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The molar hydrodynamic volume changes of factor VIIa due to GlycoPEGylation

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

[a] Department of Chemistry-NSM, 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 molar hydrodynamic volume of recombinant human rFVIIa were investigated using rFVIIa and two GlycoPEGylated recombinant human FVIIa derivatives, a linear 10 kDa PEG and a branched 40 kDa PEG, respectively. Molar hydrodynamic volumes were determined by capillary viscometry and mass spectrometry. The intrinsic viscosities of rFVIIa, its two GlycoPEGylated compounds, and of linear 8 kDa, 10 kDa, 20 kDa and branched 40 kDa PEG polymers were determined. The measured intrinsic viscosity of rFVIIa is 6.0 mL/g, while the intrinsic viscosities of 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa are 29.5 mL/g and 79.0 mL/g, respectively. The intrinsic viscosities of the linear PEG polymers are 20, 22.6 and 41.4 mL/g for 8, 10, and 20 kDa, respectively, and 61.1 mL/g for the branched 40 kDa PEG. From the results of the intrinsic viscosity and MALDI–TOF measurements it is evident, that the molar hydrodynamic volume of the conjugated protein is not just an addition of the molar hydrodynamic volume of the PEG and the protein. The molar hydrodynamic volume of the GlycoPEGylated protein is larger than the volume of its composites. These results suggest that both the linear and the branched PEG are not wrapped around the surface of rFVIIa but are chains that are significantly stretched out when attached to the protein.

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 [1,2]. 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 [1]. 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 with the use of prophylaxis giving FVIII or FIX 2-4 times weekly [3]. Recent studies have shown that prophylactic treatment with recombinant FVIIa (rFVIIa, NovoSeven®) of haemophilia patients with inhibitors reduces the frequency of bleedings significantly as compared to conventional on-demand haemostatic therapy [4]. However, based on the half-life and circulation time of rFVIIa (2–4 h), it is assumed that rFVIIa should be administered daily if used for long-term prevention [4,5]. Hence, development of rFVIIa derivatives with longer circulation time could result in both fewer administrations and better patient compliance. Modification of pharmaceutical proteins with hydrophilic polymers such as poly-ethylene-glycol (PEGylation) is an established method for prolonging circulatory half-life, reducing self-aggregation, increase water solubility and increase stability [6,7]. 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 [8]. It is assumed that the specific location of the GlycoPEGylation site and presence of small amounts of di-GlycoPEGylated species are irrelevant in relation to the overall interpretation of the results obtained in this study.

Most of the benefits of PEGylated proteins reflect the properties of the PEG polymer itself [9] and the hydration of the PEG chain determines the overall hydrodynamic properties of PEG bioconjugates [10,11]. The underlying mechanisms for the effect of PEG are not fully understood, but it is evident that the

Abbreviations: PEG, poly(ethylene glycol); rFVIIa, recombinant human coagulation factor FVIIa.

* 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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hydrodynamic radius is significantly increased by PEG leading to reduced clearance, especially of smaller proteins [12].

When developing a pharmaceutical protein solution intended for injection, the viscosity of the solution plays a key role. The final viscosity of an aqueous protein solution can be estimated from knowledge of the intrinsic viscosity or the molar hydrodynamic volume of the protein in question together with the concentration of protein in solution. Investigations regarding possible changes in the molar hydrodynamic volume of rFVIIa upon GlycoPEGylation are of high relevance in the drug development process. The present work examines the change in molar hydrodynamic volume of rFVIIa upon GlycoPEGylation by measuring the intrinsic viscosity and the molar mass of rFVIIa, PEG and GlycoPEGylated rFVIIa.

2. Materials and methods

2.1. Materials

rFVIIa, 10 kDa GlycoPEGylated rFVIIa and 40 kDa GlycoPEGylated rFVIIa were produced by Novo Nordisk A/S, Denmark, as described in [8]. L-Histidine was purchased from Aijonomoto AminoScience (Raleigh, NC), and calcium chloride-dihydrate was purchased from Merck (Germany). Barium chloride 20% (w/w) solution 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 are all from Invitrogen (Carlsbad, CA). All protein solutions were dialysed in Slide-A-Lyzer™ 30,000 MWCO dialyse cassettes against 10 mM HIS, 100 mM $CaCl_2$ solvents adjusted to pH 5.75. The PEG-rFVIIa solutions were subsequently concentrated in Amicon cells with 30,000 MWCO, while rFVIIa was concentrated and buffer exchanged with a Tangential Flow Filtration system using a 0.1 m^2 30,000 MWCO membrane. Linear polyethyleneglycol 8 kDa (7–9 kDa) was purchased from Amresco (Solon, OH), polyethyleneglycol 10 kDa (9–11.25 kDa) and 20 kDa (16 kDa–25 kDa) were purchased from Merck (Germany), branched polyethyleneglycol 40 kDa (GL2-400CA) was purchased from NOF Corporation (Japan).

