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Size of Pegylated Protein Conjugates Studied by Various Methods

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Abstract

Pegylation as the most widely used methodology for improving biopharmaceutical drugs generates considerable changes in physicochemical and biological properties of proteins. Most of the positive pharmacological effects of pegylated proteins are related to an increased hydrodynamic volume and size, respectively. To explore the size impact of polyethyleneglycol (PEG), a series of well-defined conjugates of interferon alpha 2b (IFN) were prepared with PEGs of different lengths and shapes specifically attached to the N-terminal amino group of the protein. For characterization, various methods have been used, not only common methods for estimating molecular mass and size, such as size exclusion chromatography, electrophoretic mobility in polyacrylamide gel and dynamic light scattering, but also ion exchange and reverse phase chromatographies. Although charge and hydrophobicity based separations are not directly connected with the size of molecules, their use resulted in very interesting correlations between elution times and molecular mass of the PEG-IFN conjugates.

Keywords: PEG, PEGylation, interferon alpha 2b (IFN), Dynamic Light Scattering (DLS), SE-HPLC, RP-HPLC

1. Introduction

In the last decades numerous proteins and peptides have proved as promising human therapeutics for treatment of various pathogenic states. Although very effective and safe, these macromolecules share a number of properties, which do not form a good basis for robust therapeutical agents. These, so called first-generation biopharmaceuticals, which are protein drugs mimicking the natural protein molecules, frequently display low stability and solubility, short elimination half-life, susceptibility to proteolytic enzymes, immunogenicity, toxicity and other properties that do not classify them as optimal drugs. In recent years a number of technologies have been developed, targeting at improved biomedical efficacy and better physicochemical qualities as compared to the first generation biopharmaceuticals. Among these, covalent attachment of polyethylene glycol chain(s) to a protein, usually called PEGylation, has become a widely used and acknowledged method. The first biopharmaceuticals based on PEG-protein conjugation appeared in the 1990s and currently there are six different PEG-protein products on the market, but regarding numerous new biopharmaceuticals in various development phases several new products are expected in the near future. Since it is believed that the next generation of biopharmaceuticals will be Fab fragments, protein nanobodies and protein scaffolds which need elimination half-life extension, pegylation methodology has a bright future. Covalent attachment of PEG chain to the protein substantially alters protein characteristics therefore pegylated biopharmaceuticals are treated as new chemical entities. Binding of the PEG moiety to the protein causes various effects, such as masking of surface and glycosylation function, charge modification, epitope shielding etc, but it is the size enlargement of the molecule that appears to be the main advantage,[1] resulting in reduced glomerular filtration and consequently increased elimination half life. Although PEGylation of protein is usually accompanied by decrease of biological activity (measured in vitro), this drawback is successfully compensated in vivo by improved pharmacokinetic profile, which has been confirmed in numerous clinical stud-

ies. Thus the size of the attached PEG chain represents the most important factor affecting conjugate activity as well as pharmacokinetic and pharmacodynamic properties.[2] However, determination of the size or hydrodynamic volume of the PEG-protein conjugates is not quite straightforward and additionally, the molecule size is not always directly proportional to the molecular mass of the conjugate.

PEG is usually quoted as highly hydrophilic molecule, although it is not only well soluble in water but also in various organic solvents. Its structure with numerous repeats of the ethylene oxide unit is extremely flexible and capable of coordinating numerous water molecules. Due to water binding the apparent molecular mass of the PEG-protein conjugate is substantially higher than the mass of the globular protein of the same nominal molecular mass. According to Basu et al, 2006) the PEG-protein conjugates occupy volumes 5 to 10 times bigger than proteins.[3] Considering these data it becomes questionable, if methods intended for estimating molecular mass of proteins are also appropriate for PEG-protein conjugates. As size exclusion chromatography (SEC) is also widely used for size determination of polymers, it appears as the method of choice and most comprehensive studies on PEG-protein conjugate sizes are actually based on SEC data.[4, 5]

