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Biophysical characterisation of GlycoPEGylated recombinant human factor VIIa

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ABSTRACT

The effects of GlycoPEGylation on the structural, kinetic and thermal stability of recombinant human FVIIa were investigated using rFVIIa and linear 10 kDa and branched 40 kDa GlycoPEGylated® recombinant human FVIIa derivatives. The secondary and tertiary structure of rFVIIa measured by circular dichroism (CD) was maintained upon PEGylation. In contrast, the thermal and kinetic stability of rFVIIa was affected by GlycoPEGylation, as the apparent unfolding temperature T_m measured by differential scanning calorimetry (DSC) and the temperature of aggregation, T_{agg}, measured by light scattering (LS) both increased with GlycoPEGylation. Both T_m and T_{agg} were independent of the molecular weight and the shape of the PEG chain. From the present biophysical characterisation it is concluded that after GlycoPEGylation, rFVIIa appears to be unaffected structurally (secondary and tertiary structure), slightly stabilised thermally (unfolding temperature) and stabilised kinetically (temperature of aggregation).

1. Introduction

Coagulation factor VIIa (FVIIa) is a trypsin-like serine protease, which in the presence of calcium initiates the blood coagulation when associated with its cofactor tissue factor (TF) which is exposed upon vascular injury (Davie et al., 1991; Wildgoose et al., 1993). TF-bound FVIIa activates factor IX and factor X resulting in a burst of thrombin, fibrin deposition and the formation of a haemostatic plug on the surface of activated platelets (Davie et al., 1991). Deficiencies in the coagulation system due to partial or complete deficiency of FVIII or FIX, haemophilia A or haemophilia B, respectively, can lead to severe morbidity or mortality if the bleeding is left untreated.

A safe and efficient way to prevent bleeds and joint destruction in haemophilia is prophylactically by dosing factor (F) FVIII or FIX 2–4 times weekly (Manco-Johnson et al., 2007), and recent studies have shown that prophylactic treatment with rFVIIa (NovoSeven®) of haemophilia patients with inhibitors reduces the frequency of bleedings significantly as compared to conventional on-demand haemostatic therapy (Konkle et al., 2007). However rFVIIa has a short circulation time (2–4 h in humans), and it is assumed that rFVIIa should be administered daily if used for long-term prevention (Konkle et al., 2007; Sen et al., 2010). Hence, development of rFVIIa derivatives with longer circulation time could result in both fewer administrations and better patience compliance. Modification of pharmaceutical proteins with hydrophilic polymers such as poly-ethylene-glycol (PEGylation) is an established method for prolonging circulatory half-life of proteins, reducing self-aggregation, increase water solubility and increase stability (Pasut et al., 2004; Veronese et al., 2009). PEGylation has been used successfully in several marketed proteins including Pegasys® (40 kDa PEG interferon alfa-2a, Roche), Oncaspar® (5 kDa PEG-L-asparaginase, Enzon), Cimzia® (40 kDa PEG anti-TNFα, UCB Pharma) and Neulasta® (20 kDa PEG G-CSF, AMGEN) (Bailon et al., 2001; Marshall et al., 2003; Fishburn, 2008). Due to the risk of losing activity of FVIIa because of the numerous interactions with the cell surface, TF, FIX and FX there is a limitation in the unspecific chemical modification of this protein. For this reason a novel strategy for site-directed PEGylation using glycosyltransferases to attach PEG to glycan residues, the enzyme based GlycoPEGylation™ technology, is used to covalently attach either a linear 10 kDa or a branched 40 kDa PEG polymer to rFVIIa. The site of PEG attachment to rFVIIa is demonstrated to be one of the two N-linked glycans of rFVIIa (Asn145 or Asn322) located on the light chain and heavy chain, respectively, (Stennicke et al., 2008).

The most important property of a PEGylated protein is the increased molecular size resulting form the large hydrodynamic volume of the PEG (Zalipsky, 1995). The underlying mechanisms for the effect of PEG is not fully understood, but it is evident that the hydrodynamic radius is significantly increased by PEG leading