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 per gel. The running buffer was MES running buffer. The gel was washed in 150 mL 0.1 M perchloric acid for 15 min whereupon 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 [13]. After removal of excess colouring 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. Capillary viscometry

The viscosity measurements were performed with a Micro-Ubbelohde Schott 53b 10/l capillary viscometer. The flow times varied from 90 s to 280 s, and the reproducibility was ±0.2 s for the pure PEG solutions and ±1 s for the rFVIIa and GlycoPEGylated rFVIIa solutions. The dimensions of this viscometer are such that the kinetic correction can be neglected [14]. Viscosity measurements were carried out at 25.0 ± 0.1 °C in a constant temperature water bath. The viscosities, η, of the various solutions were determined from the flow times of the solvent (t_s) and of the solutions (t) for low concentrations of the high PEG MW solutions, and higher concentration for short PEG MW solutions. The time was measured with a quartz stopwatch. 5 different concentrations of each solute were measured, and each flow time measurement was repeated three times. The concentrations of the GlycoPEGylated rFVIIa species are all based on the total weight concentration of the complex (protein and PEG).

3. Results

After dialysis the purities of the proteins were determined by SDS–PAGE and by MALDI–TOF MS.

3.1. SDS–PAGE

The apparent molar mass of GlycoPEGylated proteins was assessed by SDS–PAGE [15]. It is assumed that the higher molecular weight gel bands represent one (or more) molar incremental addition of PEG to the protein.

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 lanes 3 and 4 contain PEG-compounds. It has previously been shown, that PEG binds to SDS micelles [16] and that PEGylation decreases the mobility of the protein [13,17]. For this reason the apparent 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 plus 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 left panel) it is seen that the rFVIIa sample was pure (>95%) 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 very small amount of di-GlycoPEGylated 40 kDa GlycoPEGylated rFVIIa at (50 kDa + 2.5 × 2 × 40 kDa) ~250 kDa.

3.2. MALDI–TOF

The MALDI–TOF MS spectra of rFVIIa, 10 rFVIIa and 40 kDa GlycoPEGylated rFVIIa are shown in Fig. 2.

The MALDI–TOF MS spectrum of rFVIIa shows one dominating peak, at 49.4 kDa, equivalent to the expected sequence

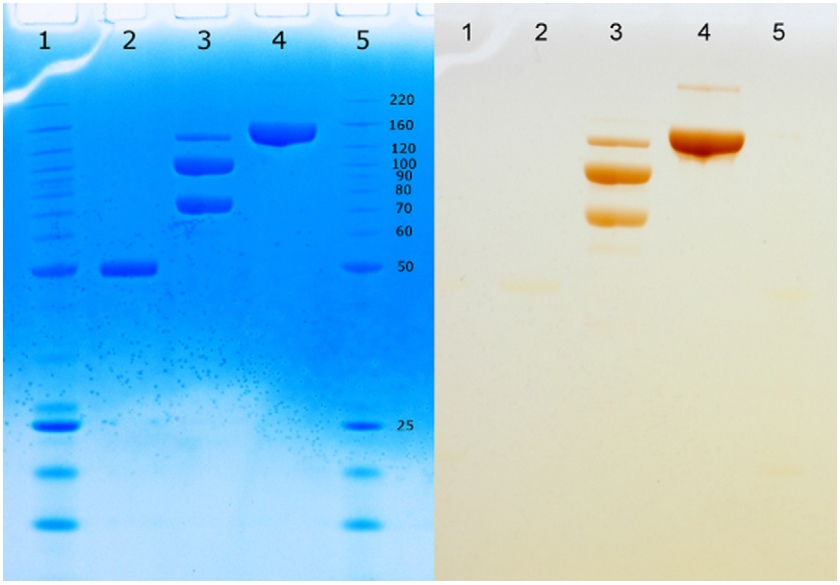

Fig. 1. Lane 1 and 5, bench mark protein ladder. Lane 2: rFVIIa. Lane 3: 10 kDa PEG-rFVIIa. Lane 4: 40 kDa PEG-rFVIIa. The gel is stained with iodine (right panel), discoloured in water and subsequently stained with Coomassie blue (left panel).

