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ScienceDirect
International Journal of Pharmaceutics 330 (2007) 89–98
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Characterisation and physical stability of PEGylated glucagon

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Received 3 October 2005; received in revised form 7 August 2006; accepted 6 September 2006
Available online 10 September 2006

Abstract

Glucagon was mono-PEGylated with PEG 5000 at Lys-12 to examine the effect on conformation and physical stability during purification and freeze-drying. The model peptide glucagon is highly unstable and readily forms fibrils in solution. Secondary structure was determined by FTIR and far-UV CD and physical stability was assessed by the Thioflavin T assay.

Glucagon samples were included, which underwent the same RP-HPLC purification and/or freeze-drying as glucagon–PEG 5000. After purification and freeze-drying glucagon samples showed formation of intermolecular β-sheet by FTIR, this correlated with shorter lag-times for fibrillation in the Thioflavin T assay. Formation of intermolecular β-sheet was less apparent for glucagon–PEG 5000 and no fibrillation was detected by Thioflavin T assay. Apparently PEGylation significantly improved the physical stability of glucagon after purification and freeze-drying, possibly by steric hindrance of peptide–peptide interactions.

Alterations in the secondary structure were observed for freeze-dried and reconstituted peptide samples by liquid FTIR. The peak for α-helix shifted to 1664 cm^{-1}, which could possibly be explained by formation of 3_{10}-helix. Neither 3_{10}-helix nor intermolecular β-sheet could be detected by far-UV CD, where all peptide samples showed similar spectra.

In conclusion, glucagon–PEG 5000 showed a significantly improved physical stability during purification and freeze-drying compared to glucagon.

Keywords: PEGylation; Glucagon; Freeze-drying; Secondary structure; Fibrillation; Physical stability

1. Introduction

The use of peptides and proteins as drug substances in the treatment of many different diseases is expanding rapidly. The advantages of peptide and protein drugs include enhanced specificity and activity compared to conventional drugs. The efficacy of peptide and protein drugs renders them attractive, but there are some limitations associated with their use as drugs. The problems include low physical and chemical stability both from manufacturing and after *in vivo* exposure, which leads to e.g. immunogenic side effects (Frokjaer and Otzen, 2005).

In the search of a method to overcome these problems, the technique of PEGylation has acquired increasing popularity since its introduction in the 1970s. PEGylation involves the covalent attachment of the non-toxic polymer polyethylene glycol to the peptides or proteins. The first PEGylated protein drug (Adagen®) was approved by the FDA in 1990 for the treatment of severe combined immunodeficiency (Harris and Chess, 2003). Numerous studies have shown impressive improvement in circulation time, combined with decreased immunogenic response, improved solubility, and a higher physical stability by attaching PEG (Nucci et al., 1991; Veronese and Harris, 2002; Bhadra et al., 2002; Harris and Chess, 2003). In the case of PEGylated insulin, the observed decreased fibrillation rate for shaken solutions was dependent on the site of PEGylation (Hinds and Kim, 2002). Also, the physical stability of epidermal growth factor was increased after PEGylation during the process of homogenisation and encapsulation in microspheres (Kim et al., 2002).

In the development of new protein-based drugs, the preparation of dry and excipient-free peptide or protein is advantageous

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doi:10.1016/j.ijpharm.2006.09.002

in fundamental studies of physical and chemical characteristics (Overcashier et al., 1997). Due to the low stability of peptides and proteins it is often necessary to apply methods such as freeze-drying to ensure a stable peptide or protein for further work. Freeze-drying of peptides and proteins often enhances the physical stability by reducing mobility as a consequence of solvent removal. This potentially results in longer shelf life due to reduced degradation rates. Even though proteins generally have higher physical stability after the freeze-drying process, the process of producing the solid protein can induce conformational change following exposure to the ice–water interface (Frokjaer and Otzen, 2005).

The effect of PEGylation on physical stability during pharmaceutical processing is investigated in this study and glucagon was chosen as a model peptide. Glucagon is considered to be a suitable model peptide since it is highly physically unstable and able to rapidly form fibrils in aqueous solution (Beaven et al., 1969). Therefore, it should be possible to detect differences in conformation and physical stability upon PEGylation and freeze-drying.

The secondary structure of native glucagon is a mixture of random structure and α-helix that changes upon fibrillation to an intermolecular β-sheet dominated structure (Gratzer et al., 1972; Panijpan and Gratzer, 1974; Gratzer and Beaven, 1969; Tran et al., 1982). Fibrillation of glucagon has recently been examined using the fluorescent probe Thioflavin T, which interacts specifically with amyloid fibrils (Onoue et al., 2004). The fluorescent probe has also been used to evaluate the ability of other therapeutic proteins to form amyloid fibrils (Harper and Lansbury, 1997; Ohnishi and Takano, 2004; Nilsson, 2004) and to study fibrillation kinetics, which has been extensively explored for insulin (Harper and Lansbury, 1997; Grillo et al., 2001; Nielsen et al., 2001). Fibrillation of PEGylated insulin has previously been determined by size exclusion chromatography, and a decrease in fibrillation rate was seen after PEGylation (Hinds and Kim, 2002).

In this study polyethylene glycol (MW 5000 Da) has been linked to the primary amino groups of glucagon with an amide linkage using an active ester PEG-reagent (Veronese, 2001). Glucagon consists of 29 amino acids including two primary amino groups (N-terminus and Lys-12).

The aim was therefore to examine the effect of PEGylation of glucagon on the conformational structure and physical stability. In this study effects of PEGylation and freeze-drying of glucagon were combined to explore the effect of PEGylation on pharmaceutically employed processes. The peptides were freeze-dried from acetonitrile containing solvent without stabilising agents. The conformational structure was determined by far-UV CD and FTIR and a Thioflavin T assay was used to compare the fibrillation properties.