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to reduced renal clearance, especially of smaller proteins (Harris et al., 2001; Marshall et al., 2003). Most of the benefits of PEGylated proteins reflect the properties of the PEG polymer itself (Zalipsky and Harris, 1997). The PEG polymer is heavily hydrated and consequently it has a large excluded volume which among other things inhibits the approach of another molecule, this could in theory result in reduced immunogenicity and decreased antibody recognition (Veronese and Morpurgo, 1999; Veronese et al., 2009). The favourable properties as postponed or prevented aggregation and thermal stabilisation are caused by the heavily hydrated PEG polymer, and the properties of the PEG polymer are transferred to the PEGylated compounds (Morar et al., 2006). Favourable pharmacokinetic and pharmacodynamic profiles are also a consequence of the improved hydrodynamic properties (Veronese et al., 2009). Other than increasing the hydrodynamic volume of the protein upon PEGylation, the conformation, physical properties and electrostatics of a PEG-conjugated protein may be altered compared with the unmodified protein (Morar et al., 2006), and the protein's biological activity is not necessarily preserved (Hinds and Kim, 2002; Harris and Chess, 2003; Veronese and Pasut, 2005). However, most studies report an unchanged secondary and tertiary structure (Hinds and Kim, 2002; Digilio et al., 2003; Meng et al., 2008; Nielsen and Rischel, 2009; Palm et al., 2009), an increased thermal stability (Dhalluin et al., 2005) and an increased temperature of aggregation (Hinds and Kim, 2002; Nielsen and Rischel, 2009) of the protein upon PEGylation. Studies regarding the stability, both thermal and structural, as well as the bioactivity of the protein are among the most fundamental when developing pharmaceutical proteins and the ideal pharmaceutical protein should have a long shelf-life and a high bioavailability. Also, the physical stability of a pharmaceutical protein has a significant impact on the ability to design a suitable liquid protein drug, as insoluble aggregates can increase immunogenic responses and are not biologically active. We study the effect of GlycoPEGylation on the thermal, kinetic and structural stability of rFVIIa with one linear 10 kDa and one branched 40 kDa PEG polymer. This provides us with a relevant pharmaceutical model system for investigating the biophysical effects of PEGylation. Biophysical characterisation tools, including circular dichroism (CD), differential scanning calorimetry (DSC) and light scattering (LS), were used to study possible changes in the secondary and tertiary structure as well as the thermal and kinetic stability of rFVIIa upon GlycoPEGylation. To the best of our knowledge no study has yet been published on a highly relevant pharmaceutical protein which combines investigations of the effect of GlycoPEGylation on the structural, thermal and kinetic stability using both linear and branched PEG polymers. This study contributes to the further understanding of the basic biophysical properties of PEGylated pharmaceutical proteins.

2. Materials and methods

2.1. Materials

rFVIIa, 10 kDa GlycoPEGylated® rFVIIa and 40 kDa GlycoPEGylated® rFVIIa were produced by Novo Nordisk A/S as described in Stennicke et al. (2008). L-Histidine was purchased from Aijonomoto AminoScience (Raleigh, N.C.), calciumchloride-dihydrate from Merck (Germany), barium chloride 20 (w/w) from Ampliqon (Denmark), 0.1 N iodine solution from Sigma–Aldrich (Germany), 70% perchloric acid from Merck (Germany), LDS sample buffer and MES SDS running buffer and Simple Blue Safe Stain all from Invitrogen (Carlsbad, CA).

All protein solutions were dialysed in Slide-A-Lyzer™ 30,000 MWCO dialyse cassettes against 10 mM HIS, 10 mM $CaCl_2$, pH 5.75.

2.2. SDS-PAGE

SDS-PAGE analysis was carried out using a 12% Bis–Tris gel from Invitrogen. The gels were loaded with an average of 5 μg per well and run at 120 mA constant current. The running buffer was MES running buffer. The gel was washed in 150 mL 0.1 M perchloric acid for 15 min until 40 mL 5% barium chloride solution and 15 mL 0.1 M iodine solution were added to detect protein bands containing PEG compounds, as described in Kurfurst (1992). After discolouring in water, the gel was coloured with Coomassie blue.

2.3. MALDI-TOF MS

Mass spectrometric analysis was performed on a Bruker Daltonics Microflex MALDI-TOF (Billerica, MA) instrument equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction, and the accelerating voltage in the ion source was 25 kV. Sample preparation was done as follows: 1 μL sample-solution was mixed with 10 μL matrix-solution (alphacyano-cinnamic acid dissolved in a 5:4:1 mixture of acetonitrile:water:3% TFA) and 1 μL of this mixture was deposited on the sample plate and allowed to dry before insertion into the mass spectrometer. Calibration was performed using external standards (a range of standard proteins) and the resulting accuracy of the mass determinations is within 0.1%.

2.4. Circular dichroism (CD) spectroscopy

Spectra of rFVIIa in the far-UV region (200–260 nm) were recorded on a Jasco J-810 CD Spectropolarimeter (Mölndal, Sweden). The light path of the cuvette was 0.05 mm and a protein concentration of 2.4 mg/mL was used. The spectra were recorded at room temperature. Each spectrum is an average of 5 scans. A value of 114 g/mol was used as a mean residue weight for rFVIIa. Spectra of rFVIIa in the near-UV region (250–350 nm) were recorded on the same Spectropolarimeter. The light path of the cuvette was 10 mm and a protein concentration of 2.4 mg/mL was used. The spectra were recorded at room temperature. Spectra at selected temperatures in the range 10–80 °C were obtained in a 2 mm cuvette. Each spectrum is an average of 5 scans. All spectra were background-corrected, smoothed and transformed into molar ellipticity (θ cm^2 $dmol^{-1}$).

2.5. Differential scanning calorimetry (DSC)

DSC experiments were performed with a MicroCal VP-DSC (Northhampton, MA). Prior to scanning, all solutions were degassed by stirring under vacuum. A pressure of 2 atm was applied over the cells during scanning, and a scan rate of 1 °C/min was used. The concentration of rFVII was 2–2.4 mg/mL and buffer scans were subtracted from rFVIIa scans. DSC data was analysed using the Origin Software from MicroCal Inc., supplied with the instrument. A baseline was subtracted prior to analysis. The apparent denaturation temperatures (T_m) values were determined as the temperature corresponding to the maximum heat capacity (C_p).

