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Biophysical characterisation of GlycoPEGylated recombinant human factor VIIa

Bitten Plesner[a,b], Peter Westh[a], Anders D. Nielsen[b,*]

[a] Department of Science, Roskilde University, P.O. Box 260, Universitetsvej 1, DK-4000 Roskilde, Denmark
[b] Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Maaloev, Denmark

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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

* Corresponding author. Tel.: +45 30 79 65 75; fax: +45 44 43 40 73.
E-mail address: ADNi@novonordisk.com (A.D. Nielsen).

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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