2. Materials and methods

2.1. Materials

Glucagon was kindly donated by Novo Nordisk A/S, Denmark. Methoxy polyethylene succinimidyl propionate 5000 (mPEG SPA 5000) was purchased from Nektar Therapeutics AL Corporation, USA. All materials were of analytical grade and were used without further purification.

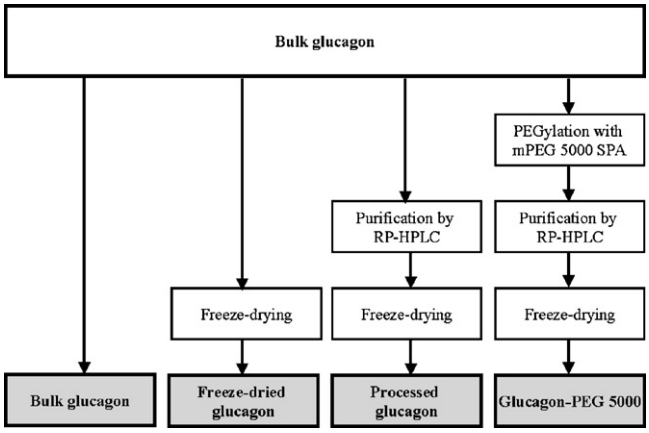

Fig. 1. Flow diagram of peptide processing.

2.2. Preparation of samples

In Fig. 1, a flow diagram for preparation of glucagon–PEG 5000 and the different glucagon samples applied in this study is shown. Glucagon–PEG 5000 was purified by RP-HPLC and freeze-drying and glucagon samples were included to explore the potential effects of the stress introduced by these methods. "Bulk glucagon" was prepared by salt-precipitation as provided by the supplier. Bulk glucagon was dissolved in 0.1% trifluoroacetic acid (TFA) and 50% acetonitrile in Milli-Q water and freeze-dried to yield "freeze-dried glucagon" and purified by RP-HPLC and freeze-dried to obtain "processed glucagon".

2.3. Mono-PEGylation of glucagon with mPEG 5000 SPA

Glucagon (20 mg) was dissolved in 1 ml 0.1 M NaOH and 4 ml *N*,*N*-dimethylformamide (DMF) to ensure dissolution of the peptide. mPEG 5000 SPA (71.6 mg) was dissolved in 2 ml borate buffer pH 9.5 and was immediately added to the glucagon solution under gentle rotation. An equivalent portion of freshly dissolved mPEG 5000 SPA solution was added after 10 min, and within a further 30 min at room temperature the reaction was considered complete. The relatively high pH and mPEG/peptide ratio were chosen after an optimisation of conditions.

The mono-PEGylated conjugate glucagon–PEG 5000 was purified by preparative RP-HPLC using a Beckman HPLC System Gold (Beckman Coulter Inc., USA) with a Daiso C-18 column (FeF Chemicals A/S, Denmark). The elution system consisted of solvent A (0.1% TFA in Milli-Q water) and solvent B (0.1% TFA in acetonitrile) in a linear gradient of 40–55% of B over 15 min at a flow rate of 3 ml/min with UV detection at 280 nm. Fractions of mono-PEGylated glucagon and processed glucagon, respectively, were collected and stored at −80 °C until freeze-drying. Peptide concentrations were determined by use of

A_{280nm} (1%) of 23 (Pace et al., 1995). For glucagon–PEG 5000 concentrations are referred to by the molar equivalent peptide content.

2.4. Freeze-drying

Freeze-drying was carried out on a Genesis 25 L SQ EL-85 (VirTis, USA). Peptides were freeze-dried from peptide concentrations 1 and 10 mg/ml in 0.1% TFA and 50% acetonitrile in order to investigate the effect on physical stability. Vials for freeze-drying were filled with 500 μl of peptide solution immediately before beginning of the freeze-drying program. Initial freezing was carried out at −45 °C for 4 h followed by primary drying at −5 °C and 8 Pa for 100 h, and secondary drying at 25 °C and 2 Pa for 30 h. The solid products were stored at −20 °C after freeze-drying.

2.5. MALDI-TOF-MS

MALDI-TOF-MS was performed on a Bruker Reflex IV (Bruker Daltonics Inc., USA) using a UV laser (N_2, λ = 337 nm, 3 ns pulse width). The samples were prepared by dissolving glucagon and glucagon–PEG 5000 in a mixture of 0.1% TFA and 50% acetonitrile in Milli-Q water to a concentration of 100 μg/ml. Equal volumes of sample solution and 30 mg/ml 2,5-dihydroxybenzoic acid matrix solution were mixed and 1 μl was added to the stainless steel MALDI plate, allowed to air dry, and inserted into the mass spectrometer for analysis. Measurements were conducted in reflector mode by accumulation of 200 spectra.

2.6. Site of PEGylation

In order to determine the site of PEG attachment, digestion with endoproteinase Asp-N was applied to the conjugate. Asp-N cleaves at the amino side of aspartic and cysteic acid and can consequently be used to distinguish between the two possible PEGylation sites, i.e. the N-terminal histidine or the Lys-12 residue. The fragments were analysed by electrospray Q-TOF MS and MALDI-TOF-MS.

Briefly, glucagon and glucagon–PEG 5000 were dissolved to a concentration of 100 μM in 0.1% formic acid to ensure dissolution. These solutions were diluted to a concentration of 10 μM in sodium phosphate buffer pH 8 and endoproteinase Asp-N was added in a ratio of 1:200 (w/w). After 1 h the samples were analysed using standard settings on a Micromass Q-TOF 2 combined with Waters CapLC (Waters Corporation, USA) and a Bruker Ultraflex MALDI TOF-TOF with enhanced Post Source Decay (Bruker Daltonics Inc., USA).