Our intention was to study the size and apparent molecular mass of the PEG-protein conjugates by different methods, and human recombinant interferon alpha2b (IFN), expressed in *E. coli* was used as a model protein. There are already two pegylated interferons on the market: PegIntron® (Shering) – interferon alpha 2b, pegylated with a linear 12 kDa PEG; and Pegasys® (Roche) – interferon alpha 2a, pegylated with a branched 40 kDa PEG. Interferon alpha 2a and interferon alpha 2b are almost identical proteins, differing only in one amino acid residue, but pharmacological behaviour, especially pharmacokinetic properties of both conjugated protein drugs are essentially different, which points out to the importance of the attached PEG. However, both marketed PEG-interferon conjugates are heterogeneous mixtures of numerous monopegylated positional isomers prepared by random pegylation.[2, 6, 7] To make the comparison of various PEG lengths and shapes easier and more precise, we have decided for N-terminal pegylation of interferon alpha 2b and employed procedure as described by Kinstler et al.[8]

We have prepared a set of well-defined PEG-interferon alpha 2b conjugates with various PEGs attached to the protein N-terminus. Interferon alpha 2b conjugates differing only in the length (and shape) of the attached PEG moiety form a good basis for size and molecular mass studies. Additionally to size exclusion chromatography that has usually been used for prediction of the viscosity radii we also applied other methods, such as dynamic light scattering (DLS), and behavior on SDS-PAGE gels. Comparison of these methods and correlation with analytic results obtained by cation exchange and reverse phase chromatographies resulted in interesting parallels between size and chromatographic behavior of the PEG-protein conjugates.

2. General Methods

Gel Filtration HMW and LMW Calibration Kits (Amersham Biosciences) were used for the size exclusion chromatography calibration and DLS measurements. Polyethylene glycol reagents (NOF, Japan), all other chemicals were purchased at Sigma (USA).

Isolation of conjugates was performed on chromatographyc system AKTApurifier (Amersham Biosciences). Buffer exchange was performed on PD10 desalting column (Amersham Biosciences) and Amicons (Millipore).

Size exclusion chromatography was performed with Agilent Technologies 1200 series analytical HPLC system, using TSKgel G4000SWxl 7,8 mm × 30 cm (Tosoh Bioscience, Japan). Mobil phase was 20 mM Na_2HPO_4, 150 mM NaCl, 1% diethylene glycol, 10% ethanol, pH 6,8. Flow rate was 0,4 ml/min Detection was performed at 215 nm and 303 nm.[9]

Reversed-phase chromatography was performed with Agilent Technologies 1200 series, analytical HPLC system, using YMC-ODS-AQ, C18, 4,6 mm × 15 cm column (YMC, Japan). Elution was carried out at flow rate of 1 ml/min with linear gradient from 10% to 100% acetonitrile in water with 0,1% TFA in 30 min, at 55 °C. Detection was performed at 215 nm.

Cationic exchange chromatography was performed with Waters analytical HPLC system W2390/5, using TSK-gel SP-5PW, 7,5 mm × 7,5 cm column (Tosoh Bioscience, Japan). Binding was performed with 34 mM sodium acetate, 10% ethanol, and 1% diethylene glycol, pH 4,4. Elution was performed with linear gradient over 105 min with 10mM K_2PO_4, 10% ethanol, and 1% diethylene glycol, pH 6,6. Flow was 1 ml/min. Detection was performed at 215 nm.[9]

Dynamic light scattering PEG standards were dissolved in water at concentration 0,5–10 mg/ml. (All PEG standards were dissolved in water at high concentration and diluted several times. Hydrodynamic result was determinated by extrapolation to 0 concentration). Addition of anorganic salts (NaCl, Na_2HPO_4) did not change results of hydrodynamic radius.[10] Hydrodynamic radius of conjugates were measured at conjugate concentration of 0,5 ± 0,15 mg/ml in 137 mM NaCl, 92 mM benzyl alcohol, 20 mM acetic acid pH 6. Conjugate concentration did not

have significant influence on the results. Protein standards were dissolved in 1% NaCl. All samples were filtrated through Ultrafree-MC microcentrifuge filters, Durapore PVDF membrane, pore size 0,22 μm.

