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Research Paper

Prevention of benzyl alcohol-induced aggregation of chymotrypsinogen by PEGylation

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Abstract

Objectives 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 (attachment of poly(ethylene glycol)) to prevent benzyl alcohol-induced aggregation of the model protein α-chymotrypsinogen A (aCTgn).

Methods 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 and the formation of insoluble aggregates was studied. The effect of benzyl alcohol on the thermodynamic stability and tertiary structure of aCTgn was also examined.

Key findings 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 two or five molecules of PEG with a molecular weight of 5000 Da were attached to the protein, whereas two or four 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.

Conclusions 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).

Keywords benzyl alcohol; PEGylatio; preservative; protein aggregation; protein formulation

Introduction

The chemical and physical instability of proteins limits 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 automated delivery from pump devices). However, after reconstitution with buffer such applications require the use of an antimicrobial preservative to prevent bacterial and other contamination. Benzyl alcohol 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 benzyl alcohol 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 benzyl alcohol-induced aggregation are highly desirable to overcome these problems.

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 matured into a go-to approach for improving the in-vivo performance of protein drugs and has resulted in at least nine US 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

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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 MW 5000 Da) and benzyl alcohol (≥99.0%) were from Sigma-Aldrich (St Louis, USA). Methoxypoly(ethylene glycol)-succinimidyl propionate (average MW 5000 Da) was purchased from Nektar Technologies (Huntsville, USA). Methoxypoly(ethylene glycol)-*N*-hydroxysuccinimide ester (685.71 g/mol) and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) were from Thermo Fisher Scientific (Hudson, USA).

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 de-ionized 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% benzyl alcohol. Samples were incubated under accelerated conditions at 45°C as 0.5-ml volumes in microcentrifuge tubes (2 ml). Samples were removed after 6, 12 and 24 h of incubation and centrifuged for 10 min (16 000*g*) to pellet insoluble protein aggregates. The samples were filtered through s 0.2 µm pore size low protein binding polyvinylidene fluoride syringe filter (Millipore, Billerica, USA), and the soluble protein concentration determined by measuring the absorbance at 280 nm of the clear solution. 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:

$$\%\ \text{Insoluble aggregates} = (1 - X) \times 100 \qquad (1)$$

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% benzyl alcohol. Spectra were recorded from 270 nm to 310 nm using 10 mm path-length quartz cells. Each spectrum was obtained by averaging five 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 nm to 450 nm. Spectra were recorded at 5°C intervals (10 min were allowed for thermal equilibration) from 25°C to 75°C. The spectral centre of mass (COM) was calculated for each spectrum from Equation 2.

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

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 the average of three independent samples ± standard deviation. All statistical analyses were done in Minitab 14 (Minitab Inc., State College, USA). Analysis of the effects of benzyl alcohol on aCTgn aggregation was performed using the Mann–Whitney *U*-test. The effect of PEGylation on benzyl alcohol-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 $P \leq 0.05$.

Results

Synthesis of PEG-aCTgn conjugates

PEG-aCTgn conjugates were synthesized by covalently attaching activated PEG with a molecular weight of 700 Da and 5000 Da to surface-exposed lysine residues. aCTgn has 14 surface-exposed lysine residues available. To systematically vary the 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 two and four chains were bound to the protein and for the 5000 Da PEG two and five were bound (henceforward 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

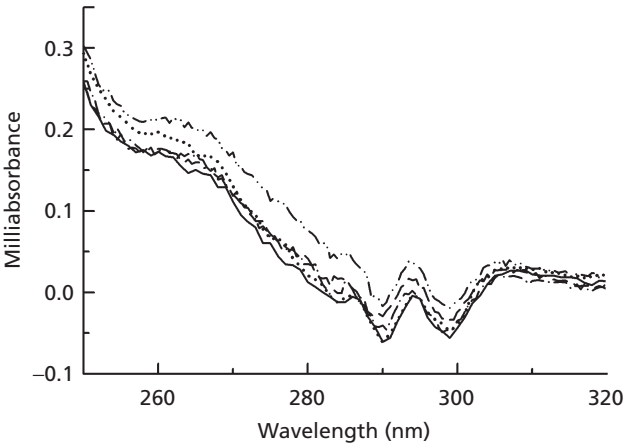

Figure 1 Near UV-CD spectra of aCTgn and PEG-aCTgn (0.6 mg/ml) in 10 mM acetate buffer at pH 5 and 25°C aCTgn (—) (PEG700)2-aCTgn (---) (PEG700)4-aCTgn (–·–·) (PEG5k)2-aCTgn (·····), and (PEG5k)5-aCTgn (–···–).