2.7. Fourier transform infrared spectroscopy

Infrared spectra were recorded on a Bomem MB 104 FTIR Spectrometer (Bomem Inc., Canada) by collecting 256 scans with a resolution of 4 cm^{-1}. Solid-state FTIR was carried out in transmission mode in a potassium bromide disk in a mixture of 1 mg peptide and 300 mg potassium bromide.

Infrared spectra in solution were obtained by placing 20 μl of samples between CaF_2 crystal windows with a path length of 6 μm. In order to measure at low peptide concentration comparable to the concentrations employed in far-UV CD, D_2O was used as solvent (Haris and Chapman, 1995). Peptides were dissolved in 0.01N DCl to obtain solutions with concentration of 1 mg/ml with pD 2 (corresponding to a pH of 2.4) (Glasoe and Long, 1960).

For spectra obtained by liquid FTIR, subtraction of vehicle and water vapour was carried out in order to obtain a straight baseline in the region from 2000 to 1700 cm^{-1} according to previously published criteria (Dong et al., 1990). Measurements in D_2O have been reported to give the same determination of secondary structure content as aqueous solution (Barth and Zscherp, 2002; Jackson and Mantsch, 1995). For the solid-state spectra only water vapour had to be subtracted. The second derivative spectra for the peptides were obtained by a nine-point Savitsky-Golay second derivative, and the baseline was corrected with a minimum of points. All spectra were normalised by area according to Kendrick et al. (1996) in the amide I region using the Bgrams software (Galactic Industries Corporation, USA).

2.8. Circular dichroism

Measurements were carried out on a Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd., UK) using a 0.1 mm circular cell and a scanning speed of 20 nm/min. An average of three scans was collected in the far-UV range (260–200 nm) with a bandwidth of 1 nm and the vehicle spectrum was subtracted. Bulk and freeze-dried peptides were reconstituted in 50 mM glycine buffer pH 2.5 with a concentration of 1 mg/ml in order to measure at a concentration level that could also be applied in liquid FTIR using deuterium oxide.

2.9. Thioflavin T assay

The fibrillation of glucagon and glucagon–PEG 5000 was examined using the Thioflavin T assay. Thioflavin T is a probe, which interacts rapidly with amyloid fibrils inducing a strong characteristic fluorescence (LeVine, 1993; Voropai et al., 2003). In the absence of amyloid fibrils the probe fluoresces weakly with excitation maximum at 350 nm and emission maximum at 438 nm. In the presence of amyloid fibrils the fluorescence changes to an excitation maximum at 450 nm and a strong emission maximum at 482 nm. Briefly, the peptides and Thioflavin T were dissolved in glycine buffer pH 2.5 to a yield a peptide concentration of 0.6 mg/ml glucagon and 0.4 μM Thioflavin T. Peptide solutions were transferred immediately to wells with 150 μl solution per well and four replicates. Development of Thioflavin T fluorescence was followed in a NOVOstar plate-reader (BMG Labtech, Germany) at 25 °C with excitation at 450 nm and emission at 480 nm employing linear shaking. The fibrillation process was fitted to a sigmoid curve according to Nielsen et al. (2001) in order to determine lag-time to use for comparison of physical stability.

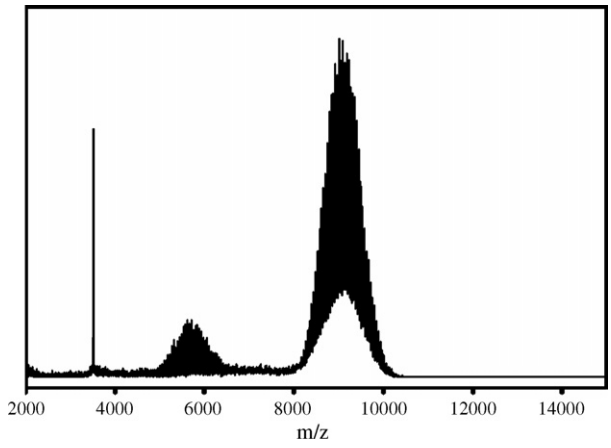

Fig. 2. MALDI-TOF-MS of glucagon–PEG 5000.

3. Results

3.1. Primary structure of glucagon–PEG 5000

The model peptide glucagon was PEGylated using mPEG 5000 SPA, purified by RP-HPLC and freeze-dried. The resultant MALDI-TOF-MS spectrum of glucagon–PEG 5000 (Fig. 2) shows a main distribution of peaks at 9000 *m/z* indicating mono-PEGylation. The peak has a normal distribution, as expected for a polymer, with peak separation of 44 mass units, corresponding to the monomer mass of PEG. Traces of unmodified glucagon and mPEG 5000 are observed in the spectrum even though baseline separation was obtained during RP-HPLC purification. Further analysis of these impurities was carried out by analytical RP-HPLC and the impurities could not be detected (data not shown). The detection limit of RP-HPLC was approximately 5%.

Analysis of digested glucagon–PEG 5000 was performed and the expected fragments from the Asp-N digestion of glucagon are shown in Table 1. The peak for the Lys-12-containing fragment (amino acids 9–14, see Table 1) was absent on analysis by electrospray Q-TOF MS and MALDI-TOF-MS. Thus, one PEG-moiety is attached to the Lys-12 residue.

3.2. Secondary structure of glucagon and glucagon–PEG 5000

The secondary structure of the peptides was analysed by FTIR and far-UV CD in order to study the effect of the PEGylation and the freeze-drying process. It is advantageous to use the two methods in combination since far-UV CD is generally less sensitive than FTIR in detecting β-sheet structure (Jackson and Mantsch, 1995).