SDS-PAGE for protein and PEG detection was performed on 4–12% gradient gels using the NuPage Novex Bis-Tris SDS-PAGE (Invitrogen) system.

SDS-PAGE gels were stained by the SimplyBlue Safe Stain (Invitrogen) for protein detection and by Iodine for PEG detection according to the method of Kurfûrst.[11]

Pegylation and Isolation of Monopegylated IFN

To the protein solution of interferon alpha 2b at 1,5 mg/ml, 10 molar excess of mPEG-CHO reagent was added and appropriate volume of 1 M $NaCNBH_3$ solution to the final concentration of 20 mM in reaction mixture. pH of the reaction mixture was adjusted to pH = 5 and pegylation reaction took place at + 4 C for 24 hours, with gentle stirring. Reaction mixture was SDS-PAGE analyzed and terminated by buffer exchange to 25 mM acetic acid pH 3,8 on PD10 desalting column (Amersham Biosciences). Separation was performed on TSK gel SP-5PW with 25 mM CH3COOH, pH 3,8 as loading buffer. Elution was performed with shallow gradient of 75 mM CH3COOH, pH 8.0. Pooled monopegylated fractions were concentrated and exchanged to 137 mM NaCl, 92 mM benzyl alcohol, 20 mM acetic acid pH 6 buffer. Concentrations were adjusted to 0,5 ± 0,15 mg/ml.

Derivatization of PEG-CHO with p-Aminobenzoic Acid (pAMBA). We have adapted the derivatization procedure described by Meyer 2001.[12] mPEG-CHO reagents of different lengths were dissolved in solution of 0,175 M p-AMBA, 0,08 M $NaCNBH_3$, 200 mM Na-phosphate, 50% acetonitrile pH 4,5 at 100 mg/ml concentration and incubated at 60 °C for 1 hour. The reaction mixture was diluted 10 times with mobile phase prior to the SEC-HPLC.

3. Results and Discussion

Covalent modification of proteins with PEG has proved a very efficient method to improve clinical efficacy of protein drugs. Such conjugates may vary substantially from the parent proteins, regarding not only biomedical but physicochemical properties as well. As mentioned in the beginning, one of the most expressed changes caused by pegylation is a larger size of the molecule and larger hydrodynamic volume, respectively. There are various methods for estimating size of proteins or their conjugates, such as mass spectrometry, electrophoretic methods, light scattering, etc, but size exclusion chromatography (SEC) as a simple and low-cost method for determining molecular mass of proteins and polymers on the basis of calibration curves appears to be most convenient.[4] For separation of proteins, aqueous mobile phases are used that enable preservation of protein native structure and salts added usually hinder undesired ionic interactions with stationary phase. However, when using such mobile phases for pegylated proteins, separation becomes less efficient due to peak broadening caused by the polydispersity of PEG. Additionally, PEG non-specific adhesion to the stationary phase frequently results into peak tailing. Both phenomena can be reduced by adding organic modifiers into the mobile phase, which also contributes to reproducibility and robustness of the method.[9, 13, 14] Although higher percentage of organic solvents might damage the protein native structure and fold, many manufacturers of SEC columns advise addition of organic modifiers for PEG-protein separations, especially for analytical purposes.