2.6. Light scattering (LS)

The LS experiments were performed with a Wyatt DynaPro Titan (Santa Barbara, CA) which employs a 829 nm laser and collects scattering intensity data at a fixed angle of 90 °C. Cuvette temperature is controlled using a thermoelectric solid-state heating module (Peltier heat pump). Solutions are examined in a quartz cuvette with 12 μL cell volume containing glass viewing windows for in situ scattering measurements. The concentration of rFVIIa

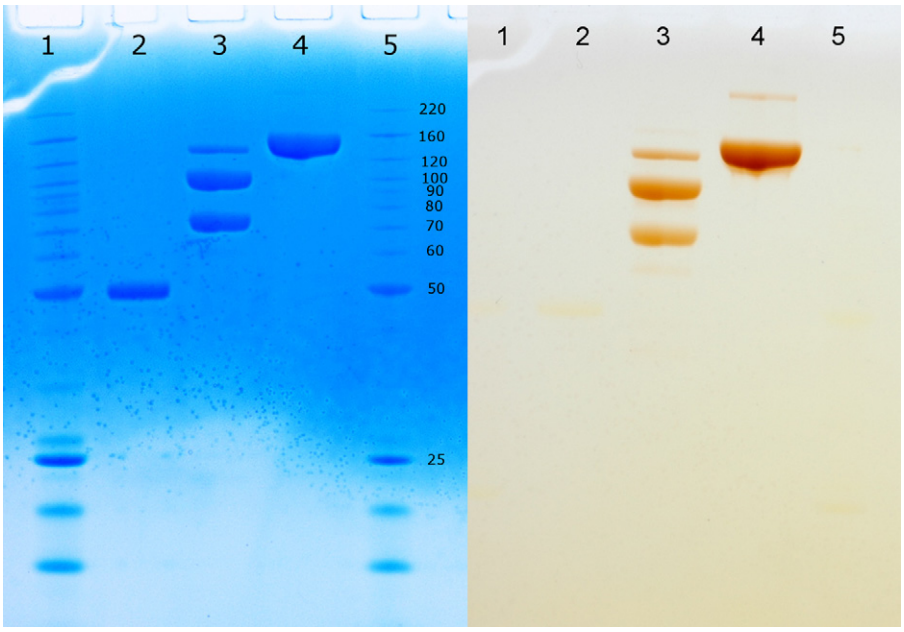

Fig. 1. The Coomassie blue stained SDS-PAGE gel (left panel). The PEG-stained SDS-PAGE gel (right panel). Lanes 1 and 5: molecular weight standard, lane 2: rFVIIa, lane 3: 10 kDa PEG-rFVIIa, lane 4: 40 kDa PEG-rFVIIa. The gel is stained with iodine (right panel), discoloured in water and subsequently stained with Coomassie blue (left panel).

was 1–2 mg/mL. 200 μL. Samples were centrifuged at 10,000 rpm for 10 min prior to analysis.

3. Results

3.1. Purity

After dialysis the purity of the protein solutions were determined by SDS-PAGE and by MALDI-TOF MS. SDS-PAGE has often been used to characterise the distribution of PEGylated proteins (Kunitani et al., 1991). It is assumed that the higher molecular weight gel band ladders represented the mono- or di-GlycoPEGylated rFVIIa compounds.

The PEG-staining gel technique is based on the complex formation between PEG and barium iodide, and the PEG-stained gel (Fig. 1 right panel) reveals that only lane 3 and lane 4 contain PEG-compounds. It has previously been shown, that PEG binds to SDS micelles (Odom et al., 1997) and that PEGylation decreases the mobility of the protein (Kurfurst, 1992; Tong et al., 2010). For this reason the molecular weight of a PEGylated protein on a SDS gel will not correspond to the actual molecular weight of the PEGylated protein. Due to the low mobility of the PEGylated protein, the location of a PEGylated protein on a SDS gel corresponds to the molecular weight of the protein added approximately 2.5 times the molecular weight of the attached PEG polymer. Comparing the results in the PEG-stained gel with the very same gel, only now Coomassie blue stained, it is evident, that the three bands in lane 3 (10 kDa PEG-rFVIIa) and the two bands in lane 4 (40 kDa PEG-rFVIIa) are GlycoPEGylated compounds of rFVIIa. From the results on the SDS-PAGE gel (Fig. 1) it is shown that the rFVIIa sample was pure and contained only rFVIIa with a molecular mass of 50 kDa. The third lane reveals that 10 kDa PEG-rFVIIa is not only mono-GlycoPEGylated. There are 3 different GlycoPEGylated species in this protein solution. These compounds have apparent molecular weights of 75 kDa, 110 kDa and 125 kDa, respectively. This corresponds to mono-GlycoPEGylated 10 kDa PEG-rFVIIa (50 kDa + 2.5 × 10 kDa) ~75 kDa, di-GlycoPEGylated 10 kDa PEG-rFVIIa (50 kDa + 2.5 × 2 × 10 kDa) ~110 kDa and tri-GlycoPEGylated rFVIIa (50 kDa + 2.5 × 3 × 10 kDa) ~125 kDa. The fourth lane contains mainly mono-GlycoPEGylated 40 kDa PEG-rFVIIa with a molecular weight of (50 kDa + 2.5 × 40 kDa) ~160 kDa but also a small amount of di-GlycoPEGylated 40 kDa PEGylated rFVIIa at (50 kDa + 2.5 × 2 × 40 kDa) ~250 kDa.