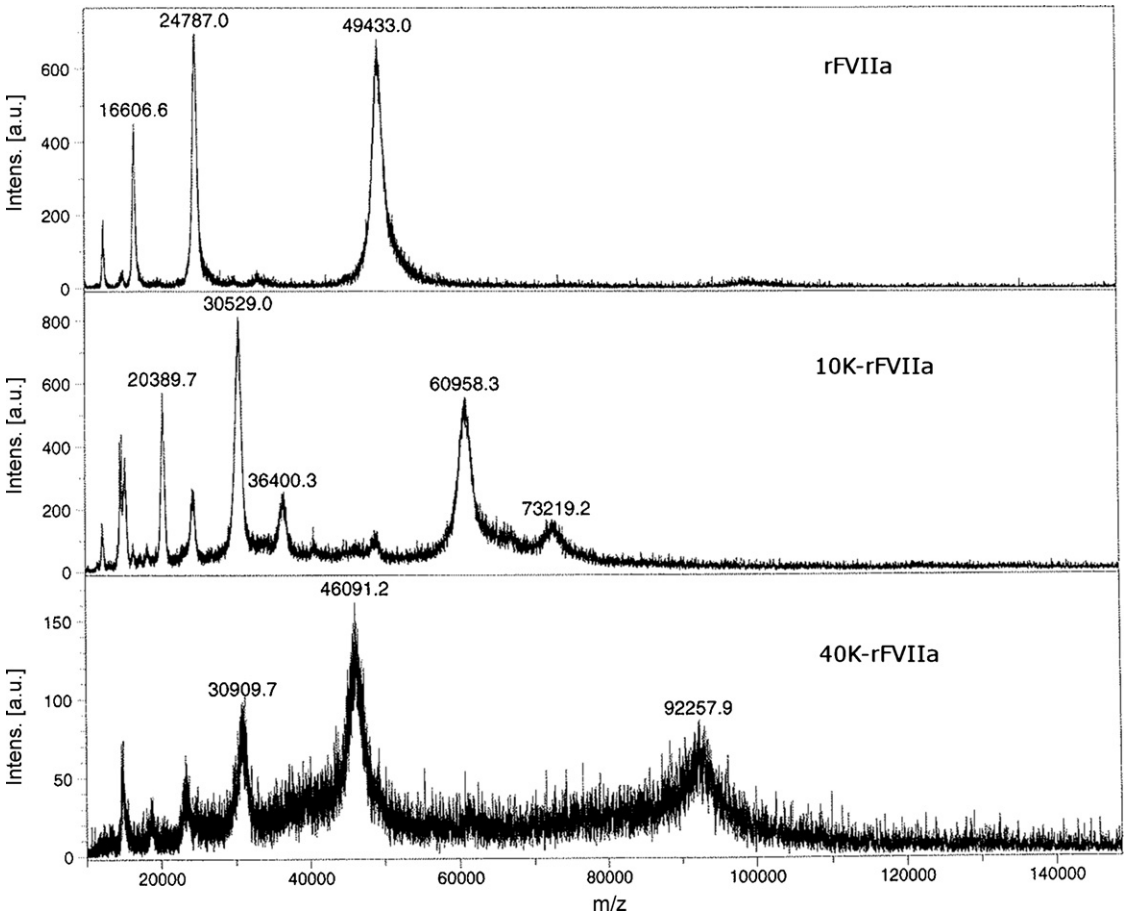

Fig. 2. MALDI–TOF spectra of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa, see text for further details.

molecular mass of rFVIIa [18]. Its peak pattern corresponds to different charges, e.g. 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 monoPEGylated, 61 kDa, and smaller amounts of a di-GlycoPEGylated compound, at 73 kDa. 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 seen as the 92 kDa

Table 1
The molecular weight, the intrinsic viscosity and the corresponding molar hydrodynamic volume of pure PEG (linear and branched), rFVIIa and GlycoPEGylated rFVIIa. The molecular mass multiplied with the intrinsic viscosity gives the molar hydrodynamic volume.

