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PEGylated recombinant von Willebrand factor analyzed by means of MALDI-TOF-MS, CGE-on-a-chip and nES-GEMMA

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ABSTRACT

Recombinant VWF (rVWF) is a candidate for therapy of von Willebrand disease and the largest known multimeric glycoprotein. In this study rVWF was covalently linked with a 20 kDa branched polyethylene glycol (PEG) to obtain PEGylated rVWF (PEGrVWF). This conjugation results in a further increase of heterogeneity besides glycoheterogeneity and a challenge in analyzing of such a bioconjugate, particular when investigated on the intact molecule level. Four different techniques including SDS-PAGE, MALDI-TOF-MS, capillary-gel-electrophoresis-on-a-chip (CGE-on-a-chip) and nano electrospray gas-phase electrophoretic mobility molecular analysis (nES-GEMMA) were applied to determine the molecular weight (MW) and the PEGylation degree of the monomeric rVWF. The degree and distribution of PEGylation of rVWF obtained by CGE-on-a-chip were in good agreement with results obtained by MALDI-TOF-MS with a special high mass detector. An average PEGylation degree of 3.1 PEG chains coupled to the monomeric glycoforms was found. MW determination by MALDI-TOF-MS (317.4 ± 1.0 kDa; 3 PEG chains attached) showed in comparison to CGE-on-a-chip (413.4 ± 2.1 kDa) the highest precision. Furthermore the orthogonal method, nES-GEMMA provided first information on the globular size (11.3 ± 0.1 nm) and based on that the MW (251 ± 7.2 kDa for the average PEGylation) of the PEGrVWF.

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1. Introduction

Von Willebrand factor (VWF) is a large multimeric glycoprotein present in human blood, plasma and inside platelets, endothelial cells and the subendothelial matrix of the blood vessel wall. The multimeric biologically active molecular structure has a varying number of subunits (monomers) from two up to 50 or even 100 repeats. This results in a molecular weight (MW) range of approximately 500–20,000 kDa (20 MDa), which makes VWF the largest known plasma protein. VWF has two essential biological functions: firstly, VWF mediates platelet adhesion and thrombus formation at sites of vascular injury and secondly, it binds and stabilizes procoagluation factor VIII (FVIII). Absence of VWF results in the most common inherited bleeding disorder, von Willebrand disease (VWD) [1,2]. VWD is classified into three categories depending on the quantitative (type 1), qualitative (type 2) and complete (type 3) deficiency of VWF. One approach to the treatment of VWD type 3 is the substitution by VWF–FVIII concentrates [3]. Nowadays, many glycoproteins isolated from blood plasma (e.g. coagulation factor IX, VWF or FVIII) are produced via biotechnological techniques. This recombinant technology has the advantage of being secure, reproducible and dependable as it does not rely on the availability of high-quality plasma supply. In addition direct isolation and purification of (glyco)proteins from human blood plasma may introduce challenges associated with proteolytic degradation of the proteins of interest, variation in the multimeric structure of VWF and, although viral removal and inactivation processes are now standard, the possibility of blood-based pathogen transmission may still exist [4]. Therefore with its similar structure and biological activity to the plasma-derived VWF (pdVWF) [5], recombinant VWF (rVWF), which is expressed in Chinese hamster ovary (CHO) cells, is a potential alternative to pdVWF.

The covalent coupling of polyethylene glycol (PEG) to therapeutic proteins and peptides, called PEGylation, is now a standard technique in pharmaceutical and biotechnological science to enhance the performance of a drug [6]. PEG as a synthetic reaction partner itself has several advantages for conjugation (1) low

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toxicity, (2) a very good solubility, (3) reduced renal clearance and (4) very low immunogenicity and antigenicity [7,8]. Altogether PEGylation improves stability against proteolytic degradation and this results in a prolonged biological effect as well as half-life time in plasma [9,10]. The covalent attachment of PEG molecules to (glyco)proteins introduces additional heterogeneity to the bioconjugates. On one hand because of the number of coupled PEG chains and on the other hand the polydispersity of the PEG molecules themselves. These facts make PEGylated (glyco)proteins very sophisticated and advanced products requiring leading edge analytical techniques.

Today matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) is a well established technique for the analysis of (glyco)proteins and PEGs [11,12]. Determination of the exact MW and degree of heterogeneity of PEGylated proteins by this technique is gaining in importance due to its high sensitivity, accuracy and speed. In the majority of studies MALDI-TOF-MS was used mainly for quality control of the chemical modification during conjugation, i.e. verification if a PEG chain was covalently linked to the protein or not. MALDI-MS was applied mainly for rather small proteins (e.g. interferon α 2a and superoxide dismutase) and hormones (e.g. salmon calcitonin and granulocyte-colony stimulating factor) where different PEGs were attached [13–16]. In the case of high MW glycoproteins such as rVWF and especially for PEGrVWF, a special new high mass detector is of interest due to the fact, that a sensitive detection up to 1 MDa was achieved [17].