The results of the MALDI-TOF MS spectra of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa are shown in Fig. 2.

The MS spectra show that the rFVIIa solution only contains a 50 kDa protein, and the peak pattern corresponds to different charges (16,606 Da peak corresponds to triple charged rFVIIa, 24,787 corresponds to double charged rFVIIa and 49,433 corresponds to single charged). The 10 kDa PEG-rFVIIa solution contains a mono-GlycoPEGylated, 60 kDa, and smaller amounts of a di-GlycoPEGylated compound, at 70 kDa, of 10 kDa PEG-rFVIIa. The peak at 36,400 corresponds to double charged di-GlycoPEGylated rFVIIa, and the 30,529 and 20,389 are double and triple charged mono-GlycoPEGylated rFVIIa, respectively. The 40 kDa PEG-rFVIIa solution contains mono-GlycoPEGylated 40 kDa PEG-rFVIIa, which is reflected at the 92 kDa peak. Peaks at 46 and 31 kDa are assigned to double and triple charged mono-GlycoPEGylated 40 kDa PEG-rFVIIa. The tri-GlycoPEGylated 10 kDa rFVIIa compound and the di-GlycoPEGylated 40 kDa rFVIIa compound are not detectable with MS.

3.2. Structural stability

The secondary structure of rFVIIa and PEGylated rFVIIa is investigated by far-UV CD in order to study the effect of GlycoPEGylation. The far-UV CD scan of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa are practically inseparable, however there is a small deviation in the far-UV signal at very low wavelength between the 10 kDa PEG-rFVIIa and the two other rFVIIa compounds. The near-UV CD scans show no change in the tertiary structure of rFVIIa upon GlycoPEGylation. The near-UV CD scans show two characteristic peaks around 286 nm and 292 nm most likely corresponding to signals from the tryptophan residues (Fig. 3).

3.3. Thermal, kinetic and structural stability

The thermal stability is investigated by three different complementing techniques: DSC, LS and CD. From the CD scans it is evident

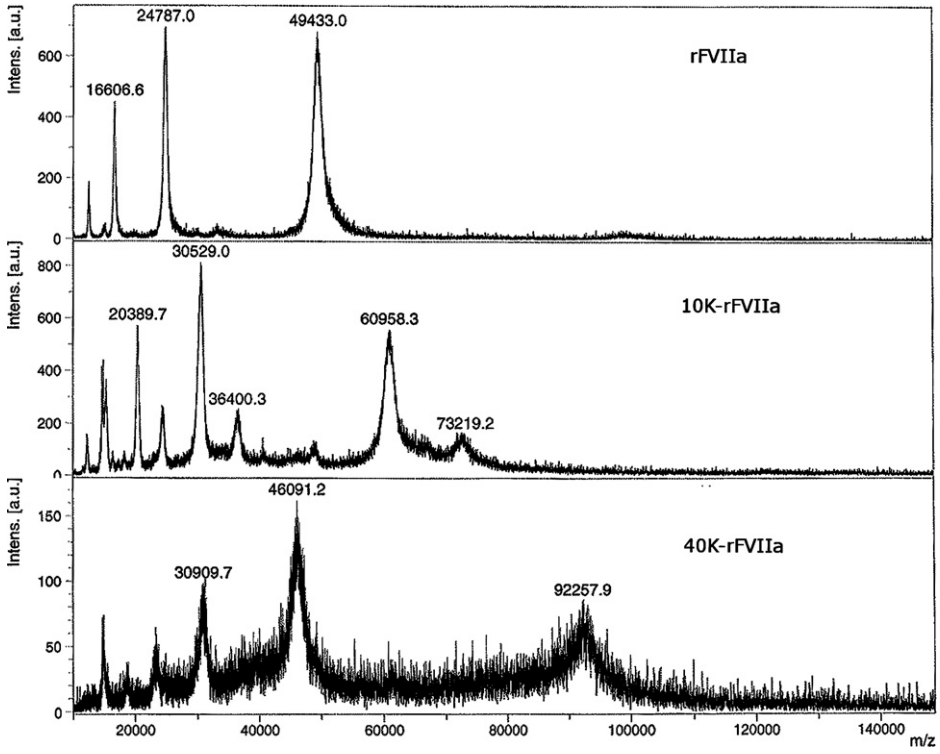

Fig. 2. MALDI-TOF spectra of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa, see text for further details.