Sample	MW (kDa)	$[\eta]$ (mL/g)	V_H (L/mol)
8 kDa linear PEG	8.9	19.8 ± 0.15	176 ± 1
10 kDa linear PEG	11.0	22.8 ± 0.8	250 ± 8
20 kDa linear PEG	23.1	41.5 ± 0.5	959 ± 12
40 kDa branched PEG	43.1	61.1 ± 1.9	2633 ± 82
rFVIIa	49.4	6.0 ± 0.3	396 ± 15
10 kDa PEG-rFVIIa	61.0	29.5 ± 0.9	1800 ± 55
40 kDa PEG-rFVIIa	92.3	79.0 ± 2.2	7292 ± 203

peak. Peaks at 46 and 31 kDa are assigned to double and triple charged mono-GlycoPEGylated 40 kDa PEG-rFVIIa.

The MS spectrum of 10 kDa PEG-rFVIIa shows two peaks, one at 61 kDa, corresponding to a mono-GlycoPEGylated 10 kDa PEG-rFVIIa, and a smaller peak at 73 kDa corresponding to a di-GlycoPEGylated 10 kDa PEG-rFVIIa. The MS spectrum of 40 kDa PEG-rFVIIa illustrates that only mono-GlycoPEGylated 40 kDa rFVIIa can be detected. The amount of di-GlycoPEGylated 40 kDa PEG-rFVIIa is too small to be detected by MS.

Measured MALDI–TOF peak molar masses of pure PEG samples are summarised in Table 1 (spectra not shown).

3.3. Intrinsic viscosity

The intrinsic viscosity of samples of pure PEG, rFVIIa and GlycoPEGylated rFVIIa compounds was determined by use of an Ubbelohde Capillary viscometer. Values of the intrinsic viscosity, $[\eta]$, were found by plotting the measured reduced viscosities either according to the Huggins method [19]:

$$\frac{\eta_{sp}}{c} = [\eta] + k_1[\eta]^2 c \quad (1)$$

or by plotting the inherent viscosities according to the Kraemer method [20]:

$$\frac{\ln \eta_r}{c} = [\eta] + k_2[\eta]^2 c \quad (2)$$

where $\eta_{sp} = (\eta - \eta_s)/\eta_s$, $\eta_r = \eta/\eta_s$, η is the viscosity of the solution, and η_s is the viscosity of the solvent, respectively. The Huggins and Kraemer constants are k_1 and k_2, respectively. Kraemer and Huggins plots were made from the measured solution viscosities at different concentrations, and the common intercept is the intrinsic viscosity $[\eta]$. The results obtained for rFVIIa and 40 kDa GlycoPEGylated rFVIIa solutions are seen in Fig. 3.

The intrinsic viscosities were determined for rFVIIa, 10 and 40 kDa GlycoPEGylated rFVIIa and the pure PEG samples of similar

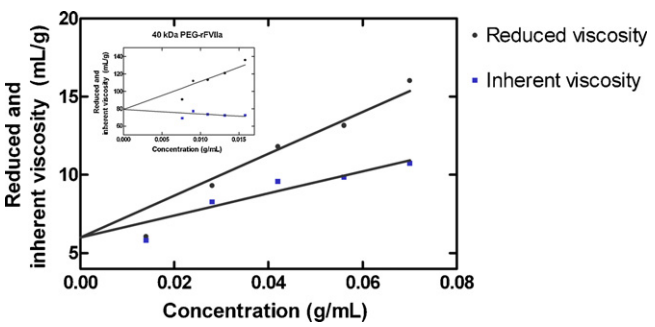

Fig. 3. The measured viscosity is plotted as a function of concentration of rFVIIa. The insert shows the viscosities of 40 kDa PEGylated rFVIIa. Kraemer and Huggins plot were made from the measured solution viscosities at different concentrations, and the common intercept is the intrinsic viscosity. Note the different y-axis ranges.