The CGE-on-a-chip system is the second promising technique for the fast analysis of PEGylated proteins allowing the determination of the heterogeneity of such compounds (based on their different electrophoretic mobility) on one hand and of the molecular mass on the other hand [18]. Only a few investigations have reported to characterizing small PEGylated proteins by CGE-on-a-chip [19] and capillary electrophoresis [20,21]. The principle of separation in the lab-on-a-chip system applied is similar to conventional SDS-PAGE. The main advantage of the on-a-chip separation with a laser-induced fluorescence detector is that protein samples can be analyzed more rapidly (60 s per sample) than with a standard CGE system or the time-consuming SDS-PAGE and furthermore, a relative low sample amount is required for on-chip analysis.

A third technique called nano electrospray gas-phase electrophoretic mobility molecular analysis (nES-GEMMA) has been gaining ground in biotechnology [22–26] for determining the size and subsequent MW of large (glyco)proteins as well as non-covalent complexes. This technique originated in the field of nanoparticle and aerosol sciences but is now increasingly used to determine the electrophoretic mobility diameter (EMD), i.e. the particle size, and derived molecular mass, especially of large (glyco)proteins, noncovalent complexes [25], aggregates, dendrimers [27], PEGs [28] and large bionanoparticles such as intact viruses or virus–antibody complexes [22–24,29]. The main advantage is that large biomolecules and bionanoparticles exceeding the molecular mass range of >400,000 Da, where standard MS techniques such as electrospray ionization (ESI) or MALDI-TOF-MS have their limitations, can be analyzed as singly charged molecular ions without any deconvolution problem. Of importance is, that a straightforward correlation between the determined EMD and the exact MW [22–24,30] is given within a ±5% molecular mass accuracy.

We present results from the determination of molecular weight and degree of PEGylation of a high molecular mass glycoprotein, namely PEGrVWF, the largest known plasma glycoprotein, generated by MALDI-TOF-MS in the linear mode, CGE-on-a-chip and nES-GEMMA. Comparison of the applied techniques in terms of achievable precision for MW determination is also given.

2. Experimental

2.1. Chemicals

The rVWF and PEGylated rVWF were produced by Baxter Innovations (Vienna, Austria) and the polymeric reagents (20 kDa PEG) were supplied by Nektar Therapeutics (Huntsville, AL, USA). Ammonium acetate (NH_4Ac), formic acid (FA) and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Sinapinic acid (SA), ferulic acid (FAc), 2,4,6-trihyhroxyacetophenon (THAP), Coomassie Brilliant Blue R-250 and sodium dodecyl sulphate (SDS) were obtained from Fluka (Buchs, Switzerland). Dithiothreitol (DTT) and human serum albumin (HSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HiMark High Molecular Weight Standard, lithium dodecyl sulphate (LDS) sample buffer (4×), NuPAGE 4–12% Bis–Tris gel (1.0 mm × 10 well) and NuPAGE MES SDS running buffer (20×) were obtained from Invitrogen (Paisley, UK).

2.2. Reduction to monomers (rVWF and PEGrVWF)

Prior to all measurements the native (multimeric) samples were denatured by heat and DTT treatment. 100 μL rVWF (0.5 mg protein content/mL) or PEGrVWF (1.3 mg/mL), both dissolved in 50 mM NH_4Ac buffer, pH 6.8, were treated with 2 μL of 1 M DTT and 10 μL of 10% SDS solution for 6 min at 95 °C on a thermomixer (Eppendorf, Hamburg, Germany) applying 400 rpm. After the samples were cooled down to RT reagents were removed by using two times Microspin G-25 columns (GE Healthcare, Uppsala, Sweden). The second filtration step was necessary to completely get rid of SDS, which otherwise would interfere with the nES process in GEMMA and with the desorption/ionization process in MALDI-TOF-MS.

2.3. SDS-PAGE

SDS-PAGE analysis was carried out under standard Laemmli conditions [31] and protein was visualized by Coomassie Brilliant Blue R-250 staining. Electrophoresis was performed under reducing conditions and therefore a mixture containing 6 μL sample solution (3 μg rVWF and 2.6 μg PEGrVWF, respectively), 3 μL of 4× LDS buffer and 3 μL of 100 mM DTT solution, was heat denatured for 1 min at 90 °C and cooled down to RT. Afterwards denatured and reduced samples were separated on a 4–12% Bis–Tris–polyacrylamide gel using MES SDS running buffer and the voltage was set to constant 200 V for 40 min. HiMark molecular weight marker was run next to the protein lanes for estimation of MW.

