trp-mPEGs) of 2 and 5 kDa molecular weight and suggest their use in protein formulation. The synthesis and physico-chemical characterization of the excipients are described. Possible cytotoxic and hemolytic activities of the Trp-mPEGs were examined. Turbidity, 90° static light scatter, intrinsic fluorescence, fluorescence after staining the samples with Nile Red and fluorescence microscopy were used to study the inhibitory effect of the Trp-mPEGs on the aggregation of salmon calcitonin (sCT) in different buffer systems and at various molar ratios. Aggregation of sCT was reduced significantly with increasing concentrations of Trp-mPEG 2 kDa. A 10-fold molar excess of Trp-mPEG 2 kDa suppressed almost completely the aggregation of sCT in 10 mM sodium citrate buffer (pH 6) for up to 70 h. Trp-mPEG 5 kDa also reduced the aggregation of sCT, though less pronounced than Trp-mPEG 2 kDa. Low aggregation of sCT was measured after approximately 10 days in 10 mM sodium citrate buffer, pH 5, with a 10-fold molar excess of Trp-mPEG 2 kDa. This paper shows that Trp-mPEGs are potent excipients in reducing the aggregation of sCT. Trp-mPEGs are superior to dansyl-PEGs concerning the stabilization of sCT in a harsh environment, wherein sCT is prone to aggregation. Trp-mPEGs might therefore also be used for stabilization of other biopharmaceuticals prone to aggregation.

1. Introduction

Biologics gained considerably increased market share during the last decade [1–3]. However, chemical and physical degradation, among which aggregation is one of the main concerns, limit the rapid commercialization of protein-based pharmaceuticals [4]. During their manufacturing and formulation processes, proteins are subjected to various stresses, which may lead to aggregation of the biopharmaceutical drug. Processing steps, e.g., freeze drying, pumping, agitation, shear stress, changes in temperature and environmental conditions like buffers, formulation pH or additives, or packaging materials are a few parameters that may affect the stability of a protein [4–6]. Due to the many factors triggering aggregation, and which vary from drug to drug, suppression or reduction in aggregation is most often obtained experimentally by testing various parameters, such as different buffer systems or formulation pH values, under accelerated aggregation conditions [4,7].

For many proteins, aggregation starts by the formation of partially unfolded intermediates. The fraction of unfolded intermediates is usually relatively small but favors protein association, because of an increased amount of exposed hydrophobic patches [8–10]. Native monomers may also aggregate, if 'sticky' patches are present on the protein's surface. Aggregation can then proceed through hydrophobic or electrostatic forces between those patches [9,11].

Covalent conjugation of poly(ethylene glycol), commonly referred to as PEGylation, has been successfully applied to reduce the aggregation of biopharmaceuticals [12–14]. The protective effect of PEG on protein aggregation may be explained by sterical shielding of hydrophobic patches on the protein's surfaces. Further benefits that may be obtained by PEGylation are an increased half-live *in vivo* and a decreased *in vivo* immunogenicity of the biopharmaceutical [15–17]. These positive attributes led to the successful approval of different PEGylated biopharmaceuticals that hold

Abbreviations: Trp, L-tryptophan; Trp-mPEG, tryptophan-mPEG; mPEG, methoxypoly(ethylene glycol); pnp-PEG, *p*-nitrophenyl carbonate mPEG; sCT, salmon calcitonin.

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important therapeutic value in the treatment of severe diseases like cancer (Oncaspar®), hepatitis C (PEGintron®) or severe combined immunodeficiency disease (Adagen®) since the beginning of the 1990s [18].

However, several challenges concerning covalent PEGylation remain, which are (i) the chemical reaction needed in order to attach the PEG-polymer and (ii) the loss of *in vivo* bioactivity observed after PEGylation. Although various techniques have been developed for covalent PEGylation, none has been found that circumvents the chemical reaction and subsequently needed purification. These additional processes during the preparation of the final drug product represent additional stresses for biopharmaceuticals and may lead to aggregation, resulting in a partial or complete loss of *in vivo* bioactivity and increased *in vivo* immunogenicity.

In order to reduce the imposed stresses on biopharmaceuticals during formulation, we presented in a recent publication the new method of stabilization against aggregation by hydrophobic interaction through "non-covalent PEGylation" [19]. The concept is based on non-covalent interaction between the hydrophobic headgroup of a newly synthesized PEG derivative and hydrophobic patches on the surface of biopharmaceuticals. A sterical shielding of the latter by the PEG moiety shall be obtained. Protein/protein interactions should thus be rendered less probable, and consequently aggregation of the biopharmaceutical in liquid formulations should be reduced. A patent application related to this technology has recently been filed [20].

In the previous publication, we were able to show that aggregation of salmon calcitonin (sCT) in liquid formulations was reduced after the addition of dansyl-PEGs of varying molecular weights [19]. In this publication, we present the preparation and physicochemical characterization of PEG conjugates with the amino acid tryptophan (Trp) as hydrophobic headgroup. Although in our studies no hemolysis was observed for both the Trp-PEGs and the dansyl-PEGs, Trp holds the advantage of being used as nutritional supplement for humans [21]. The influence of the monovalent Trp-mPEG polymers of 2 and 5 kDa on the aggregation of sCT was evaluated by performance of accelerated aggregation studies. Trp-mPEGs were superior to dansyl-PEGs in stabilizing sCT against aggregation in a harsh environment, in which sCT in the absence of stabilizing excipients aggregates very fast.