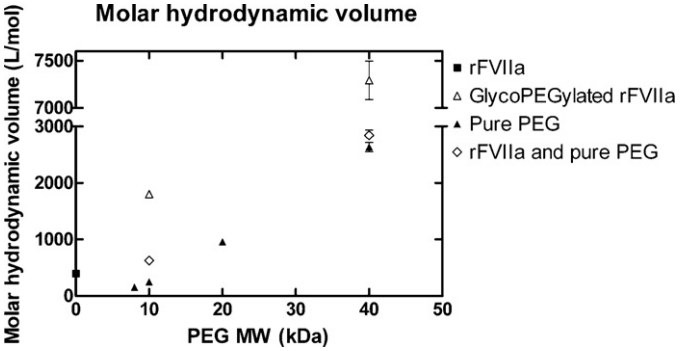

Fig. 4. The molar hydrodynamic volume of rFVIIa, the two GlycoPEGylated rFVIIa compounds and pure PEG polymers are plotted as a function of the PEG molar mass. The native protein is placed at PEG molar mass zero. The diamonds are the total molar hydrodynamic volume of rFVIIa and pure PEG. The error bars are generally so small that they are hidden behind the symbols.

molecular weight; see Table 1 and Fig. 4. The intrinsic viscosities, $[\eta]$, were found by the common intercepts from the Kraemer and Huggins plot and are an average of two intercepts. The deviations in the determination of the molar hydrodynamic volume are based on the deviations in the determination of the intrinsic viscosity, which is the effective volume in solution per mass of solute. The molar mass of a solute multiplied by its intrinsic viscosity equals the effective molar hydrodynamic volume of the solute independently of shape or flexibility of the solute.

From the results in Table 1 it is evident, that the molar hydrodynamic volume (V_H) of the conjugated protein is not just an addition of the molar hydrodynamic volume of the PEG polymer and the protein. In Fig. 4 the molar hydrodynamic volumes of rFVIIa, the two GlycoPEGylated rFVIIa compounds and pure PEG are plotted as a function of PEG molecular weight. In this plot non-modified rFVIIa thus has zero molecular weight.

The molar hydrodynamic volumes, V_H, plotted in Fig. 4 illustrate that V_H of the GlycoPEGylated rFVIIa is larger than the total V_H of its composites. The molar hydrodynamic volume of the protein–PEG complex is approximately 2.5 times larger than the sum of its two components.

4. Discussion

From the intrinsic viscosity and the molar mass measured by MALDI–TOF, the molar hydrodynamic volume was calculated. The molar hydrodynamic volume of rFVIIa increases markedly with GlycoPEGylation, and the molar hydrodynamic volume of 40 kDa PEG-rFVIIa is larger than that of 10 kDa PEG-rFVIIa. When dissolving a PEG polymer or a protein in an aqueous solution, the molecule occupies a certain hydrodynamic volume determined by the molecular weight and the intrinsic viscosity of the molecule. By covalently attaching a PEG moiety to the protein the molar hydrodynamic volume of the protein–PEG complex will increase unavoidably. Our intrinsic viscosity and MALDI–TOF data indicate that the molar hydrodynamic volume is not just an addition of the molar hydrodynamic volume of PEG and protein, respectively. This observation is in accordance with studies done on PEGylated haemoglobin [21]. From Table 1 it is evident that the V_H of both 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa is more than 2.5 times larger than the total V_H of rFVIIa and linear 10 kDa PEG or branched 40 kDa, respectively.

One of the basic characteristics of the PEG polymer is its ability to bind two to three water molecules per ethylene glycol unit [22]. These properties are transferred to the protein–PEG complex upon PEGylation, leading to a molecule with an effective molar hydrodynamic volume that increases up to 2–3 times in this study. We suggest that this significant increase in molar hydrodynamic

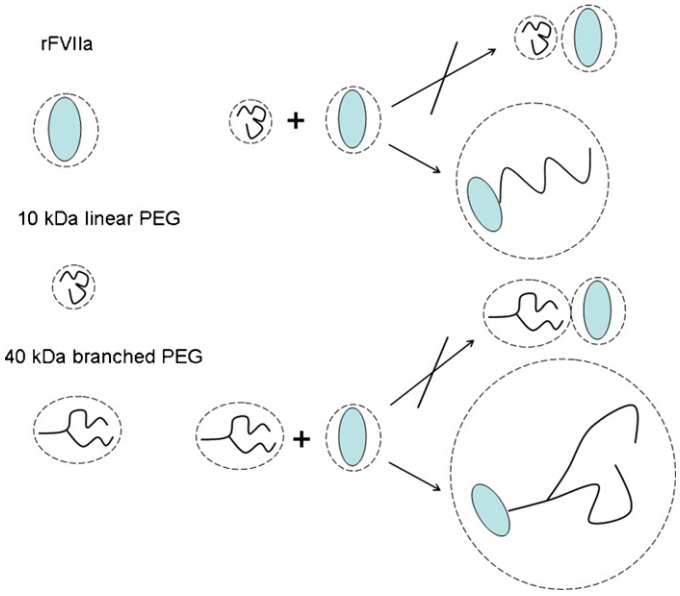

Fig. 5. The molar hydrodynamic volume of rFVIIa, pure PEG and GlycoPEGylated rFVIIa. The molar hydrodynamic volumes of the conjugated protein indicate that the PEG polymer is not wrapped around the protein, on the contrary it appears to be stretched out from the protein surface.