2.4. MALDI-TOF-mass spectrometry

Positive ion mass spectra measured in the linear mode were obtained on an AXIMA CFR+ (Shimadzu Biotech, Manchester, UK) instrument retrofitted with a moveable high mass ion conversion detector (ICD HM1, CovalX AG, Zuerich, Switzerland). The principle of the ICD HM1 is that ions are converted into secondary ions on a dynode. These secondary ions are then post-accelerated with −20 kV before entering a special secondary electron multiplier [32]. By means of this type of detector a better signal-to-noise ratio and absolute signal intensity for high molecular mass compounds above 100 kDa can be achieved. All mass spectra shown represent the accumulation of 100 unselected single laser shots smoothed via company-supplied Gauss algorithm. External calibration was used by applying 1 pmol HSA on the target. The monomeric ion and multimeric cluster ions of HSA as well as the doubly charged ion of the monomer were selected for external mass calibration. Five samples

spots were prepared according to the procedure given below and subsequently analyzed.

2.5. Sample preparation for MALDI-TOF mass spectrometry

The following MALDI-MS preparations were used for rVWF and PEGrVWF analysis: 10 mg/mL THAP, FAc or SA were dissolved in an ACN/aqueous 1% FA (50:50, v/v) mixture, whereas 1 μL of matrix solution and 1 μL of the reduced and purified sample solution were mixed together, and 1 μL of this mixture was deposited on the sample plate. The final amount on the target was 0.2 μg for rVWF and 0.7 μg for PEGrVWF. All samples were air dried at ambient temperature prior to MS analysis.

2.6. CGE-on-a-chip

All measurements were carried out on an Agilent 2100 CGE-on-a-chip (Agilent Technologies, Waldbronn, Germany) in combination with the Protein 230 assay kit and the non-commercial modified software for measuring and data analysis. The Protein 230 assay kit was used with a modified run time script to optimize the electrophoretic separation for proteins greater than a MW of 230 kDa.

Sample preparation, chip priming and loading were carried out as recommended by the Agilent Technologies' assay kit guide, except that a denaturing time of 8 min was used before chip loading. The absolute amount of protein deposited into the CGE-on-a-chip well was 130 ng for rVWF and 350 ng for PEGrVWF. At least five aliquots were analyzed for molecular mass determination.

2.7. nES-GEMMA

The GEMMA system used consists of the following parts: (a) a nano electrospray source (nES, model 3480), which generates multiply charged bionanoparticles, (b) a ^{210}Po unit for charge reduction to neutral and single charged particles, (c) a nano differential mobility analyzer (nDMA, model 3980) for particle separation based on the electrophoretic mobility in air, and (d) an ultrafine condensation particle counter (model 3025) for detection (all parts from TSI Inc., St. Paul, MN, USA). The particle size range (scan range) was adjusted from 3 to 40 nm for rVWF and PEGrVWF and scan time was 2 min/scan. Ten scans were averaged for all shown GEMMA spectra and five aliquots were injected for size determination. MW was determined using the conversion equation (EMD to MW) for this nES-GEMMA instrument from Laschober et al. [30].

All nES conditions were identical for all samples. Measurements were performed in the stable cone-jet mode applying a voltage of 1.7 kV, and a sheath gas flow composed of 0.25 L/min CO_2 (99.5% quality for technical use, Air Liquide, Schwechat, Austria) and 1.5 L/min compressed particle-purified air. A pressure of 4 psi was used for sample introduction resulting in a sample solution flow of approximately 70 nL/min through an uncoated fused silica capillary (25 cm length, 40 μm ID and 160 μm OD, TSI Inc., St. Paul, MN, USA). The nDMA was run at a flow rate of 17 L/min compressed particle-purified air.

2.8. Sample preparation for nES-GEMMA

All reduced and purified sample solutions were directly diluted with a 50 mM aqueous NH_4Ac buffer (pH 6.8) prior to analysis. The concentration for the nES-GEMMA measurements was optimized, approximately 100 μg/mL for each sample, to avoid the formation of concentration-dependent cluster ions (e.g. $[2M]^+$ or $[3M]^+$).

3. Results and discussion

3.1. SDS-PAGE

Briefly, SDS-PAGE analyses (see inset in Fig. 1A) showed a MW of approximately 270 ± 10 kDa for rVWF which is in good agreement with the literature [33, obtained by agarose/polyacrylamide gel electrophoresis]. A broad smear band in the range of 300–600 kDa was found for PEGrVWF (see inset in Fig. 1B). The mass difference observed is due to PEGylation of rVWF.

