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Prevention of benzyl alcohol-induced aggregation of chymotrypsinogen by PEGylation

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Abstract

Addition of the antimicrobial preservative benzyl alcohol to reconstitution buffer promotes the formation of undesirable aggregates in multidose protein formulations. Herein we investigated the efficiency of PEGylation to prevent benzyl alcohol-induced aggregation of the model protein α -chymotrypsinogen A (aCTgn). Various PEG-aCTgn conjugates were prepared using PEG with a molecular weight of either 700 or 5000 Da by varying the PEG-to-protein ratio during synthesis. When the model protein was reconstituted in buffer containing 0.9% benzyl alcohol, copious amounts of buffer-insoluble aggregates formed within 24 h (>10%). Benzyl alcohol-induced aggregation was completely prevented when 2 or 5 molecules of PEG with a molecular weight of 5000 Da were attached to the protein, whereas 2 or 4 molecules of bound 700 Da PEG were completely inefficient in preventing aggregation. Mechanistic investigations excluded prevention of structural perturbations or increased thermodynamic stability by PEGylation from being responsible for the prevention of aggregation. Simple addition of PEG to the buffer was also inefficient and PEG had to be covalently linked to the protein to be efficient. The most likely explanation for the protective effect of the 5000 Da PEG is shielding of exposed hydrophobic protein surface area and prevention of protein-protein contacts (molecular spacer effect).

INTRODUCTION

The chemical and physical instability of proteins has limited their full potential as drugs.¹ Proteins are constantly exposed to many stress factors during their production, purification, packaging, and storage.^{2,3} In many instances, physical protein instability causes substantial protein aggregation which has to be kept at the minimum in a pharmaceutical product.

Multidose protein formulations are attractive for a variety of reasons, e.g., cost efficiency of the product and possibility of automatized delivery from pump devices. However, after reconstitution with buffer such applications require the use of an antimicrobial preservative to prevent bacterial and other contaminations. Benzyl alcohol (BA) is the most frequently employed antimicrobial preservative in multidose protein formulations, typically at a concentration of 0.9%.⁴ Previous studies have shown that the presence of BA leads to the formation of insoluble aggregates of recombinant human growth hormone (rhGH),⁵ human insulin-like growth factor I (hIGF-1),⁶ recombinant human interferon- γ (rhIFN- γ),⁷ interleukin-1 receptor (IL-1R),⁸ and recombinant human granulocyte colony stimulating factor (rhGCSF).⁹ Strategies that prevent BA-induced aggregation are highly desirable to overcome these problems.

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Covalent attachment of poly(ethylene glycol) to biomolecules (PEGylation) was originally performed to improve blood circulation half life and to reduce immunogenic reactions.^{10,11} PEGylation has since then matured into a go-to approach for improving the *in vivo* performance of protein drugs and has resulted in at least nine U.S. FDA approved protein products and more are in clinical trials.¹² An additional benefit of protein PEGylation is the potentially increased *in vitro* stability, e.g., higher thermodynamic stability,¹³ prevention of temperature-induced aggregation of insulin,¹⁴ rhGCSF,^{15,16} and glucagon,¹⁷ and increased protein stability when encapsulated in polymer microspheres for sustained release applications.^{18,19} Herein we tested PEGylation as an approach to prevent antimicrobial preservative-induced protein aggregation.

MATERIALS AND METHODS

Materials

α -Chymotrypsinogen A (aCTgn) from bovine pancreas, methoxypoly(ethylene glycol) (average M_w of 5,000 Da) and benzyl alcohol (BA) ($\geq 99.0\%$) were from Sigma-Aldrich (St. Louis, MO). Methoxypoly(ethylene glycol)-succinimidyl propionate (average M_w of 5,000 Da) was purchased from Nektar Technologies (Huntsville, AL). Methoxypoly(ethylene glycol)-N-hydroxysuccinimide ester (685.71 g/mol) and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) were from Thermo Fisher Scientific (Hudson, NH).