Table 1
Expected fragments from Asp-N digest of glucagon with amino acids containing primary amino groups highlighted in bold

Fragment	MH^+	Sequence
Amino acids 1–8	864.38	**His**-Ser-Gln-Gly-Thr-Phe-Thr-Ser
Amino acids 9–14	788.38	Asp-Tyr-Ser-**Lys**-Tyr-Leu
Amino acids 15–20	732.37	Asp-Thr-Asn-Met-Leu-Trp
Amino acids 21–29	1153.53	Asp-Phe-Val-Gln-Ala-Arg-Arg-Ser

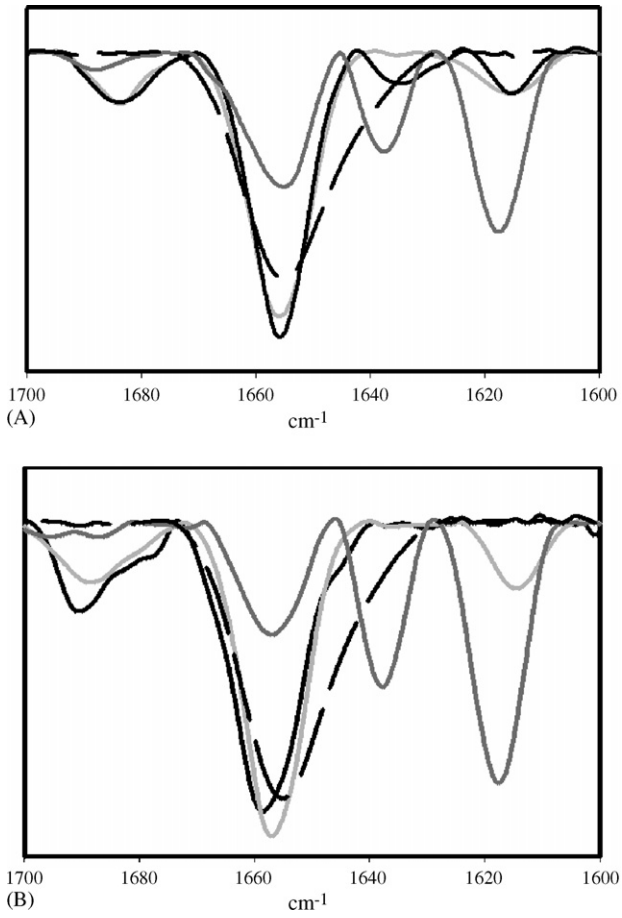

Fig. 3. (A) Solid-state FTIR spectra of peptides freeze-dried from 1 mg/ml. (B) Solid-state FTIR of peptides freeze-dried from 10 mg/ml. Bulk glucagon (black dashed line), freeze-dried glucagon (light grey solid line), processed glucagon (dark grey solid line) and glucagon–PEG 5000 (black solid line).

Solid-state FTIR spectra of the peptides were recorded (Fig. 3). The spectrum of bulk glucagon showed α-helix and the spectrum is shown as a reference, with peak assignments in Table 2.

Changes in secondary structure are seen after freeze-drying glucagon and glucagon–PEG 5000 (both 1 mg/ml), where formation of intermolecular β-sheet is detected. Additional processing causes larger changes with relatively larger content of intermolecular β-sheet. The structural changes are relatively smaller for glucagon–PEG 5000, where a major α-helix peak dominates the spectrum. From peptides samples freeze-dried from a higher concentration (10 mg/ml) only small changes appear for freeze-dried glucagon and glucagon–PEG 5000 and again more pronounced intermolecular β-sheet content is observed for processed glucagon.

Liquid FTIR spectra of reconstituted peptides were obtained at low peptide concentration in D_2O (Table 3 and Fig. 4). The bands were assigned according to Barth and Zscherp (2002). Using these band assignments, bulk glucagon showed structure of α-helix and/or random coil in solution. All freeze-dried peptides showed altered spectra upon reconstitution with a shift in α-helix peak to 1664 cm^{-1}, which can possibly be assigned 3_{10}-helix (Haris and Chapman, 1995). Additionally processed

Table 2
Band assignments[a] in the amide I region of FTIR spectra (Fig. 3) of freeze-dried glucagon, glucagon–PEG 5000, processed glucagon and bulk glucagon obtained in solid-state (1 mg peptide in 300 mg KBr tablet)

Peptide sample	Low frequency β-sheet	Random coil and/or α-helix	3_{10}-Helix	High frequency β-sheet
Solid-state FTIR spectra of peptides freeze-dried from 1 mg/ml				
Bulk glucagon		1655		
Freeze-dried glucagon	1616	1657		1683
Processed glucagons	1618, 1637	1655		1688
Glucagon–PEG 5000	1616, 1635	1657		1684
Solid-state FTIR spectra of peptides freeze-dried from 10 mg/ml				
Bulk glucagon		1655		
Freeze-dried glucagon	1614	1658		1688
Processed glucagon	1618, 1637	1657		1681
Glucagon–PEG 5000		1659		1678 (shoulder), 1690

[a] According to Jackson and Mantsch (1995) and Kennedy et al. (1991).

glucagon freeze-dried from 1 mg/ml contains intermolecular β-sheet structure.

More pronounced changes could be detected after freeze-drying of glucagon from 10 mg/ml compared to 1 mg/ml. Both freeze-dried glucagon and processed glucagon contain intermolecular β-sheet structure at this higher concentration level. This is not the case for glucagon–PEG 5000, where no content of intermolecular β-sheet could be determined in any of the concentration levels applied.