Comparing sizes of proteins and PEG-protein conjugates it is generally anticipated that PEG chain can bind water molecules resulting in an increased apparent molecular mass. However, how big this effect is, cannot be simply predicted. Fig 1 shows a SEC chromatogram of 3 different molecules having approximately the same nominal molecular mass: linear 40 kDa mPEG-CHO reagent (derivatized with pABA to permit UV215 detection), 38 kDa PEG-IFN conjugate and 43 kDa ovalbumin. As expected, molecules exhibit distinctly different retention times, 40 kDa PEG reagent behaving as the biggest molecule and protein without conjugation as the smallest. When analyz-

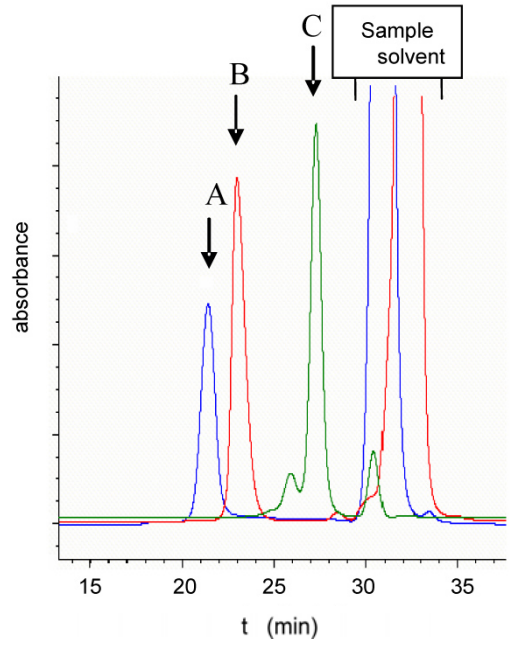

Figure 1: SEC analysis of mPEG-CHO reagent derivatized with pAB (40 kDa) (A – blue), IFN-20PEG, (38 kDa) and (B – red) ovalbumin (43 kDa) (C – green) on TSKgel G4000SWxl (Tosoh Bioscience, Japan). Main peaks are marked with arrows.

ing the same molecules on SDS-PAGE gels (Fig 2, lanes 4, 8 and 9) the apparent size order is preserved. The marked entities having approximately the same nominal molecular mass possess distinctly different positions on the gel. PEGs as well as PEG-protein conjugates are in complete disagreement with protein molecular mass standard (Fig 2, lane 1). As already observed by Kurfürst,[11] for rough estimation the apparent molecular mass of PEG-protein conjugates are better comparable to PEG standards than protein standards. This clearly demonstrates the substantial impact of PEG on the hydrodynamic volume or viscosity radius of the PEG-protein conjugate.

gates fit well into the same line, irrespective of the PEG shape. Surprisingly it was found that plotting the retention times (or distribution coefficients Kav) of the PEG-IFN conjugates as a function of nominal molecular mass of the PEG moiety, leaving out the molecular mass of the protein, the line obtained almost matches with the trend line corresponding to PEG reagents themselves. This clearly indicates that the PEG portion in the PEG-IFN conjugates predominantly contributes to the total size of the conjugate molecule.

In SEC it is a usual approach to calibrate columns using molecular mass standards in terms of distribution