3.2. MALDI-TOF-MS

Three different matrices THAP [34], FAc and SA [35] were investigated for MALDI-MS analysis of rVWF and PEGrVWF. All sample spots were prepared by the volume technique (see Fig. 1 with SA; for THAP and FAc, data not shown). The volume technique was used as the favored preparation technique because it has been reported to provide the best results in high molecular weight protein analysis [32]. In case of THAP the multiple charged molecules (dominated by $[M]^{2+}$ and $[M]^{3+}$) were observed as the preferred ion species in positive ion MALDI mass spectra. The use of FAc and SA yielded similar mass spectra but, in contrast, the doubly charged ions dominated in FAc matrix, while in the case of SA the singly charged ions were the predominant molecular ions (Fig. 1A). Therefore the optimal matrix for rVWF and PEGylated rVWF samples turned out to be SA in combination with the volume technique. This preparation method should be recommended for MALDI-TOF-MS of such types of high molecular mass and heterogeneous molecules.

For obtaining the exact MW and extent of PEGylation MALDI-TOF mass spectra as shown in Fig. 1 were used (Fig. 1A rVWF before and Fig. 1B after PEGylation reaction). Based on such mass spectra (singly charged molecules), the MWs were determined by external calibration and are presented in Table 1. The achieved resolution for singly-charged rVWF molecule was around 18.4 at FWHM. This relative low value is related to the fact that rVWF exhibits a high degree of glycan heterogeneity. A MW of 256 ± 0.9 kDa ($n = 5$) was found for rVWF which was significantly less than obtained by SDS-PAGE (270 kDa). A molecular mass accuracy for unmodified proteins up to 160 kDa of approximately ±0.06% can be achieved by the used system with the described detector. This is a general observation concerning glycoproteins in SDS-PAGE. Due to the considerable glycan moiety of rVWF (15%) [36], electrophoretic migration is slowed down and the MW observed is too high. This is a well known phenomenon observed in SDS-PAGE [37,38]. Size and charge-to-mass ratios of glycoproteins are not comparable directly with those of standard proteins used as MW marker. The electrophoretic mobility of PEGrVWF is even more slowed down because the additionally attached PEG chains increase the already large hydrodynamic radius and molecular mass [39]. This result indicates that SDS-PAGE gives only a rough estimate of MW and is not useful either for exact MW determination or for the fast characterization of large PEGylated glycoproteins.

In a previous study, plasma-derived VWF was analyzed by MALDI-TOF-MS equipped with a cryodetector resulting in a MW of 260 kDa [40]. This result indicates that the plasma-derived and recombinant VWF corresponds very well in terms of molecular mass in its reduced form. For PEGrVWF (Fig. 1B and C) the total number of attached PEG chains went up to 5 PEG chains per glycoprotein monomer with the most abundant signal corresponding to 3 PEG chains linked to the rVWF molecule. A molecular mass precision of ±0.5% could be reached by means of external calibration. A low signal-to-noise m/z value was detected near the predicted m/z value of the unreacted starting material, which may indicate