No fibril content was detected by the Thioflavin T assay of any of the reconstituted peptides (data not shown).

Secondary structure was also determined by far-UV CD (Fig. 5). The peptides were dissolved in 50 mM glycine buffer pH 2.5 in a concentration of 1 mg/ml. All spectra display minima at 222 and 203 nm, possibly due to a mixed structure of random coil and α-helix (Onoue et al., 2004) or 3_{10}-helix (Haris and Chapman, 1995). It is not possible to detect neither any β-sheet structure nor any significant changes from the far-UV CD spectra.

The structural changes after purification and freeze-drying were investigated by comparing peptide containing RP-HPLC-fractions and dissolved bulk glucagon using far-UV CD. The peptides eluted in 0.1% TFA and 50% acetonitrile. As seen in Fig. 5C, all peptides have identical α-helix structure as demonstrated by the two minima at 208 and 222 nm. In addition, no fibrils were detected by the Thioflavin T assay (data not shown).

3.3. Physical stability of glucagon and glucagon–PEG 5000

Physical stability of the peptides was assessed by the Thioflavin T assay in order to compare ability to fibrillate. Lag-times for fibrillation of peptides reconstituted in 50 mM glycine buffer pH 2.5 at 0.6 mg/ml are shown in Fig. 6. For bulk glucagon a fibrillation lag-time of 17.1 ± 0.6 h was determined. Glucagon freeze-dried from 1 mg/ml had a significantly longer lag-time (35.2 ± 4.0 h) compared to 11.1 ± 0.1 h when freeze-dried from 10 mg/ml. Processed glucagon showed shorter lag-times of 7.7 ± 0.3 h (freeze-dried from 1 mg/ml) and 7.4 ± 0.2 h (freeze-dried from 10 mg/ml). In the case of glucagon–PEG 5000 it was not possible to detect any fibril formation during the 120 h at either concentration level. Statistical analysis using analysis of variance at 5% level of confidence showed that all fibrillation lag-times were significantly altered compared to the lag-time for bulk glucagon.

4. Discussion

4.1. Primary structure of glucagon–PEG 5000

The selected model peptide glucagon is a 29 amino acid peptide containing two primary amino groups, one in the Lys-12 and one at the N-terminus. Glucagon was modified by conjugation

Table 3
Band assignment[a] in the amide I region of liquid FTIR spectra (Fig. 4) of freeze-dried glucagon, glucagon–PEG 5000, processed glucagon and bulk glucagon obtained in 0.01 N DCl, pD 2 (peptide concentration of 1 mg/ml)

Peptide sample	Low frequency β-sheet	Random coil and/or α-helix	3_{10}-Helix	High frequency β-sheet
Liquid FTIR spectra of peptides freeze-dried from 1 mg/ml				
Bulk glucagon		1650		
Freeze-dried glucagon			1664	
Processed glucagon	1616		1664	
Glucagon–PEG 5000	1637 (broad)		1664	
Liquid FTIR spectra of peptides freeze-dried from 10 mg/ml				
Bulk glucagons		1650		
Freeze-dried glucagons	1616		1664	
Processed glucagons	1620		1664	
Glucagon–PEG 5000	1637 (broad)		1664	

[a] According to Barth and Zscherp (2002).

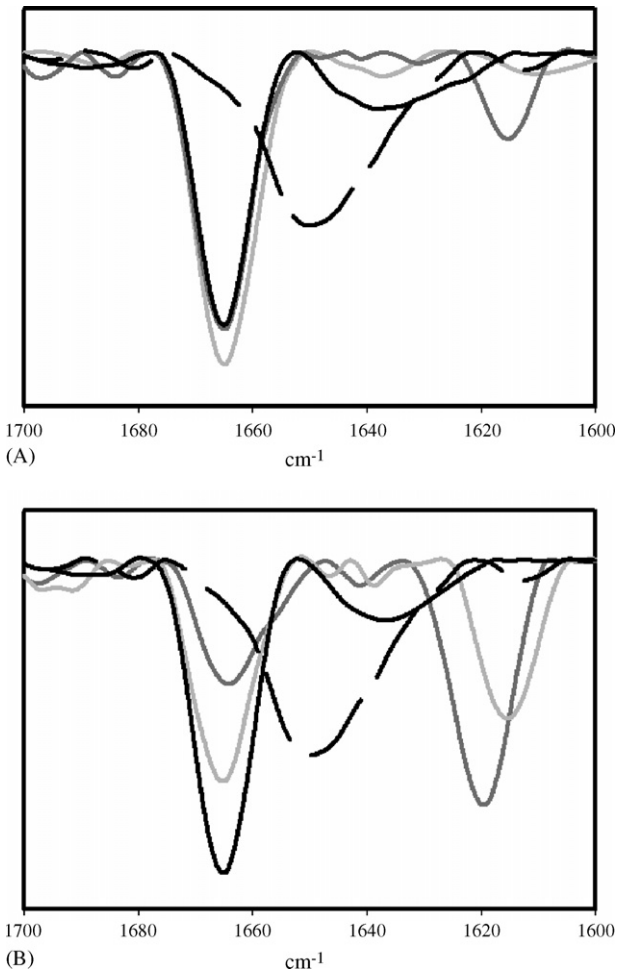

Fig. 4. (A) Liquid FTIR spectra of peptides freeze-dried from 1 mg/ml reconstituted in D_2O, pD 2 with peptide concentration of 1 mg/ml. (B) Liquid FTIR of peptides freeze-dried from 10 mg/ml reconstituted in D_2O, pD 2 with peptide concentration of 1 mg/ml. Bulk glucagon (black dashed line), freeze-dried glucagon (light grey solid line), processed glucagon (dark grey solid line) and glucagon–PEG 5000 (black solid line).