volume indicates that the grafted PEG polymer is not wrapped around the protein; on the contrary it appears as though it is stretched out from the protein to which it is attached. This hypothesis is illustrated in Fig. 5. If the very hydrophilic PEG was wrapped around the protein, then it would have only little effect on the total molar hydrodynamic volume of the PEGylated protein. On the other hand, if the PEG polymer was located as a 'polymer cloud' next to the protein and thus stretched out from the protein, then the polymer would contribute significantly to the intrinsic viscosity.

The increase in molar hydrodynamic volume of rFVIIa upon GlycoPEGylation seems to be independent of whether the PEG polymer is linear or branched, as the molar hydrodynamic volumes of both GlycoPEGylated species appear to be approximately 2.5 times larger than its composites.

To illustrate the change in viscosity of rFVIIa with GlycoPEGylation, the viscosity of rFVIIa, GlycoPEGylated rFVIIa (10 kDa and 40 kDa) and pure PEG at 10 mg/mL is plotted as a function of PEG molar mass (Fig. 6). The viscosity at 10 mg/mL based on the experimental measurements (Huggins and Kraemer constants) described in Section 2.

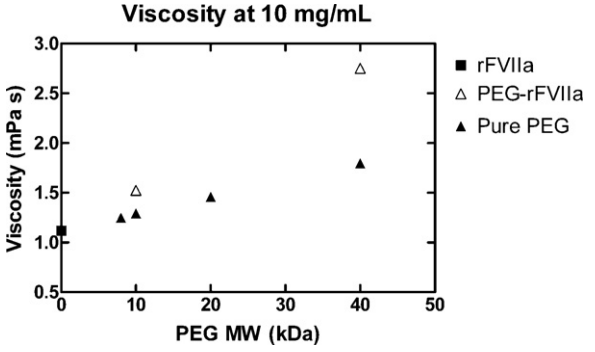

Fig. 6. The viscosities of pure PEG, unmodified rFVIIa and GlycoPEGylated rFVIIa at 10 mg/mL are plotted as a function of PEG molar mass. The concentrations for the two PEG-protein solutions are based on protein concentrations in the complex. The rFVIIa at zero molar mass is the unmodified rFVIIa. All viscosities at 10 mg/mL are calculated from the Ubbelohde viscometer measurements.

From the results presented in Fig. 6, it is clear that the viscosity of a 10 mg/mL protein solution increases significantly with GlycoPEGylation. The critical over-lap concentration, $C \approx (1/[\eta])$, for 10 kDa GlycoPEGylated rFVIIa is approximately 33 mg/mL and 12 mg/mL for 40 kDa PEG-rFVIIa. Above this concentration the viscosity of the solution increases markedly with concentration as the solution reaches the semi-dilute regime.

If the overall purpose of PEGylating a pharmaceutical protein is increasing the circulation time while administration is through injection, a difficult balancing act arises. A large attached PEG will favourably increase the circulation time more than a smaller PEG, while the overall viscosity of the solution will increase unfavourably. The improved molar hydrodynamic properties of the protein conjugate will often result in an increased stability and thus favourable pharmacokinetic and immunological properties as compared to the unmodified protein [23]. However, the size and shape of the used PEG should be chosen on the basis of which gives the best biological effect.

5. Conclusion

From the current study of the molar hydrodynamic volume of rFVIIa, pure PEG and GlycoPEGylated rFVIIa in solution it is evident, that the molar hydrodynamic volume of the conjugated protein is not just an addition of the molar hydrodynamic volume of the PEG and the protein. The molar hydrodynamic volume of the GlycoPEGylated protein is larger than the volume of its composites. These results suggest that both the linear and the branched PEG are not wrapped around the surface of rFVIIa but chains are significantly stretched out when attached to the protein.

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