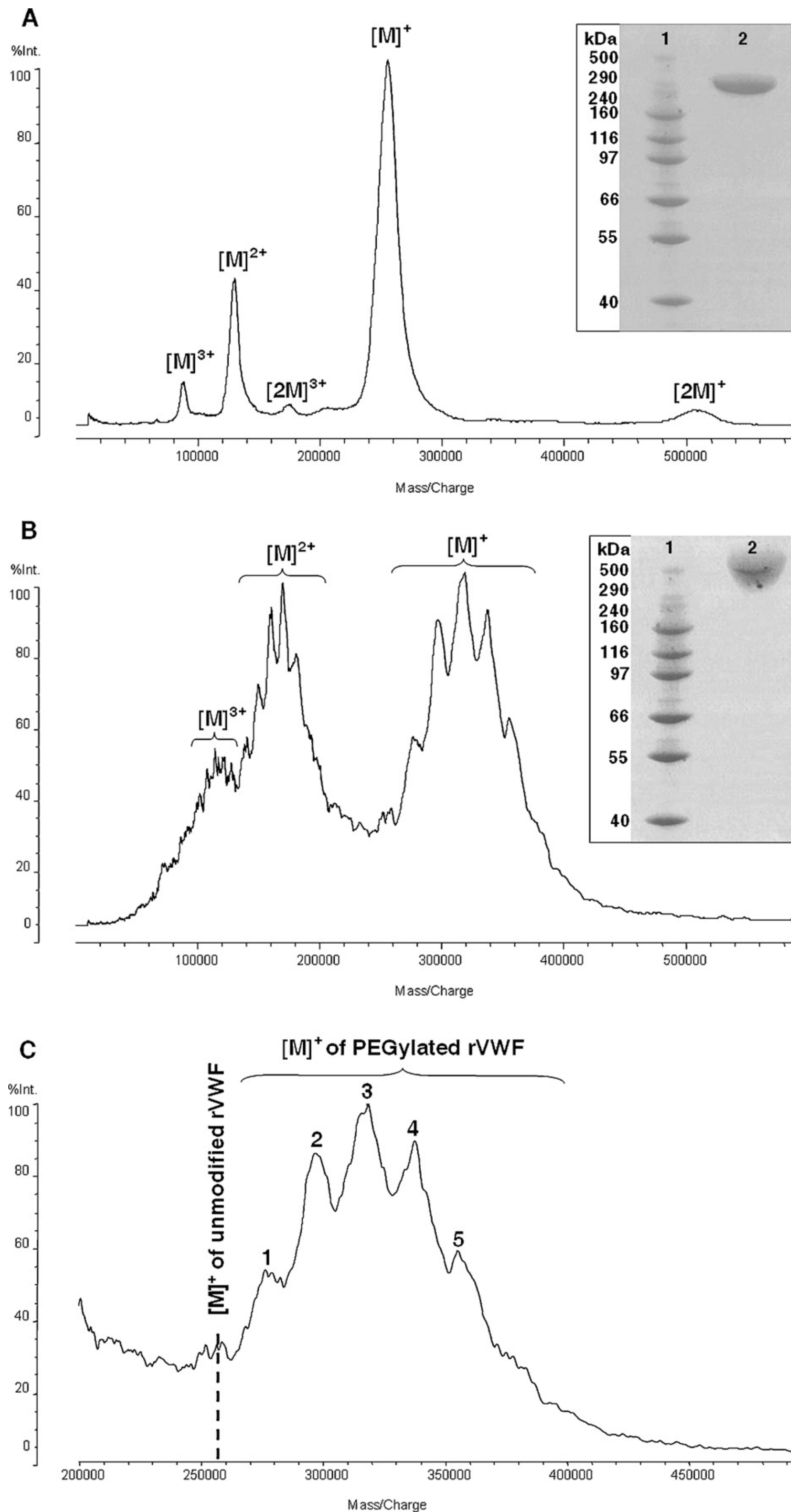

Fig. 1. Positive ion MALDI-TOF mass spectra of denatured and reduced (A) rVWF, (B) PEGrVWF and (C) upper m/z range of the singly charged PEGrVWF molecule. Numbers on top of the peaks (1–5) represent the different PEGylated forms of rVWF corresponding to the number of covalent linked PEG chains. Final concentration on the target was 200 ng for rVWF and 700 ng for PEGrVWF. The labels $[M]^+$, $[M]^{2+}$, $[2M]^+$ etc. stand for protonated, sodiated or potassiated species due to the fact that these peaks cannot be assigned unambiguously. Insets: SDS-PAGE electrophoretic images of rVWF and PEGrVWF under reducing conditions [lane 1: high MW standard and lane 2: (A) rVWF (3 μg) and (B) PEGrVWF (2.6 μg)].

Table 1
MWs for rVWF and PEGrVWF obtained by MALDI-MS, CGE-on-a-chip and nES-GEMMA. All denoted values are in kDa and all values are from 5 individual measurements. n.c.d., not clearly detectable, i.e. not sufficient abundant signal-to-noise ratio.

	MALDI-MS [kDa]	CGE-on-a-chip [kDa]	nES-GEMMA [kDa]
Unmodified rVWF	256.0 ± 0.9	305.6 ± 1.9	225 ± 6.7
rVWF + 1PEG	278.8 ± 0.8	344.3 ± 1.4	
rVWF + 2PEG	299.4 ± 1.1	375.9 ± 1.4	
rVWF + 3PEG	317.4 ± 1.0	413.4 ± 2.1	Peak maximum 251 ± 7.2
rVWF + 4PEG	334.3 ± 0.6	435.9 ± 2.6	
rVWF + 5PEG	354.7 ± 1.1	452.2 ± 3.4	
rVWF + 6PEG	n.c.d.	466.9 ± 2.5	

the presence of unreacted product in the mixture or a combination of starting and degradation material (see vertical broken line in Fig. 1C). This explanation was corroborated by the CGE-on-a-chip data shown later. A peak corresponding to an rVWF molecule with six and even seven PEG chains might be hidden in the high m/z tail of the mass spectrum in Fig. 1C but could not be resolved due to a low signal-to-noise ratio by the linear time-of-flight analyzer used. The mass increments between the different PEGylated