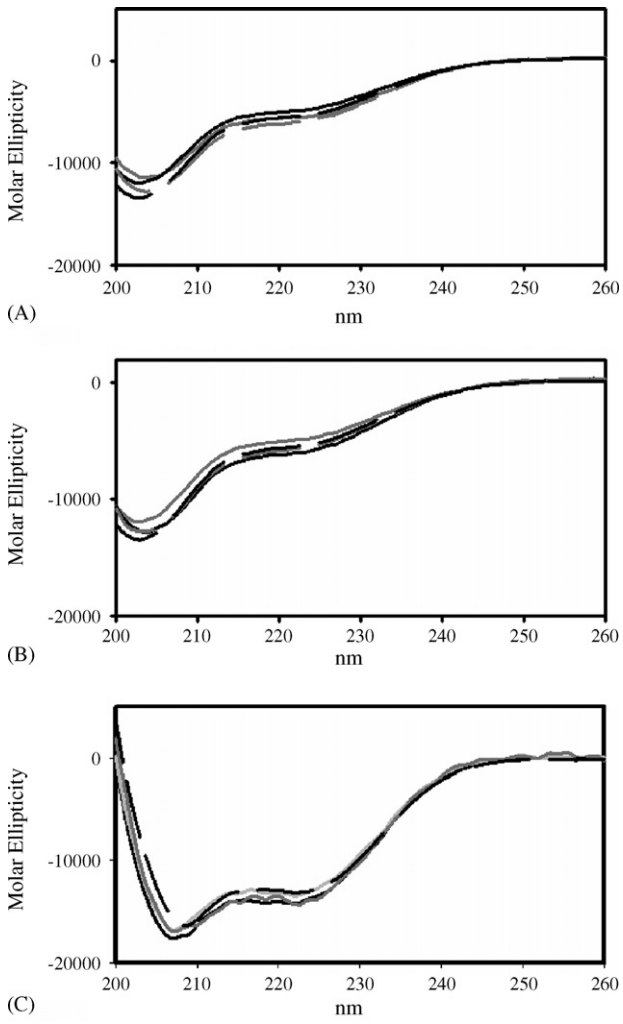

Fig. 5. (A) Far-UV CD spectra of peptides freeze-dried from 1 mg/ml, reconstituted in 50 mM glycine buffer pH 2.5 with peptide concentration of 1 mg/ml. (B) Far-UV CD spectra of peptides freeze-dried from 10 mg/ml, reconstituted in 50 mM glycine buffer pH 2.5 with peptide concentration of 1 mg/ml. (C) Far-UV CD spectra of 1 mg/ml peptides in 0.1% TFA and 50% acetonitrile. Bulk glucagon (black dashed line), freeze-dried glucagon (light grey solid line), processed glucagon (dark grey solid line) and glucagon–PEG 5000 (black solid line).

of mPEG SPA reagent that reacts predominantly with primary amino groups yielding a stable amide bond (Morpurgo and Veronese, 2004). The reaction mixture was purified using RP-HPLC (chromatograms not shown) and the conjugate was analysed by MALDI-TOF-MS to determine the degree of PEGylation.

The PEGylation site was determined to be the Lys-12 residue by peptide digestion and MALDI-TOF-MS, which is a widely used technique (Clark et al., 1996; Kinstler et al., 1996; Lee and Park, 2002; Na et al., 2001). This is in agreement with the expected rank of reactivity, where the ε-amino group (Lys-12) is more reactive than the α-amino group (the N-terminus) for PEGylation with amino groups at pH values above 8 (Morpurgo and Veronese, 2004).

Impurities consisting of glucagon and mPEG 5000 were detected in glucagon–PEG 5000. This could potentially interfere with the results obtained, however, the presence of these impurities are below 5% by RP-HPLC. Possible reasons could be insufficient purification by RP-HPLC, slow degradation of the linkage between glucagon and PEG 5000 prior to MALDI-TOF-MS or cleavage of the bond during MALDI-TOF-MS measurement.

The detection limits for MALDI-TOF-MS are typically in the sub-picomole range and thus the technique is inherently sensitive to low concentrations of impurities (Johnsson, 2001). Additionally it is difficult to interpret MALDI-TOF-MS quantitatively (Veronese, 2001; Veronese and Morpurgo, 1999; Johnsson, 2001). It is unlikely that the peaks are derived from cleavage of the glucagon–PEG 5000 during MALDI-TOF-MS analysis due to the low laser power and the soft nature of the ionisation process used to generate the ions under standard analysis conditions (Johnsson, 2001). Degradation of the stable amide linkage formed between glucagon and mPEG 5000 is not expected to occur.

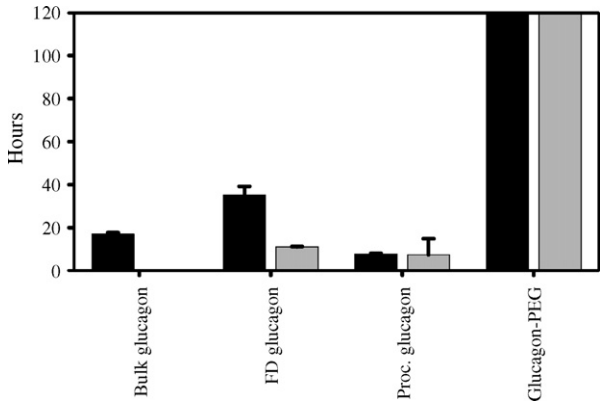

Fig. 6. Lag-times of peptide fibrillation determined by Thioflavin T-assay. Peptide concentration was 0.6 mg/ml in glycine buffer pH 2.5. Black bars are peptides freeze-dried from 1 mg/ml and grey bars peptides freeze-dried from 10 mg/ml. Bars from left are: Bulk glucagon, freeze-dried glucagon (FD glucagon), processed glucagon (Proc. glucagon) and glucagon–PEG 5000 (glucagon–PEG).