Synthesis of PEG- α -CT Conjugates

Chemical modification of surface exposed lysine ϵ -amino groups of aCTgn with activated PEG was carried out as reported.¹³ In brief, 100 mg of aCTgn and the desired amount of activated PEG were dissolved in 100 mM borate buffer at pH 9.2. Different mPEG-to-aCTgn molar ratios were employed to vary the extent of PEGylation. The reaction solution was stirred for 3 h at 4°C and the reaction was stopped by lowering the pH to 5 with 1 M HCl. Free PEG and buffer salts were removed by dialysis (molecular weight cut-off 20,000 Da) against deionized water. The PEG-aCTgn conjugates were subsequently freeze-dried and stored at -20°C. The extent of protein PEGylation was determined by colorimetric titration of free (unreacted) amino groups with TNBSA.^{13, 20}

Aggregation Studies

aCTgn and PEG-aCTgn conjugates were dissolved in 10 mM acetate buffer (pH 5.0) to a final concentration of 1 mg/ml with or without 0.9% BA. Samples were incubated under accelerated conditions at 45°C as 0.5 mL aliquots in microcentrifuge tubes (2 mL). Samples were removed after 6, 12, and 24 hours of incubation and centrifuged for 10 min ($16000 \times g$) to pellet insoluble protein aggregates. The samples were filtered through a 0.2 μ m pore size low protein binding polyvinylidene fluoride syringe filters from Millipore, Billerica, MA, and the soluble protein concentration determined by measuring the absorbance at 280 nm of the clear solutions. Protein loss due to centrifugation and filtration was negligible. The soluble protein fraction (X) was calculated as the ratio of soluble protein concentration at a given time over the initial protein concentration. Percentages of insoluble aggregates were calculated using the following formula:

$$\% \text{ insoluble aggregates} = (1 - X) * 100 \quad (1)$$

Circular Dichroism (CD) Measurements

Near-UV CD spectra were measured using an Olis DSM-10 UV-Vis CD spectrophotometer equipped with a temperature controlled (0.1°C) cell holder. The protein concentration was

adjusted to 0.6 mg/mL in 10 mM acetate buffer (pH 5.0) with or without 0.9% BA. Spectra were recorded from 270 to 310 nm using 10 mm path-length quartz cells. Each spectrum was obtained by averaging 5 scans at 0.5 nm resolution. Subtraction of the respective buffer blank spectrum was performed digitally.

Fluorescence Spectroscopy

Fluorescence spectra of aCTgn and PEG-aCTgn were acquired using a Varian Cary Eclipse spectrophotometer. Samples at a protein concentration of 0.05 mg/ml in 10 mM acetate buffer (pH 5.0) were excited at 280 nm and the fluorescence emission was recorded from 300 to 450 nm. Spectra were recorded at 5°C intervals (10 min were allowed for thermal equilibration) from 25 to 75°C. The spectral center of mass (COM) was calculated for each spectrum from eq. 2.

$$COM = \frac{\sum_{\lambda} (1/\lambda) I_{\lambda}}{\sum_{\lambda} I_{\lambda}} \quad (2)$$

where λ is the wavelength, and I_{λ} is the emission intensity at λ .²¹ The COM was plotted as a function of temperature and a sigmoidal curve was fitted to it using Sigma-Plot.²² The melting temperature (T_m) was determined from the maxima of the first-derivative plot of the fitted sigmoid curve.

Statistical Methods

Reported values are averages of three independent samples $\pm$ standard deviation. All statistical analyses were done in Minitab 14 (Minitab Inc., State College, PA). Analysis of the effects of BA on aCTgn aggregation was performed using the Mann-Whitney U-test. The effect of PEGylation on BA-induced aggregation of aCTgn was evaluated with the Kruskal-Wallis test. Post-hoc comparisons of the means of individual groups were performed using Dunn's test. Statistical significance was accepted at the $p \leq 0.05$ level.

RESULTS

Synthesis of PEG-aCTgn Conjugates

PEG-aCTgn conjugates were synthesized by covalently attaching activated PEG with M_w of 700 and 5000 Da to surface exposed lysine residues. aCTgn has 14 surface exposed lysine residues available. To systematically vary to amount of PEG bound to the protein, different PEG-to-aCTgn molar ratios were used in the reaction. Four preparations were accomplished: for the 700 Da PEG 2 and 4 chains were bound to the protein and for the 5000 Da PEG 2 and 5, respectively (furtheron simply referred to as (PEG700)2-aCTgn etc.).

To test whether the PEGylation reaction caused changes to the protein tertiary structure, aCTgn and PEG-aCTgn were characterized by near-UV CD spectroscopy (Figure 1). The spectra of PEG-aCTgn conjugates were similar to the spectrum of unmodified aCTgn which shows that PEGylation did not alter the tertiary structure substantially.