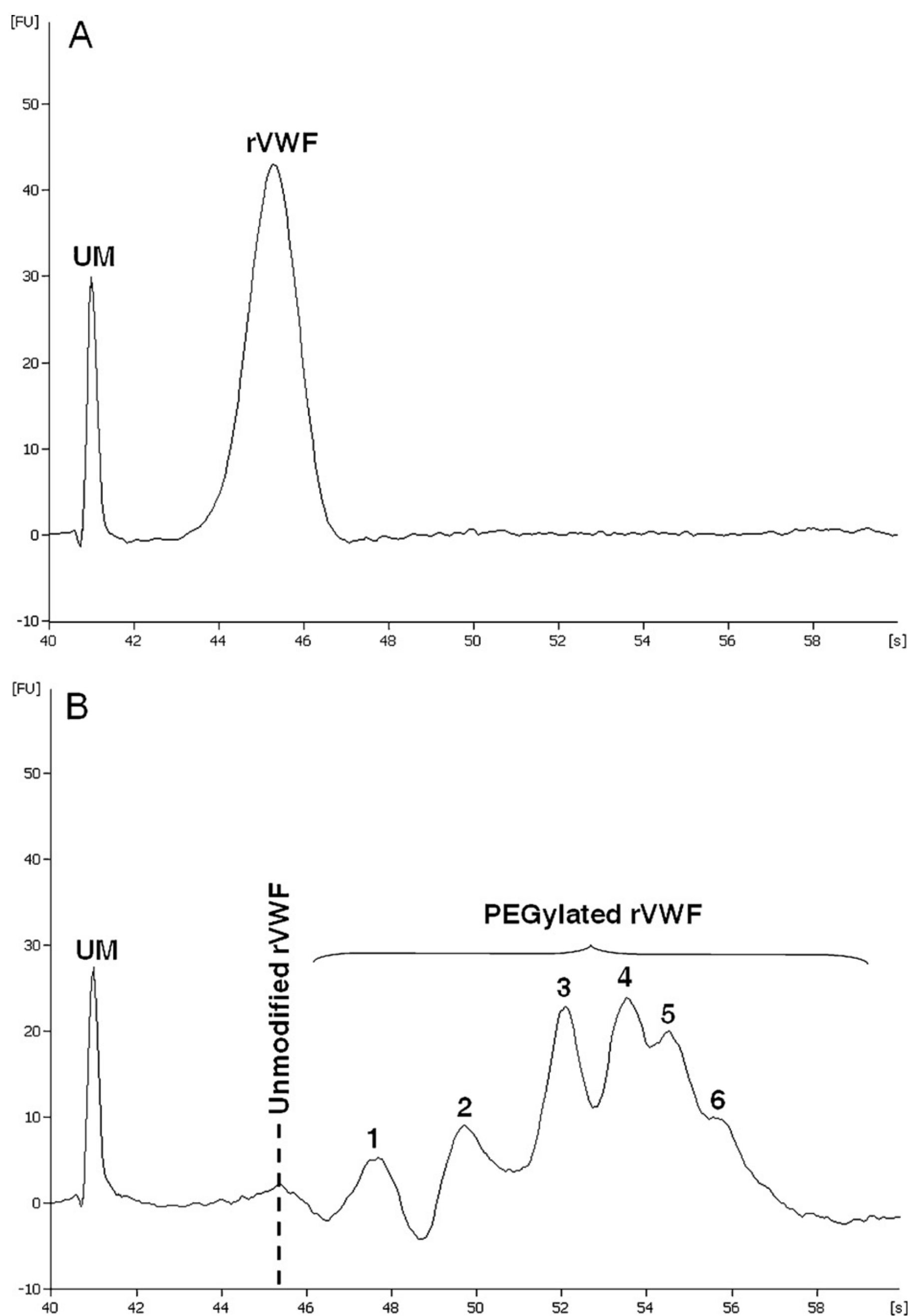

Fig. 2. CGE-on-a-chip electropherograms of (A) rVWF (130 ng) and (B) PEGrVWF (350 ng) under denaturing and reducing conditions. Numbers on top of the peaks indicate the PEGylation degree (UM, upper marker of the P230 protein kit).

rVWF monomers were found to be approximately 21 kDa. This corresponds with the average MW of the applied 20 kDa branched PEG (the molecular mass maximum of the starting material was also validated by MALDI-TOF-MS, too; data not shown) used for the conjugation experiments to obtain PEGrVWF.