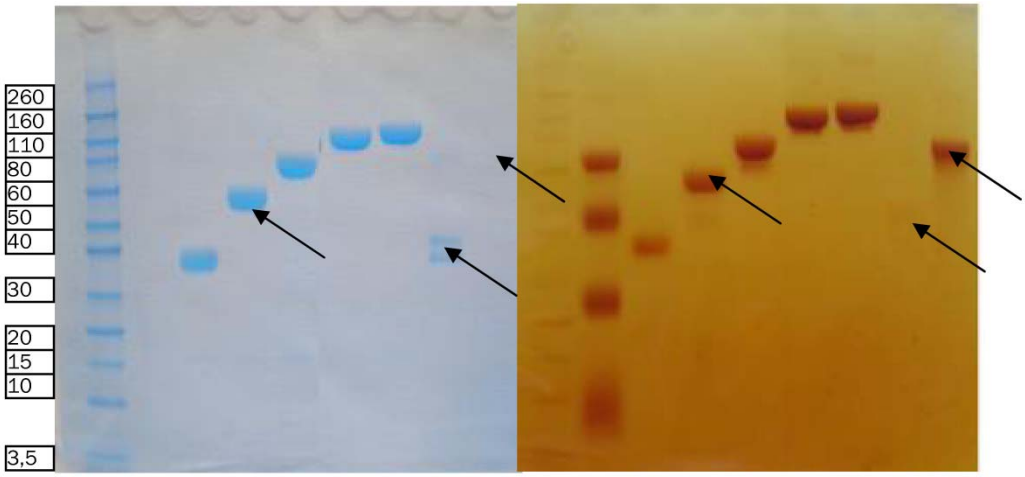

Figure 2: SDS-PAGE of mPEG-OH standards, PEG-IFN conjugates and ovalbumin – Simply Blue Safe stained (a), iodine stained (b)

(Lane 1: Novex Sharp Mw standard; Lane 2: PEG MIX standard (5, 10, 20, and 30 kDa); Lane 3: IFN-10PEG; Lane 4: IFN-20PEG; Lane 5: IFN-30PEG; Lane 6: IFN-40PEG branched; Lane 7: IFN-45PEG branched; Lane 8: Ovalbumin; Lane 9: 40 kDa PEG)

SEC on TSKgel G4000SWxl was also used to determine retention times of protein standards, PEG reagents and various PEG-IFN conjugates bearing PEGs of different lengths and shapes (Fig 3). As expected from previous reports,[4, 5] three distinctly different calibration curves were obtained. Again, regarding the nominal molecular mass, PEG reagents occupy the largest volume and proteins the smallest, while PEG-protein conjugates as hybrid molecules are somewhere in the middle. The same behaviour was found by Fee and Van Alstine (2004).[4] They obtained separate calibration curves not only for PEGs and proteins, but also for pegylated conjugates of each individual protein. In their studies various randomly pegylated conjugates of three different proteins were used, while in our case a series of monopegylated conjugates of human recombinant interferon alpha2b (IFN) were prepared with PEGs of different lengths and shapes attached specifically to the N-terminus of IFN. As already documented by Fee (2007), there is no difference between linear and branched PEGs on SEC, therefore, it is not unexpected that branched and linear PEGs fit well into the same trend line (Fig 3).[5] Similarly PEG-protein conju-

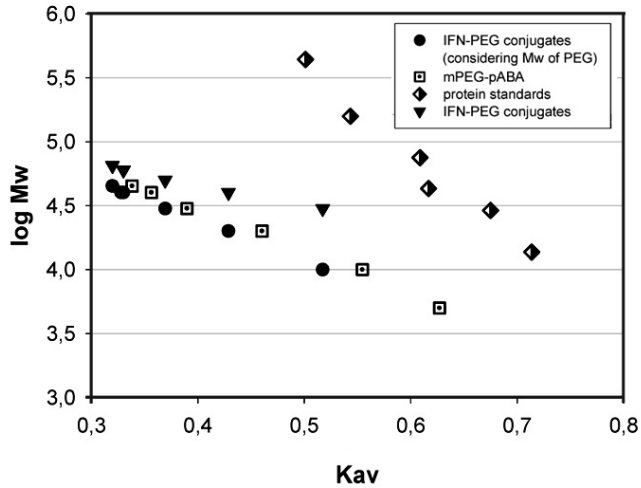

Figure 3: Calibration curves on TSKgel G4000SWxl (Tosoh Bioscience, Japan) for PEG-IFN conjugates (IFN-10PEG, IFN-20PEG, IFN-30PEG, IFN-40PEG branched, IFN-45PEG branched), mPEG-pABZ (5, 10, 20, 30, 40 kDa and branched 45 kDa) and protein standards (Amersham Biosciences) of molecular mass: 13,7, 29, 43, 75, 158 and 440 kDa.

coefficient Kav versus natural logarithm of molecular mass. As the distribution coefficient is not governed by molecular mass but by the size (hydrodynamic volume or viscosity radius) of the molecule, the viscosity radii of proteins and PEG polymer can be calculated on the basis of experimentally defined equations and the same approach was extended to PEG-protein conjugates.[4]