2. Materials and methods

2.1. Materials

The chemicals employed to prepare the buffer solutions, Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one), anhydrous toluene and trifluoroacetic acid (TFA) were supplied by Sigma–Aldrich (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland). The used buffers were constituted of: (i) acetic acid–sodium acetate, pH 5; (ii and iii) citric acid–sodium citrate, pH 5 and pH 6, respectively; (iv) sodium phosphate monobasic–sodium phosphate dibasic, pH 8. Anhydrous DMSO, anhydrous dichloromethane, and L-tryptophan were purchased from Fluka (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland). Chloroform was supplied by Chimie-Plus (Chimie Plus Laboratoires, Denicé, France). Concentrated HCl and anhydrous Na_2SO_4 were obtained from Riedel de Haën (Sigma–Aldrich Laborchemikalien, Seelze, Germany). Diethylether, dichloromethane, anhydrous triethylamine, iso-propanol, and *p*-nitrophenyl chloroformate were provided by Acros (Acros Organics BVBA; Geels, Belgium). The mPEG-OH 2 kDa and 5 kDa were obtained from Iris Biotech (Iris Biotech GmbH, Marktredwitz, Germany). All solvents and compounds used were of analytical grade. The salmon calcitonin was provided by Therapeomic (Therapeomic Inc., Basel, Switzerland). UV transparent 96-well or 384-well Costar® Corning microplates and UV–Vis transparent and pressure sensitive Corning® Universal Optical sealing tape were purchased from Corning (Corning Life Sciences, Schiphol, The Netherlands).

2.2. Characterization of tryptophan-PEGs

All polymers were dissolved in deuterated DMSO and analyzed on a Varian VXR 300 MHz spectrometer (Varian, Switzerland) to obtain ^{1}H NMR and ^{13}C NMR spectra. MALDI-TOF mass spectrometry was performed on an Axima CFR + Shimadzu mass spectrometer, using 2-(4-hydroxyphenylazo)-benzoic acid (HABA) as matrix. A Perkin-Elmer 100 FT-IR spectrometer (Perkin-Elmer, Switzerland) was used to measure FTIR spectra in the range of 4000–400 cm^{-1}. The used pellets were made of 1% w/w of product in KBr. UV spectra were obtained on a Varian Cary 50 spectrophotometer (Varian, Switzerland). The tryptophan-mPEGs were further analyzed for their specific optical rotation properties according to European Pharmacopeia 5.6 using a Perkin-Elmer 241 Polarimeter.

2.3. Synthesis of mPEG-p-nitrophenyl carbonate 2 kDa

The method was adapted from [21]. In short, 1.76 mmol of dried mPEG-OH 2 kDa were dissolved in anhydrous dichloromethane and 5.27 mmol of *p*-nitrophenyl chloroformate and 3.52 mmol of dry triethylamine were added (1:3:2 ratio). The pH was adjusted to a value between 7.5 and 8, and the reaction was left to proceed at room temperature for 24 h. The reaction was stopped by adding several drops of TFA until the solution became colorless; then, dichloromethane was partially evaporated and precipitation from cold diethylether was performed. The solid collected by filtration was twice redissolved in dichloromethane, precipitated from cold diethylether, and collected by filtration. A white powder was obtained and dried under vacuum.

^{1}H NMR (300 MHz, DMSO-d-6): 3.23 ppm, PEG CH_3—O— (s); 3.50 ppm, PEG —O—CH_2— (m); 7.55 ppm, p-nitrophenyl-aromate (d); 8.31 ppm, p-nitrophenyl-aromate (d). ^{13}C NMR (300 MHz, DMSO-d-6): 58.06 ppm, PEG CH_3—O—; 69.52 ppm, PEG —O—CH_2—; 122.59 ppm, p-nitrophenyl-aromate; 125.34 ppm, p-nitrophenyl-aromate; 144.21 ppm, PEG —O—*CH_2*—C=O; 151.99 ppm, aromatic C_5H_4=*C*—NO_2; 155.27 ppm, PEG —CH_2—*OCO*—. FTIR: 3435; 2888; 2739; 2678; 2493; 1967; 1769; 1617; 1594; 1527; 1468; 1360; 1343; 1281; 1242; 1113; 1060; 963; 841; 663; 529 cm^{-1}. MS (MALDI-TOF): *m/z* 2201 (M^+).

2.4. Synthesis of tryptophan-mPEG 2 kDa

The method was adapted from [22]. In anhydrous DMSO, 0.018 mol L-tryptophan was dissolved and the pH was adjusted to a value of ~8.3. Then, 1.76 mmol of dried mPEG-*p*-nitrophenyl carbonate 2 kDa was added. The pH was maintained at a value of approximately 8.3, and the reaction was left to proceed at room temperature for 4 h. The reaction was stopped by cooling to 0 °C and adjusting the pH to a value of 3 with 2 M HCl. The aqueous phase was extracted with chloroform. The obtained organic phase was dried over anhydrous Na_2SO_4 and partially evaporated. Precipitation from cold diethylether was performed and the solid collected by filtration. The solid was once reprecipitated from cold diethylether, and twice from cold iso-propanol. A white powder was obtained and dried under vacuum.

^{1}H NMR (300 MHz, DMSO-d-6): 3.17 ppm, Trp indole—*CH_2*—CH_2— (d); 3.24 ppm, PEG —CH_3—O— (s); 3.51 ppm, PEG —O—CH_2— (m); 4.17 ppm, Trp indole—CH_2—*CH_2*— (q); 6.98 ppm, Trp-indole (t); 7.06 ppm, Trp-indole (t); 7.16 ppm, Trp-indole (s); 7.32 ppm, Trp-indole (d); 7.51 ppm, Trp-indole (d); 10.82 ppm