3.3. CGE-on-a-chip

CGE-on-a-chip electropherograms of rVWF and PEGrVWF are shown in Fig. 2A and B, respectively. All electropherograms exhibit peaks beyond the so-called upper marker protein due to size/molecular mass of reduced rVWF/PEGrVWF. They show a peak pattern which is similar to MALDI-TOF mass spectra indicating a size/molecular mass correlating separation. The electropherogram in Fig. 2A demonstrates the homogeneity of the rVWF preparation (i.e. the starting material for conjugation) but on the other hand the peak width is characteristic for the high degree of glycosylation (15%). The achieved capillary electrophoretic peak resolution turned out to be 1.1. Fig. 2B exhibits the electrophoretic separation of PEG rVWF, indicating a total number of attached PEG chains up to at least 6 PEG chains per monomer (migration time 56.0 s) with the most abundant signal corresponding to 4 PEG chains per rVWF molecule (migration time 53.9 s). This observation was also described in case of conventional CE [20,21,41] and CGE-on-a-chip [19] analysis of small PEGylated proteins. These data confirmed the results from MALDI-TOF-MS analysis (compare the peak pattern in Figs. 1C and 2B). Again probably a tiny amount of glycoprotein starting material was detected.

3.4. Molecular weight determination by CGE-on-a-chip

MW determination of proteins using the CGE-on-a-chip is in principle similar to that of SDS-PAGE. In contrast to SDS-PAGE, the CGE-on-a-chip provides a fully automated system allowing MW determination in a very short time (several seconds after the separation run). A protein standard mixture (ladder) is run on each chip and the software automatically generates a standard calibration for proteins in the range of 5–200 kDa (recombinant proteins without modifications). Accuracy is dependent on the protein characteristics as for example the amino acid sequence, isoelectric point and structure. For these reasons not all proteins migrate according to their actual (primary structure-based) MW value [42,43]. In the case of rVWF and PEGrVWF, a MW determination was only possible by extrapolation of the standard calibration (different sized recombinant proteins) beyond the upper marker. The assigned MWs are shown in Table 1 and have to be considered with caution due to the extrapolation performed. For both samples, rVWF and PEGrVWF the MWs were determined to be too high compared with MWs derived from MALDI-TOF-MS for reasons similar to those described in the case of SDS-PAGE (see above). A molecular mass precision of ±0.8% (n = 5) was found. The mass increments determined between the different PEGylated glycoprotein monomers varied from approximately 14 to 39 kDa for PEGrVWF. Mass differences decrease with increasing degrees of PEGylation and this coincides with the reduced migration time differences from 2.4 s to 1 s with increasing sizes of the PEGylated species. Therefore the more PEG chains that are attached to the rVWF the less it contributes to the increase of the hydrodynamic radius [19]. These results clearly show that CGE-on-a-chip can be used for rapid analysis of high mass glycoproteins and its multiple PEG conjugates, even beyond the instrument company's stated limits. Monitoring the quality of the starting rVWF as well as the formation of PEGrVWF during production steps is feasible in a high throughput way (60 s per analysis). MWs determined by CGE-on-a-chip in the range of the glycoproteins shown should be considered with caution, particularly if no orthogonal method (e.g. MALDI-TOF-MS) was applied in early stages of method development.

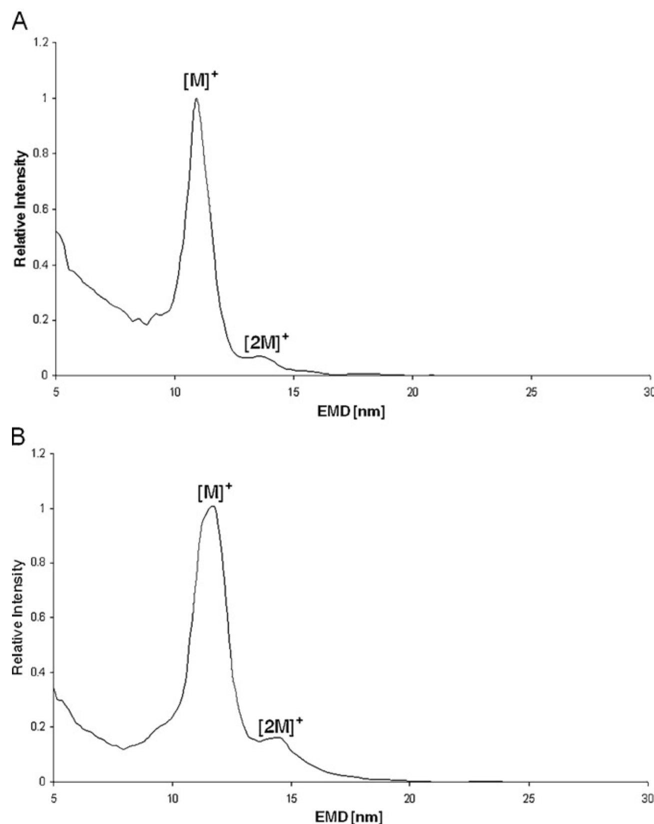

Fig. 3. Nano ES-GEMMA spectra of reduced (A) rVWF and (B) PEGrVWF. An amount of 140 ng per sample was used for obtaining the GEMMA spectra corresponding to ten averaged scans.