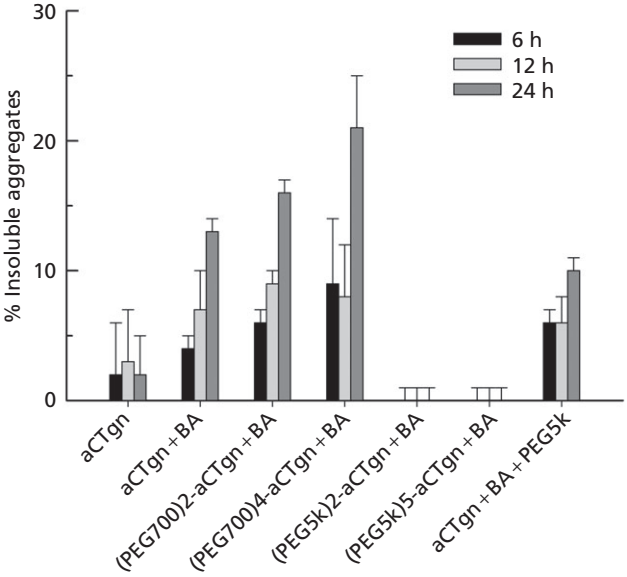

Figure 2 Benzyl alcohol-induced aggregation of aCTgn and PEG-aCTgn conjugates after different incubation times. Samples were incubated at 45°C in 10 mM acetate buffer (pH 5.0).

spectrum of unmodified aCTgn, which shows that PEGylation did not alter the tertiary structure substantially.

Benzyl alcohol-induced aggregation studies

The effect of benzyl alcohol on the aggregation of aCTgn was investigated by incubating aCTgn solutions without and with 0.9% benzyl alcohol at 45°C. At different time points (6, 12 and 24 h), samples were removed and the amount of insoluble aggregates was determined (Figures 2 and 3). After 24 h of incubation, the solution containing 0.9% benzyl alcohol contained 13% of insoluble aggregates. Incubation of chymotrypsinogen and PEG-aCTgn conjugates in the absence of benzyl alcohol caused the formation of no more than 2% of

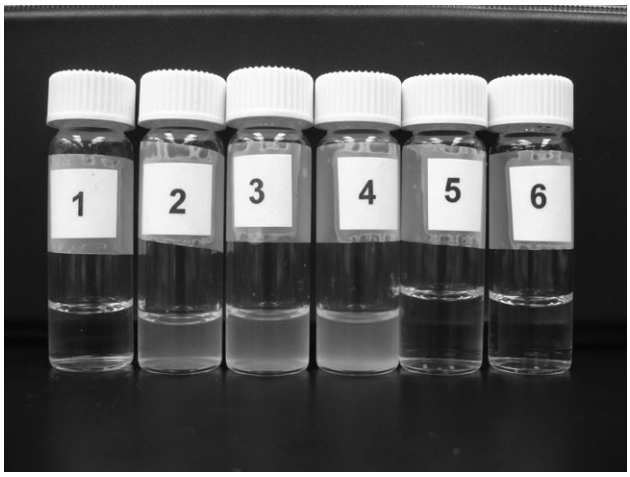

Figure 3 Samples of aCTgn and PEG-aCTgn conjugates after 24 h of incubation at 45°C in 10 mM acetate buffer (pH 5). 1, aCTgn; 2, aCTgn + 0.9% benzyl alcohol; 3, (PEG700)2-aCTgn + 0.9% benzyl alcohol; 4, (PEG700)4-aCTgn + 0.9% benzyl alcohol; 5, (PEG5k)2-aCTgn + 0.9% benzyl alcohol; 6, (PEG5k)5-aCTgn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

insoluble aggregates. Statistical analysis demonstrated that benzyl alcohol-induced aggregation of aCTgn was significant after 24 h of incubation ($P < 0.01$).