We also investigated influence of PEG on hydrodynamic radius by dynamic light scattering (DLS) measurements, which are shown in Fig 4. Similarly to behaviour on SEC, hydrodynamic radii of PEG standards and PEG-protein conjugates differ substantially from radii of proteins having similar molecular mass. Again three separate calibration curves are obtained for proteins, PEGs, and PEG-protein conjugates. Interestingly, the experimentally determined equation of the trendline for linear PEG standards (eq 1) is rather similar to the published equation (eq 2) that was established on the basis of SEC data.[4]

$$R_{h,PEG} = 0{,}0201\ Mw^{0,556} \quad (1)$$

$$R_{h,PEG} = 0{,}0191\ Mw^{0,559} \quad (2)$$

In contrast, our DLS measurements of protein standards and PEG-IFN conjugates do not match so well with literature results obtained on the basis of SEC. One of the possible explanations for this disagreement could be the solvent used in our DLS measurements, which is probably not optimal and appropriate for all proteins.

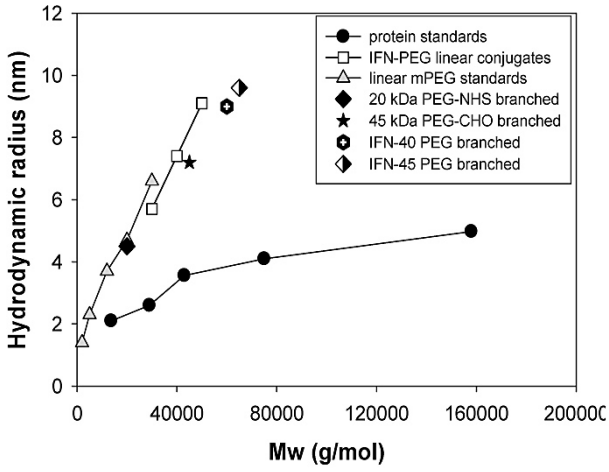

Figure 4: DLS measurements of hydrodynamic radius of linear mPEG standards, branched PEG reagents, protein standards and PEG-IFN conjugates.

Experimentally determined radii of 20 kDa and 45 kDa branched PEG reagents appear somewhat smaller than expected on the basis of the curve for linear PEGs. This phenomenon is even more expressed in PEG-IFN conjugates: DLS measurements of conjugates with branched PEGs do not match well with conjugates containing linear PEGs (Fig 4). In DLS, protein conjugates with branched structured PEGs behave as entities of smaller size than the conjugates with linear PEGs of the same nominal molecular mass. This is in contrast to SEC results where no difference in size between conjugates with branched and linear PEGs has been observed (Fig 3). Also according to the literature data,[4 5] there is no difference among linear and branched PEGs on SEC, and the size of the PEG-protein conjugate is determined by molecular mass of the protein and total weight of the grafted PEG. Smaller size of branched PEG conjugates determined by DLS might suggest a more compact structure than in the case of conjugates with linear PEGs. However, to make valuable conclusions about influence of PEG structure on shape and size of PEG-protein conjugates more DLS measurements (also with other proteins) should be performed.

Random pegylation as the most common type of PEG-protein conjugation is usually performed with PEG reagents suitably activated to react with amino groups on the protein surface, most frequently with the ε-amino groups of the lysine residues. Due to abundance of lysines, random pegylation usually proceeds quickly and results in heterogeneous mixtures of various pegylated species. Even monopegylated conjugates containing only a single PEG moiety in the molecule can differ significantly depending on the position and environment of the lysine residue that has reacted with PEG. All methods mentioned so far, SEC, SDS-PAGE and DLS, separate or characterize molecules according to the number of the attached PEG chains, but they are quite ineffective for resolving such positional isomers. For this purpose, ion exchange chromatography appears to be the method of choice. Using cation exchange chromatography (CEX) mixtures of randomly mono-pegylated IFN can typically be resolved in numerous peaks.[9 15] Different lysines as the main objects of random conjugation have different contributions to overall net charge of the conjugate, or interpreted differently, the pegylated lysine residues contribute specific changes to the microenvironment charge. The resolving power of cation exchange chromatography has already been observed in numerous studies and well exploited for isolation and analysis of pegylated proteins.[9, 16, 17]