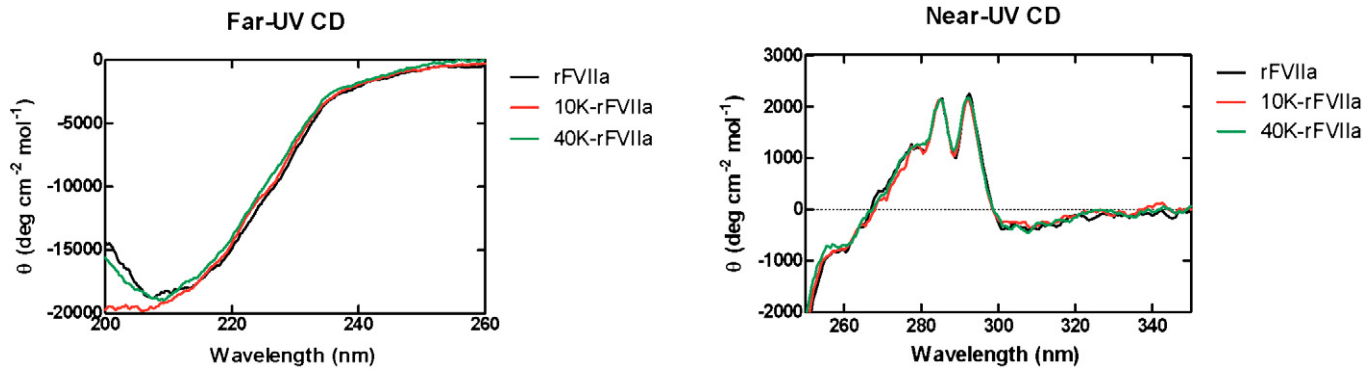

Fig. 3. Far-UV CD scan and near-UV CD scan of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa. The far-UV CD scans show only little variation between the spectrum of 10 kDa PEG-rFVIIa and the two spectrums of rFVIIa and 40 kDa PEG-rFVIIa, and the near-UV CD scans show that GlycoPEGylation has no notable effect on the tertiary structure (near-UV CD) of rFVIIa.

that the GlycoPEGylated rFVIIa compounds maintain some residual tertiary structure at high temperatures (>70 °C) (Fig. 4). The appearance of the near-UV CD heating scan is different from the unmodified protein to the two GlycoPEGylated protein compounds. It is evident that the tertiary structure of the GlycoPEGylated rFVIIa molecules is less affected by the increase in temperature than the unmodified protein.

We chose to follow one characteristic wavelength, 286 nm, throughout the CD heating scan (Fig. 5). This wavelength was chosen as the CD signal at this specific wavelength changes significantly upon heating, as illustrated in Fig. 4, and corresponds to the first of the two characteristic peaks.

The overall CD signal at 286 nm is different for the unmodified protein compared to the GlycoPEGylated protein compounds. The low temperature range CD signal for all the three compounds is around 2000° cm^{-2} mol^{-1} and decreases slowly until 50 °C where the CD signal decreases significantly for all the three compounds to around −1000° cm^{-2} mol^{-1}. For rFVIIa there is a drop in the CD signal around T_m, whereupon the signal increases and stabilises around 0° cm^{-2} mol^{-1}. The GlycoPEGylated rFVIIa compounds, on the other hand, demonstrate a more continuous course. The CD signals were differentiated with respect to the temperature in order to obtain the transition midpoint (T_m) value, which is defined as the point where the tangent has the largest slope, see Table 1.

The DSC thermograms and the LS intensity data are plotted in the same figure (Fig. 6) to illustrate the difference between the thermal and kinetic changes. Three different characteristic temperatures are found in Fig. 6. The onset temperature, T_{onset}, corresponding to the temperature where the unfolding of the unmodified rFVIIa or GlycoPEGylated rFVIIa initiates. The apparent unfolding temperature, T_m, where theoretically half of the unmodified rFVIIa or GlycoPEGylated rFVIIa molecules are folded, the other half unfolded, and the temperature of aggregation, T_{agg}, the aggregation onset temperature. There is a clear difference between the appearance of the DSC scan of the unmodified rFVIIa and the Gly-