To test whether PEGylation of aCTgn could prevent benzyl alcohol-induced aggregation, all PEG-aCTgn conjugates obtained were incubated at 45°C in the presence of 0.9% benzyl alcohol and the amount of insoluble aggregates determined (Figures 2 and 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 5000 Da, either with two or five PEG molecules attached to aCTgn, aggregation was completely inhibited (Figures 2 and 3).

In addition, it was tested whether co-dissolved PEG (5000 Da) could also prevent benzyl alcohol-induced aggregation. Even though a reduction in the amount of insoluble aggregates was observed when PEG (5000 Da) was co-dissolved with aCTgn at a 5:1 molar ratio, the difference compared with the sample without PEG was statistically not significant.

Thermostability of aCTgn and PEG-aCTgn conjugates

The effect of benzyl alcohol 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 benzyl alcohol, the apparent T_m of aCTgn and PEG-aCTgn was 58 ± 1°C. In the presence of 0.9% BA benzyl alcohol the T_m was lowered by 5°C to 53 ± 1°C. Under the conditions

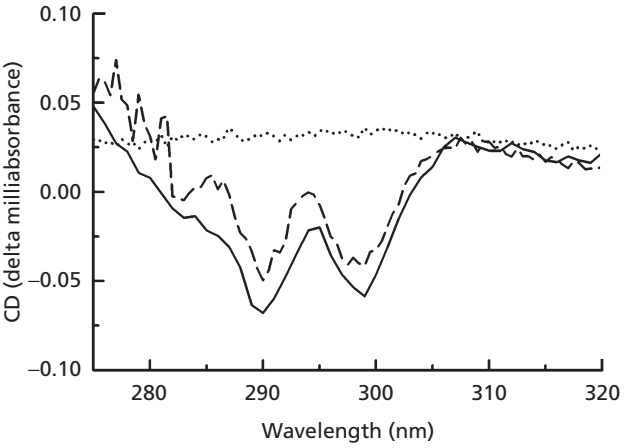

Figure 4 Near UV-CD spectra of aCTgn (0.6 mg/ml) in 10 mM acetate buffer (pH 5.0) (—) and with 0.9% benzyl alcohol added (---) at 45°C. The baseline of 0.9% benzyl alcohol in acetate buffer is the dotted line (·····).

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% benzyl alcohol.

Near-UV CD studies

Near-UV CD spectra were collected to characterize the effect of benzyl alcohol 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% benzyl alcohol are shown in Figure 4. The spectrum of aCTgn shows two minima at 290 nm 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% benzyl alcohol 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 benzyl alcohol. The spectra for PEG-aCTgn conjugates in buffer alone and with 0.9% benzyl alcohol are shown in Figure 5. Similar to unmodified aCTgn, the near-UV CD spectra of PEG-aCTgn conjugates demonstrate minor structural perturbation induced by benzyl alcohol. It is obvious from this that benzyl alcohol-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. Benzyl alcohol 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% benzyl alcohol in the reconstitution buffer does destabilize aCTgn, demonstrated by a reduced thermostability (the T_m was decreased by 5°C). Current strategies to stabilize proteins against benzyl alcohol-induced aggregation include the employment of excipients. For example, Frason *et al*.[6] used mannitol to reverse the destabilizing effects of benzyl alcohol and prevent aggregation. Zhang *et al*.[26] reported that sucrose inhibited benzyl alcohol-induced aggregation of rhIL-1ra by being excluded from the protein surface, thus favoring a more compact and stable conformation.

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 benzyl alcohol 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 minimizing protein–protein interactions that lead to the formation of aggregates. Protein glycosylation also prevented protein aggregation in a size-dependent way. Solá *et al*.[31] reported that chymotrypsin was stabilized against heat-induced aggregation when covalently modified with large sugars (dextran MW 10 kDa), whereas when chymotrypsin was modified with smaller glycan (lactose MW ~500Da) aggregation still occurred.