BA-induced Aggregation Studies

The effect of BA on the aggregation of aCTgn was investigated by incubating aCTgn solutions without and with 0.9% BA at 45°C. At the different time points (6, 12, and 24 h), aliquots were removed and the amount of insoluble aggregates was determined (Figures 2, 3). After 24 h of incubation, the solution containing 0.9% BA contained 13% of insoluble aggregates. Incubation of chymotrypsinogen and PEG-aCTgn conjugates in the absence of

BA caused the formation of no more than 2% of insoluble aggregates. Statistical analysis demonstrated that BA-induced aggregation of aCTgn was significant after 24 h of incubation ($p < 0.01$).

To test whether PEGylation of aCTgn could prevent BA-induced aggregation, all PEG-aCTgn conjugates obtained were incubated at 45°C in the presence of 0.9% BA and the amount of insoluble aggregates determined (**Figures 2, 3**). PEGylation of aCTgn with PEG of 700 Da did not prevent protein aggregation. On the contrary, the PEG700-aCTgn conjugates seemed to contain a higher amount of aggregates than unmodified aCTgn, but this effect was not statistically significant ($p > 0.05$). When modified with PEG of 5,000 Da, either with 2 or 5 PEG molecules attached to aCTgn, aggregation was completely inhibited (**Figures 2, 3**).

In addition, it was tested whether co-dissolved PEG (5,000 Da) could also prevent BA-induced aggregation. Even though a reduction in the amount of insoluble aggregates was observed when PEG (5,000 Da) was co-dissolved with aCTgn at a 5:1 molar ratio, the difference the the sample without PEG was statistically not significant.

Thermostability of aCTgn and PEG-aCTgn Conjugates

The effect of BA on the thermostability of aCTgn and PEG-aCTgn was studied using fluorescence spectroscopy. Under the conditions employed, the thermal unfolding of aCTgn is irreversible due to aggregate formation. Thus, only apparent T_m values can be reported. Thermal unfolding of aCTgn and PEG-aCTgn conjugates led to a shift of the maximum wavelength of fluorescence emission from 340 ± 1 nm at 25°C to 349 ± 1 nm at 75°C. In the absence of BA, the apparent T_m of aCTgn and PEG-aCTgn was 58 ± 1 °C. In the presence of 0.9% BA, the T_m was lowered by 5°C to 53 ± 1 °C. Under the conditions used in this study, PEGylation of aCTgn did not have a significant effect on the protein thermostability and all PEGylated samples had a T_m of 53 ± 1 °C in the presence of 0.9% BA.

Near-UV CD Studies

Near-UV CD spectra were collected to characterize the effect of BA on the tertiary structure of aCTgn and PEG-aCTgn conjugates. Spectra were collected at 45°C unless otherwise stated. The near-UV CD spectra of aCTgn in 10 mM acetate buffer (pH 5) with and without 0.9% BA are shown in Figure 4. The spectrum of aCTgn shows two minima at 290 and 299 nm. When the tertiary structure of chymotrypsinogen is significantly perturbed, e.g., by thermal unfolding, both peaks in the near-UV CD spectra disappear.¹⁸ In the presence of 0.9% BA there is a small decrease in the intensity of both of these minima. These changes in the spectrum are caused by minor tertiary structural perturbations of aCTgn induced by BA. The spectra for PEG-aCTgn conjugates in buffer alone and with 0.9% BA are shown in Figure 5. Similar to unmodified aCTgn, near-UV CD spectra of PEG-aCTgn conjugates demonstrate minor structural perturbation induced by BA. It is obvious from this that BA-induced unfolding and its prevention are not responsible for the observed aggregation and its prevention by PEGylation.

DISCUSSION

The objective of this study was to investigate protein PEGylation as a potential stabilization strategy against protein aggregation induced by antimicrobial preservatives commonly employed in multidose formulations. BA induces minor perturbations to the protein tertiary structure and decreases protein thermostability.^{5,23,24} Previous studies have demonstrated that hydrophobic interactions are dominant between proteins and benzyl alcohol. These interactions shift the equilibrium from the natively folded protein to partially unfolded,

aggregation-prone species.²⁵ In agreement with past studies, this report shows that 0.9% BA in the reconstitution buffer does destabilize aCTgn, evidenced by a reduced thermostability (the T_m was decreased by 5°C). Current strategies to stabilize proteins against BA-induced aggregation include the employment of excipients. For example, Frason et al. used mannitol to reverse the destabilizing effects of BA and prevented its aggregation.⁶ Zhang et al. reported that sucrose inhibited BA-induced aggregation of rhIL-1ra by being excluded from the protein surface, thus favoring a more compact and stable conformation.²⁶