It is possible that impurities derive from insufficient purification and is in a quantity below the detection limit of preparative and analytical RP-HPLC, even though glucagon and glucagon–PEG 5000 elute 2 min apart in the RP-HPLC chromatogram with baseline separation. Analytical RP-HPLC had a detection limit of approximately 5% and did not show any detectable amounts of impurities. Such a low amount of impurities is not expected to interfere with the analyses applied in this study.

4.2. Changes in secondary structure

The secondary structure of the glucagon–PEG 5000 was examined and compared to three glucagon samples (Fig. 1). Native glucagon has a very flexible secondary structure, which varies with the peptide concentration and solvent to give three different conformational states (Beaven et al., 1969). In dilute aqueous solution (less than 1 mg/ml) glucagon is in a random coil with reported α-helix content of 10–15% (Wu et al., 1982). Self-association occurs in pH ranges of 2–4.5 and 9–11 at glucagon concentrations above 3 mg/ml, accompanied by increased α-helix content of up to 35%. Under these conditions equilibrium exists between monomers and trimers, and the association is stabilised by hydrophobic interactions and hydrogen bonds (Blundell, 1983). Structural changes can easily occur because of the low physical stability in solution of glucagon, leading to intermolecular β-sheet structure and fibrillation.

The bulk glucagon used in this study was salt-precipitated and has an ordered secondary structure similar to the crystalline structure of glucagon (Blundell, 1983). Freeze-drying of peptides and proteins is known to result in an amorphous structure, which is less ordered and less crystalline-like (Wang, 2000).

Solid-state FTIR revealed changes in secondary structure of the different glucagon samples. These changes were most apparent for freeze-dried and processed glucagon resulting in formation of intermolecular β-sheet. All peptide samples showed some content of α-helix and the proposed 3_{10}-helix structure of the reconstituted peptides is not found in the solid-state. This indicates that this change in conformation occurs upon reconstitution and not during the freeze-drying process.

Liquid FTIR of reconstituted peptides shows a surprising shift in peak from regular α-helix to a peak, which possibly could be assigned to 3_{10}-helix. Content of 3_{10}-helix has been determined in the crystal structure of glucagon comprising of residues 5–11 (Blundell, 1983) and this structural element has previously been reported to yield a sharp peak in 1662–1666 cm^{-1} (Kennedy et al., 1991; Haris and Chapman, 1995). By far-UV CD, it is not possible to detect any differences between the peptides. The far-UV CD spectra showed large similarities to what has previously been reported to be a mixed α-helix and random coil structure for glucagon (Chou and Fasman, 1975; Wu and Yang, 1980; Onoue et al., 2004). However, far-UV CD spectra with identical characteristics were found for 3_{10}-helix structure (Haris and Chapman, 1995; Toniolo et al., 2004). Therefore, using far-UV CD it is difficult to distinguish the two proposed structures. Additionally thermal scans were conducted by far-UV CD and no differences in the unfolding of the peptides could be detected (data not shown).

Conformational changes for freeze-dried and processed glucagon were determined in liquid FTIR by appearance of intermolecular β-sheet. The intermolecular β-sheet content could not be detected by far-UV CD. Therefore, far-UV CD is considered to be an unsuitable method for detection of structural changes for glucagon.

It has been stated that PEGylation typically changes the physicochemical properties of the peptides and proteins compared to the native drug and that the process of PEGylation may induce conformational changes (Bailon and Berthold, 1998). In many studies no changes in the conformation have been observed upon PEGylation (Hinds and Kim, 2002). In this study the effect of processing by RP-HPCL and freeze-drying after PEGylation was assessed. For glucagon–PEG 5000 the conformational changes after purification and freeze-drying were less apparent than for glucagon samples by FTIR. Formation of intermolecular β-sheet could be detected in relatively smaller amounts, but no changes between the concentration levels could be detected. Therefore, PEGylation protects against formation of intermolecular β-sheet during purification and freeze-drying. Many investigations only include determination by CD, which is less sensitive to the β-sheet components of peptides (Barth and Zscherp, 2002). It is generally recommended that FTIR and CD should be used as complementary tools to reveal more information about the secondary structure (Jackson and Mantsch, 1995; Barth and Zscherp, 2002).

4.3. Physical stability

The Thioflavin T assay is used widely to examine the presence and the kinetics of amyloid fibrils (LeVine, 1993). It has not yet been determined exactly how Thioflavin T interacts with amyloid fibrils, but the characteristic change in fluorescence is not induced by native β-sheet structure (LeVine, 1993). The Thioflavin T assay has previously been applied to detect amyloid fibril formation in glucagon (Onoue et al., 2004). To our knowledge, no report has been published so far that shows the

detection of fibrillation of PEGylated peptides or proteins by Thioflavin T. However, it has previously been shown by the less sensitive method of size exclusion chromatography that PEGylation of proteins decreases the fibrillation rate (Hinds and Kim, 2002; Kim et al., 2002).

The results from the Thioflavin T assay are consistent with results from liquid FTIR. Intermolecular β-sheet in liquid FTIR spectra is observed in the same glucagon samples that show a decrease in fibrillation lag-times (processed glucagon from 1 to 10 mg/ml and freeze-dried glucagon from 10 mg/ml). Furthermore, no intermolecular β-sheet formation was detected in reconstituted glucagon–PEG 5000 and no fibrillation was observed in the Thioflavin T assay. This indicates a correlation between FTIR spectral changes and the Thioflavin T assay.