3.5. nES-GEMMA

3.5.1. Size determination by nES-GEMMA

GEMMA spectra exhibiting the EMDs of rVWF and PEGrVWF are shown in Fig. 3. In the case of the rVWF (Fig. 3A) and PEGrVWF (Fig. 3B) monomers were found with an average diameter of 10.9 nm and 11.3 nm, respectively. A diameter precision of ±1% (n = 5) was determined. The resulting shift of peak maximum due to PEGylation was 0.4 nm. The peak of PEGrVWF showing a higher width at half maximum also suggests greater heterogeneity than rVWF based on the additional presence of the PEG chains. But in contrast to MALDI-TOF-MS and CGE-on-a-chip, no separation of the individual PEGylated rVWF forms (isoforms) was achieved due to the limited resolving power of the nDMA applied. A resolution of 9.1 at FWHM was achieved for rVWF in our case. The peak maximum (diameter of 11.3 nm) in the case of PEGrVWF (Fig. 3B) corresponds to particles with an average degree of PEGylation. In addition to the singly charged species, a second peak with a higher EMD value with much lower intensity indicates the presence of singly charged concentration-dependent dimers ($[2M]^+$) in both cases. The method turned out to be fast for determining the size of the rVWF and PEGrVWF molecules directly out of a solution in the nanometer range.

3.5.2. Molecular weight determination by nES-GEMMA

In addition to the size determination, a MW determination was carried out by applying a conversion consisting based on well-defined standard proteins [30]. nES-GEMMA MW determination works well for globular (glyco)proteins providing molecular

masses close to the expected values within the instrument accuracy (±5% [23]). In the case of rVWF a diameter of 10.9 nm relates to 225 kDa and for PEGrVWF, 11.3 nm corresponds to 251 kDa (Table 1). As mentioned above, the broader peak of PEGrVWF also suggests a broader MW distribution than in plain rVWF. Again the instrumental resolution of the nES-GEMMA system used was not sufficient to differentiate between PEGrVWF molecules with different extents of PEGylation. The GEMMA spectra of rVWF and PEGrVWF (Fig. 3) showed a shift in the average diameter of 0.4 nm corresponding to an apparent total mass shift of 26 kDa due to PEGylation. This mass shift was much lower than expected based on MALDI-TOF-MS and CGE-on-a-chip results. According to the average degree of 3.1 PEGs for PEGrVWF monomers by MALDI-MS, a mass shift of about 60 kDa would have been expected compared with the starting material (plain rVWF). The behavior of PEGylated glycoproteins in gas phase at atmospheric pressure is not known and therefore an interpretation of the result is difficult [28]. Furthermore, the issue of different densities of the PEG and protein moiety of the PEGylated molecules is unresolved and this plays an important role in the MW determination by nES-GEMMA. Thus, the situation is problematic for MW determination of PEGrVWF by nES-GEMMA, similar to that for CGE-on-a-chip and SDS-PAGE.

4. Conclusions

MALDI-TOF-MS in the linear mode and CGE-on-a-chip turned out to be the techniques of choice for the analysis of PEGrVWF, providing complementary results due to their orthogonal concepts and their high resolving power to separate different PEGylated glycoproteins. The speed of analysis and the mass accuracy obtainable are the major advantages of MALDI-MS. Using the high mass ion conversion detector with MALDI-MS a sensitive detection in the high m/z range was possible for rVWF and its PEGylated forms (200 ng for rVWF and 0.7 μg for PEGrVWF) that exhibit an extremely high heterogeneity (N/O-glycosylation and PEGylation at multiple sites). Important to mention is that sample preparation in MALDI-MS is more time consuming and sensitive to variations. Nevertheless, CGE-on-a-chip's performance in terms of its power to resolve the PEGylated species was surprising good for such a large and complex glycosylated molecule as PEGrVWF. Sample preparation and injection is compared to MALDI-MS very simple and straightforward. Thus, the combination of the two techniques makes an interesting and cost-effective tool for rapid analysis of complex PEGylated glycoproteins (e.g. for monitoring the PEGylation process or batch-to-batch control). By contrast, nES-GEMMA's strength is that information about the exact size of the PEGylated glycoprotein in the gas-phase as well as in starting material can be obtained easily but at low resolution and with low content of nonvolatile contaminants. The combination of these liquid- and gas-phase electrophoretic techniques with MALDI-TOF-MS can be considered a useful toolkit for the fast characterization and process control of PEGylated therapeutic glycoproteins such as the recombinant VWF.

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