Protein PEGylation has been used to stabilize proteins against heat induced aggregation.¹⁴⁻¹⁶ Computer models and low-resolution structural data suggest that PEG molecules fold upon themselves and closely interact with the protein surfaces, thus shielding hydrophobic regions on the protein surfaces.²⁷ Small-angle X-ray diffraction experiments support the fact that PEG molecules closely interact with the protein surface.²⁸ Therefore, it is reasonable to hypothesize that PEG molecules would act as molecular spacers and inhibit protein-protein interactions that lead to protein aggregation. In addition, PEG molecules can shield partially exposed hydrophobic regions that accelerate protein aggregation. However, protein PEGylation is usually accompanied by a loss in activity, the magnitude of which will depend on the number, size, and location of the PEG molecules.²⁹ Specifically, a systematic study of the effects of PEG molecular weight and PEGylation extent on the enzymatic activity of alpha-chymotrypsin was reported by the Griebenow laboratory.³⁰ This fact should lead the formulation scientist to consider these PEGylation parameters when developing particular PEG-protein conjugate formulations.

In our studies, aggregation of aCTgn was completely prevented by PEGylation with 5000 Da PEG, whereas a 700 Da PEG did not prevent aggregation. It is important to note that PEGylation did not have an effect on protein thermostability when BA was present, so stabilization by increasing protein thermostability was discarded. Since only the larger PEG was able to inhibit protein aggregation it is reasonable to hypothesize that PEG is acting as a molecular spacer which minimizes protein-protein interactions that lead to the formation of aggregates. Protein glycosylation also prevented protein aggregation in a size-dependent way. Solá et al. reported that chymotrypsin was stabilized against heat-induced aggregation when covalently modified with large sugars (dextran M_w 10 kDa), whereas when chymotrypsin was modified with smaller glycan (lactose M_w ~500Da) aggregation still occurred.³¹

CONCLUSIONS

It was shown that by conjugating PEG molecules to aCTgn it was possible to stabilize it against antimicrobial preservative-induced aggregation. While modification with PEG of 5000 Da completely inhibited aCTgn aggregation, modification with PEG of 700 Da did not. This observation suggests that steric shielding of exposed hydrophobic patches plays an important role in inhibiting protein aggregation. These results should help the design of stabilizing strategies for multidose protein formulations.

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Figure 1. Near UV-CD spectra of aCTgn and PEG-aCTgn (0.6 mg/ml) in 10 mM acetate buffer at pH 5 and 25°C. aCTgn (solid line), (PEG700)2-aCTgn (dashed line), (PEG700)4-aCTgn (dash-dot-dash line), (PEG5k)2-aCTgn (dotted line), and (PEG5k)5-aCTgn (dash-dot-dot line).

Figure 2. Benzyl alcohol-induced aggregation of aCTgn and PEG-aCTgn conjugates after different incubation times. Samples were incubated at 45°C in 10 mM acetate buffer (pH 5.0).

Figure 3.

Samples of aCTgn and PEG-aCTgn conjugates after 24 h of incubation at 45°C in 10 mM acetate buffer (pH 5). **1.** aCTgn **2.** aCTgn + 0.9% BA **3.** (PEG700)2-aCTgn + 0.9% BA **4.** (PEG700)4-aCTgn + 0.9% BA **5.** (PEG5k)2-aCTgn + 0.9% BA **6.** (PEG5k)5-aCTgn + 0.9% BA.

Figure 4. Near UV-CD spectra of aCTgn (0.6 mg/ml) in 10 mM acetate buffer (pH 5.0) (solid line) and with 0.9% BA added (dashed line) at 45°C. The baseline of 0.9% BA in acetate buffer is the dotted line.

Figure 5.

Near UV-CD spectra of PEG-aCTgn conjugates in 10 mM acetate buffer (solid line) and in 0.9% BA in 10 mM acetate buffer (pH 5.0) (dashed line) at 45°C. **A.** (PEG700)2-aCTgn; **B.** (PEG700)4-aCTgn; **C.** (PEG5k)2-aCTgn + 0.9% BA; **D.** (PEG5k)5-aCTgn + 0.9% BA.