The fibrillation lag-time for glucagon freeze-dried from 1 mg/ml was extended compared to bulk glucagon. It seems surprising that exposing glucagon to physical stress of freeze-drying yields longer lag-times before fibrillation. This could be caused by two options: Firstly, the structural rearrangement for the proposed 3_{10}-helix formation could increase the physical stability of glucagon. Secondly, the presence of TFA may affect the fibrillation rate. Addition of 0.1% TFA in the Thioflavin T assay yielded an increase in fibrillation lag-times by a factor of 2 for bulk glucagon (data not shown). It has previously been described that using TFA as counter ion can affect fibrillation, both increasing and decreasing lag-times (Nilsson and Raleigh, 1999). If all TFA remained after freeze-drying this would result in concentration of 0.06%. It is, however, unlikely that trace amounts of TFA could be the explanation of longer lag-times of glucagon freeze-dried from 1 mg/ml, since TFA is volatile and will be removed during freeze-drying. Therefore, it is likely that the longer lag-time is caused by structural rearrangement prior to fibrillation.

In the Thioflavin T assay applied in this study it was not possible to detect any fibrillation of glucagon–PEG 5000, hereby showing the physically stabilising effect of PEGylation. This decreased ability to fibrillate could be due to steric hindrance by the conjugated polymer, which lowers the peptide-peptide interaction. Thioflavin T assay of glucagon–PEG 2000 (also mono-PEGylated at the Lys-12 residue) showed that a smaller PEG was less effective in reducing the fibrillation rate (data not shown).

4.4. Effect of peptide concentration and processing

The different glucagon samples were included in order to determine the effect of the stress induced by purification and freeze-drying, which glucagon–PEG 5000 underwent. Both RP-HPLC purification and freeze-drying are commonly used methods in the development and formulation of peptide and protein drugs that can cause conformational changes. Upon PEGylation, glucagon–PEG 5000 showed relatively small changes in secondary structure compared to glucagon samples, indicating to an increased robustness during purification and freeze-drying.

Freeze-dried and processed glucagon samples showed changes in secondary structure, particularly by formation of intermolecular β-sheet. To determine whether these structural changes occurred during purification, far-UV CD spectra were acquired of RP-HPLC fractions before freeze-drying. All spectra exhibited α-helix structure, and consequently purification did not cause any detectable structural differences between glucagon and glucagon–PEG 5000. The mobile phase consisted of 0.1% TFA and 50% acetonitrile at the time of elution of the peptides, and the high content of α-helix in the RP-HPLC fractions was likely solvent-induced.

Acetonitrile is one of the widest used solvents in RP-HPLC for the purification and analysis of peptides and protein. Acetonitrile has been shown to induce α-helix in glucagon (Hearn and Zhao, 1999). This shows that glucagon can adopt α-helical structures when an organic solvent is added to an aqueous solution. However, the introduction of α-helical structure during freeze-drying did not protect the conformation of glucagon samples during freeze-drying.

The effect of freeze-drying from two concentration levels was also investigated. For glucagon samples the starting concentration had an effect on conformational changes and fibrillation lag-times. Processed and freeze-dried glucagon from 10 mg/ml displayed shorter lag-times in the Thioflavin T assay and showed a higher content of intermolecular β-sheet in liquid FTIR spectra. In contrast, freeze-dried glucagon from 1 mg/ml showed the slowest fibrillation of all glucagon samples in the Thioflavin T assay combined with no detection of intermolecular β-sheet by liquid FTIR.

For glucagon–PEG 5000 no differences between the concentration levels could be distinguished, thus peptide concentration had no detectable effect on conformation.

Freeze-drying of a peptide or protein solution induces stresses that can induce conformational changes as observed for glucagon in this study. However, it can be beneficial to employ freeze-drying on peptides or proteins in order to ensure sufficient long-term storage. Results in this study show that by PEGylating an unstable peptide the robustness of the peptide is increased and harsher pharmaceutical methods can be employed in the formulation.

5. Conclusions

In the present study the model peptide glucagon was PEGylated with PEG 5000 in order to investigate the effect of PEGylation on physical stability in different processing methods. Glucagon–PEG 5000 showed significant resistance towards fibrillation compared to glucagon by Thioflavin T assay. Glucagon and glucagon–PEG 5000 underwent chromatographic purification and freeze-drying, which caused formation of intermolecular β-sheet for glucagon after reconstitution, combined with a decrease in fibrillation lag-time. Glucagon–PEG 5000 did not form intermolecular β-sheet and fibrillation could not be detected in the Thioflavin T assay. This shows an improvement in physical stability after PEGylation of glucagon and shows the increased robustness in pharmaceutical processes after PEGylation.

A correlation was found between detection of intermolecular β-sheet of reconstituted peptides and decreased physical

stability by Thioflavin T assay. After freeze-drying and reconstitution both glucagon and glucagon–PEG 5000 showed a shift from α-helix to 3_{10}-helix structure.

This increased robustness after PEGylation is advantageous if an unstable peptide should be formulated by methods such as freeze-drying.

Acknowledgements

The authors would like to acknowledge Anne Merete Svendsen (Novo Nordisk A/S, Denmark) for help with identification of PEGylation site and Erik Frederiksen and Lene Kragelund (both Novo Nordisk A/S, Denmark) for help with freeze-drying. Apotekerforeningen af 1991 is acknowledged for financially supporting the Bomem Infrared Spectrometer. This work was partly financed by grants from Novo Nordisk A/S, Denmark (Pernille Stigsnaes) and the Alfred Benzon Foundation (Marco van de Weert).

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