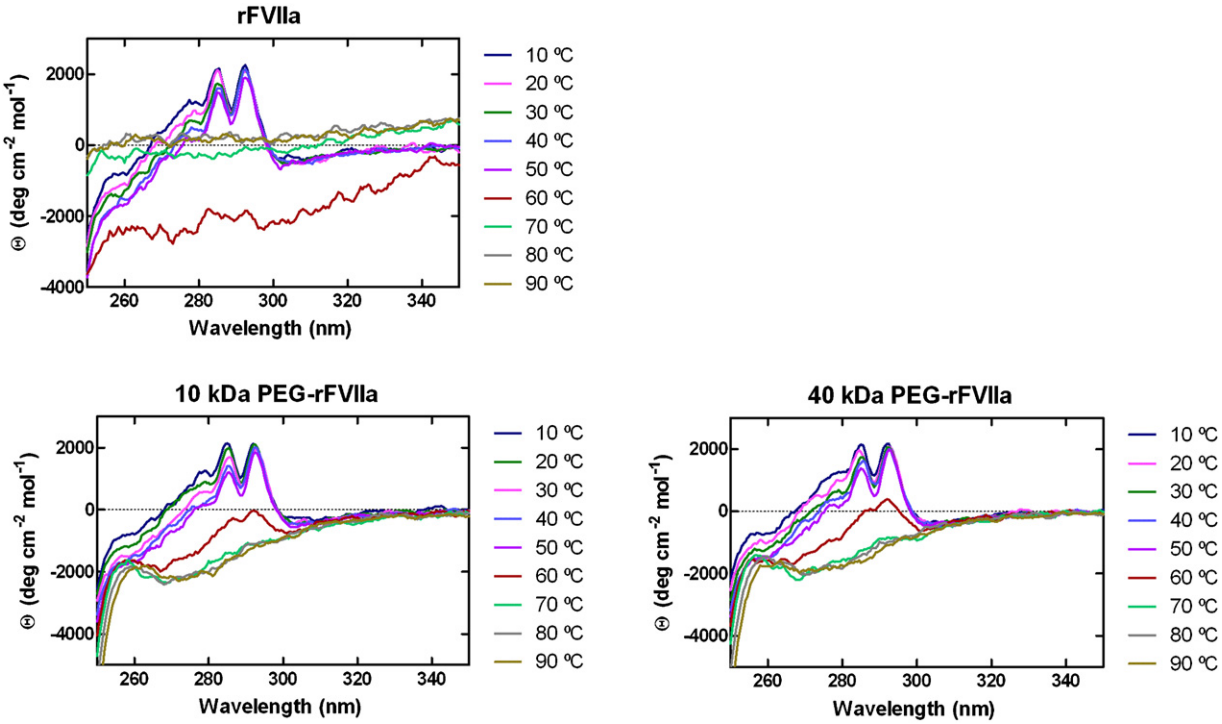

Fig. 4. Near-UV CD at different temperatures (see legends) of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa. The spectra show that the unfolding process differs from the non-modified protein to the GlycoPEGylated proteins. The GlycoPEGylated proteins maintain some residual tertiary structure at 70 °C and above, whereas CD signal from the unmodified protein is showing no tertiary structural characteristics at these temperatures. The native rFVIIa solution was milk-white upon heating whereas the solutions of the GlycoPEGylated rFVIIa compounds were transparent.

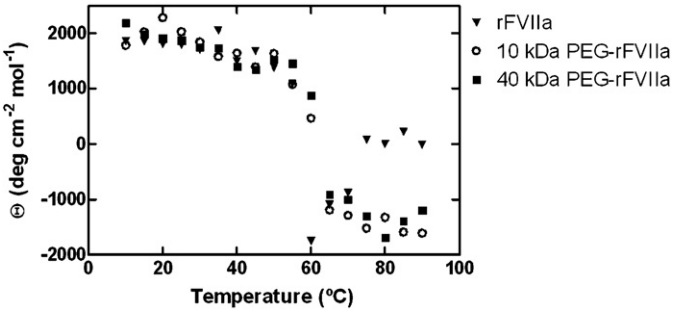

Fig. 5. Near-UV CD signals of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa versus temperature at 286 nm. Triangles, circles and squares are chosen in favour of solid lines to clearly illustrate the differences around 60 °C, and the error bars are hidden under the symbols. From the results represented in this figure it is clear that the temperature dependent CD signal at 286 nm is different from the unmodified protein to the GlycoPEGylated proteins. The two GlycoPEGylated proteins are alike.

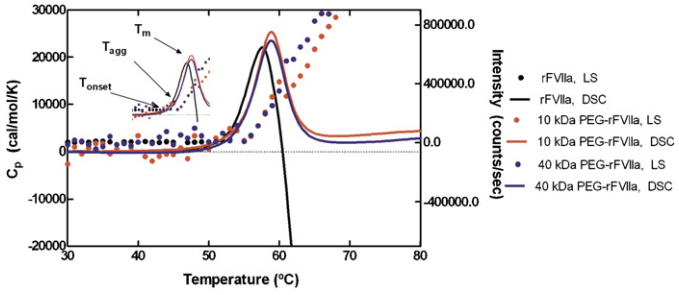

Fig. 6. DSC thermograms (primary axis) and SLS intensity measurements (secondary axis) of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa as a function of temperature. The dotted line corresponds to the LS intensity (the error bars are hidden under the symbols), the solid line corresponds to the DSC thermogram. The insert illustrates where the onset temperature, T_{onset}, the temperature of aggregation, T_{agg}, and the apparent unfolding temperature, T_m, are found. The rFVIIa aggregates formed close to T_{agg} were too large for the LS detector to measure, and continuous intensity measurements as a function of temperature were impossible after 53 °C for rFVIIa.

coPEGylated rFVIIa compounds as the former is showing signs of aggregation identified by a sharp drop in C_p and large, immeasurably aggregates in the LS experiment. The aggregation of the two GlycoPEGylated compounds appears to happen at a slower rate, detectable by LS.