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

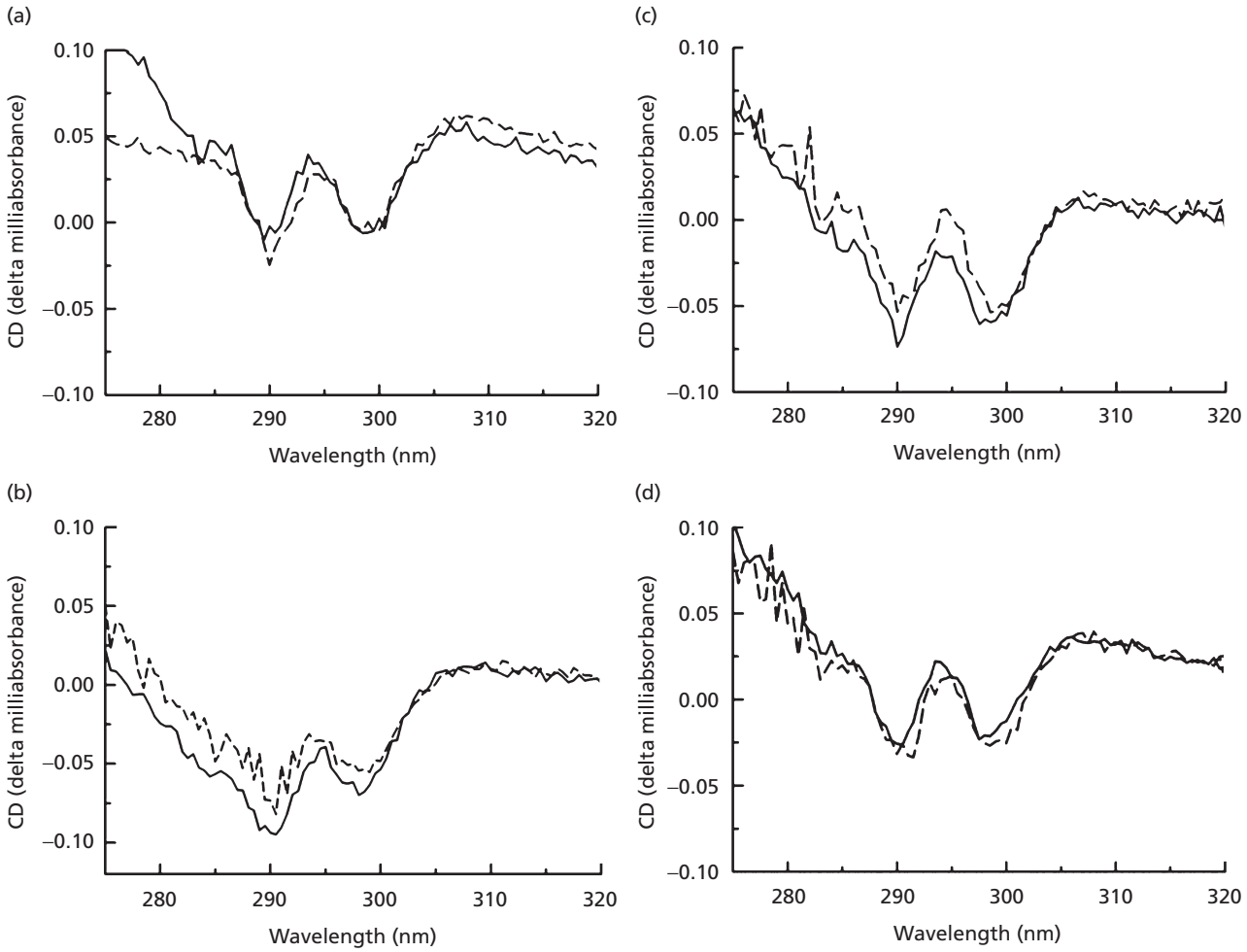

Figure 5 Near UV-CD spectra of PEG-aCTgn conjugates in 10 mM acetate buffer (—) and in 0.9% benzyl alcohol in 10 mM acetate buffer (pH 5.0) (---) at 45°C. (a) (PEG700)2-aCTgn; (b) (PEG700)4-aCTgn; (c) (PEG5k)2-aCTgn + 0.9% benzyl alcohol; (d) (PEG5k)5-aCTgn + 0.9% benzyl alcohol.

should help the design of stabilizing strategies for multidose protein formulations.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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