The DSC thermograms reveal that GlycoPEGylation increases the apparent unfolding temperature, T_m. The temperature of aggregation, T_{agg} is found by differentiating the intensity of the LS signal with respect to the temperature. Linear regression was performed

Table 1
The unfolding temperature measured by CD, the onset temperature of the peaks in the DSC heating scan, the apparent unfolding temperature, T_m, measured by DSC, the aggregation temperature, T_{agg}, measured by LS and the difference between T_{onset} and T_{agg}. The insert in Fig. 6 illustrates where T_{onset}, T_{agg} and T_m are found. The extraction of the enthalpy of unfolding of rFVIIa is not possible due to the irreversibility of the denaturation process.

	T_m °C[a] (CD)	T_{onset} °C (DSC)	T_m °C (DSC)	ΔH (T_m) (kJ/mol)	T_{agg} °C (LS)	$T_{agg} - T_{onset}$
rFVIIa	60.3 ± 0.2	50.9 ± 0.2	57.7 ± 0.2	NA	50.8 ± 0.4	−0.1
10 kDa PEG-rFVIIa	59.9 ± 0.2	53.3 ± 0.1	59.0 ± 0.2	140	57.1 ± 0.2	3.8
40 kDa PEG-rFVIIa	60.7 ± 0.2	52.9 ± 0.1	59.0 ± 0.2	134	57.9 ± 0.9	5

[a] Based on the CD scans in Fig. 5. The uncertainties are calculated based on the resolution of the original CD scan.

on this last part of the curve, where the intensity increases due to formation of aggregates. The calculated intercept with the *x*-axis, where the scattering intensity is zero, is named T_{agg}, temperature of aggregation in the following. The LS measurements demonstrate that the aggregation temperature, T_{agg}, increases with GlycoPEGylation (Table 1).

4. Discussion

4.1. Secondary and tertiary structure

The structural information obtained with far-UV and near UV-CD scans show that both the secondary and the tertiary structure of rFVIIa are maintained upon GlycoPEGylation and the structures do not change with PEG chain length nor with the PEG conformation (linear or branched). Minor differences in the CD scans are observed around 210–200 nm for the 10 kDa GlycoPEGylated rFVIIa compound compared to the 40 kDa GlycoPEGylated rFVIIa compound and the unmodified rFVIIa, the origin of this small difference is not known but it could reflect changes in β-turns (Kelly et al., 2005).

4.2. Structural, kinetic and thermal stability

The DSC, LS and CD heat scans were all performed at the same scan rate, which makes it possible to compare the kinetics. The three different measurement types are complementary and they illustrate each different aspects of the unfolding or aggregation processes. The unfolding and aggregation pathway can be described by a model put forward by Lumry and Eyring (1954). According to the Lumry Eyring model, irreversible protein denaturation involves at least two steps. The first step is the reversible unfolding of the native protein (N), characterised by the rate constants, k_1 and k_2. This step is followed by an irreversible change of the unfolded protein (D), into an inactivated, irreversible aggregate (A), characterised by the rate constant k_3. The unfolded state, D, is characterised by having some tertiary structure.

$$N \underset{k_2}{\overset{k_1}{\rightleftarrows}} D \xrightarrow{k_3} A \quad (1)$$

From our experimental results it is suggested that the covalent attachment of a PEG polymer to rFVIIa influences at least two of the three rate constants mentioned above. The aggregation of the protein depends on the unfolding of the protein to occur first. The aggregation is measured by LS; the unfolding is measured by CD, whereas DSC measures unfolding, an endothermic event, as well as aggregation, which here is an exothermic event. There is an equilibrium between the native (N) and the unfolded (D) state, which shifts towards unfolding as the temperature increases. This process is followed by the irreversible step from unfolding (D) to aggregation (A), which is also promoted with increasing temperature.

From the results shown in Fig. 6 it is evident that the apparent unfolding temperature, T_m, of the GlycoPEGylated rFVIIa compounds is slightly higher than that of rFVIIa. This is in accordance with previous studies, which have reported that PEGylation can increase the apparent unfolding temperature, T_m, compared to the unmodified protein (Harris et al., 2001; Dhalluin et al., 2005). For rFVIIa it seems as though a longer, and branched, PEG chain does not contribute more to the thermal stability than a shorter, linear PEG chain. Taking a closer look at the DSC thermograms and the LS intensities for the GlycoPEGylated rFVIIa compounds (Fig. 6) it is evident that GlycoPEGylation delays the thermally induced aggregation of rFVIIa, and that the thermally induced aggregation event begins while endothermic processes dominates. The formation of aggregates will contribute exothermally to the total heat signal. As the heat measured by DSC is the total contribution from both unfolding and aggregation, an extensive formation of aggregates beginning shortly after T_{onset} may shift the location of the peak in the thermogram towards a lower temperature. Thus GlycoPEGylation may not directly stabilise rFVIIa thermally, but could affect the value of T_m indirectly by postponing the formation of the thermally induced aggregates. The hypothesis that GlycoPEGylation prevents or postpones the aggregation of rFVIIa is supported by the results of the CD heating scans. The changes in the CD signal at 286 nm in the heating scan (Fig. 5) indicate that the non-modified rFVIIa undergo a conformational change in the tertiary structure around the apparent unfolding temperature and no tertiary structure can be detected at higher temperature. As the physical appearance of the rFVIIa solution upon heating was milk-white, the non-detectable tertiary structure above 70 °C could be a sign of aggregation and precipitation. The GlycoPEGylated rFVIIa compounds, on the other hand, do maintain some non-native residual tertiary structure upon unfolding, even at 90 °C, as the CD signal stabilises around 1500° cm^{-2} mol^{-1} (Fig. 5). These observations support the general conception that PEGylation may not necessarily prevent the protein from unfolding (Dhalluin et al., 2005; Nielsen and Rischel, 2009), but it hinders protein–protein interactions, which can lead to aggregation and precipitating.

Nielsen and Rischel (2009) have investigated the thermal stability of rFVIIa and 40 kDa PEG-rFVIIa with DSC and high resolution ultrasonic spectroscopy (HR-US) (Nielsen and Rischel, 2009). On the basis of the DSC measurements they conclude, that the denaturation of rFVIIa is irreversible. In contrast they find that the unfolding of 40 kDa PEG-rFVIIa is characterised by being partly reversible, as approximately 60% reversibility is observed after heating to 60 °C. Based on HR-US experiments they report that the post-denaturation aggregation process is delayed upon GlycoPEGylation. In addition it is suggested, that the GlycoPEGylated protein demonstrates a less pronounced aggregation. All these conclusions are supported by the results obtained in this study.

Based on the results obtained in this study it seems reasonable to assume that the equilibrium constant, k_1/k_2, reflecting the equilibrium between the native and the unfolded protein, is independent of GlycoPEGylation, since unfolding process for rFVIIa seems to be similar, whether the protein is GlycoPEGylated or not. The rate constant k_3, on the other hand, describing the step from unfolding to aggregation, decreases when the protein is GlycoPEGylated. The thermally induced rFVIIa aggregates formed close to T_{agg} were too large for the LS detector to measure, and continuous intensity measurements as a function of temperature were impossible after 53 °C for rFVIIa. As the light scattering increases with the sizes of the formed aggregates (Bloomfield, 2000), a high intensity count most likely corresponds to large aggregates. The increase in the LS intensity is much slower for the GlycoPEGylated rFVIIa compounds, thus the formation of aggregates is reduced and happens at a lower rate. The fact that PEGylating a protein postpones the aggregation has also been reported previously (Hinds and Kim, 2002; Rajan et al., 2006; Nielsen and Rischel, 2009). By comparing the difference between the onset of unfolding, T_{onset}, with the onset of aggregation, T_{agg}, (Fig. 6 and Table 1), it is evident that ($T_{agg} - T_{onset}$) is of the same order of magnitude for both for 10 kDa PEG-rFVIIa and 40 kDa-PEG-rFVIIa. Given that both GlycoPEGylated compounds postpone the aggregation behaviour of rFVIIa, but not the thermally induced unfolding, indicates that PEGylating a protein may affect the aggregation properties of a protein but not through thermal stabilisation of the protein. This is in agreement with previously reported studies on insulin (Hinds and Kim, 2002).

It is noticeable that the PEG molecular weight and shape has no effect on the thermal, the structural or the kinetic stability of rFVIIa. All of the results obtained in this study indicate that attaching a PEG moiety of similar size as rFVIIa appears to have the same effect as attaching a smaller 10 kDa PEG to rFVIIa. From a pharmaceutical

formulation point of view these results indicate that the decision of what shape and size PEG to use for GlycoPEGylation of rFVIIa depends on other factors than those currently investigated. Instead, the size and shape PEG should be chosen as that which gives the best biological effect.

Favourable properties, such as postponed or prevented aggregation and thermal stabilisation are caused by the heavily hydrated PEG polymer, and the properties of the PEG polymer are transferred to the GlycoPEGylated compounds. In particular, the postponed and decreased aggregation is crucial in protein drugs as this increases the stability of the protein.

5. Conclusion

Recombinant human factor VIIa was mono-GlycoPEGylated with two different PEG polymers, a 10 kDa linear PEG and a 40 kDa branched PEG. The CD measurements suggest that the secondary and tertiary structure of rFVIIa is maintained upon GlycoPEGylation. The DSC and LS measurements suggests that GlycoPEGylation delays the thermally induced aggregation, and slightly increases the unfolding temperature of the protein (increase from $57.7 \pm 0.2\,^{\circ}C$ to $59.0 \pm 0.2\,^{\circ}C$), both of which are independent of the molecular weight and size of the attached PEG polymer.

From the biophysical characterisation of rFVIIa and GlycoPEGylated rFVIIa in the present study it is concluded, that rFVIIa after GlycoPEGylation appears to be unaffected structurally (secondary and tertiary structure), slightly stabilised thermally (unfolding temperature), and stabilised kinetically (temperature of aggregation).

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