On the other hand, our purified conjugates were monopegylated only at one, well-defined attachment site, the N-terminus of interferon alpha 2b (confirmed by peptide mapping, results not shown). Theoretically the N-terminal pegylation should not significantly change the charge of the protein.[18] Furthermore, the conjugates of the same protein bearing different PEGs, but all attached site-specifically to the N-terminus, have the same net charge and according to the principles of ion exchange chromatography no charge-based separation are possible. If

the net charge of the conjugate were the only factor determining the eluting behaviour, our N-terminally pegylated conjugates would have the same retention times. Fig 5 shows the CEX chromatograms of various pegylation mixtures after reaction of IFN with PEG-CHO reagents of different lengths and shapes. In each chromatographic profile, the largest peak corresponds to the N-terminally pegylated IFN conjugate. Peaks eluted earlier in the gradient are higher pegylated (tri-pegylated, di-pegylated) or belong to IFN species pegylated at lysines and not at the N-terminus. The last eluted peak is always the un-pegylated IFN. Fig 5 indicates that in the case of IFN, PEG size substantially affects the retention time: the longer the PEG chain of the N-terminally monopegylated IFN, the shorter the retention time. This is another evidence that PEG exerts a strong shielding and/or steric effect resulting in weakening of the interaction between the protein and the chromatographic medium. As already observed by Seely and coworkers,[16, 19] in separations of pegylated proteins PEG-to protein mass ratio primarily rules the elution order.

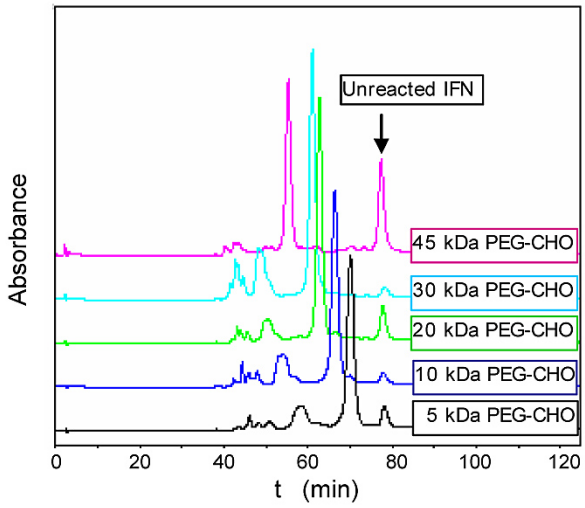

Figure 5: Chromatograms of pegylation mixtures of IFN with various mPEG-CHO reagents of different lengths and shapes (5 kDa linear, 10 kDa linear, 20 kDa linear, 30 kDa linear and 45 kDa branched) on TSK-gel SP-5PW column (Tosoh Bioscience, Japan).

Even more interestingly, on cation exchange column the relationship between the retention time and logarithm of molecular mass has been found almost linear for the whole series of N-terminally pegylated IFNs (Fig 6). The only exception is the PEG-IFN conjugate bearing a 45 kDa branched PEG. If we omit this conjugate, the linear calibration curve with r^2 of 0.999 is obtained, which is almost incredible for a chromatographic method not related to the size of the molecules. The branched PEG-IFN conjugate shows shorter retention than expected on the basis of its molecular mass. Most probably, the branched structured PEG shields protein surface more effectively than a linear PEG of the same molecular mass, which also explains the frequently mentioned superior biological effects of branched PEG conjugates.[20, 21]

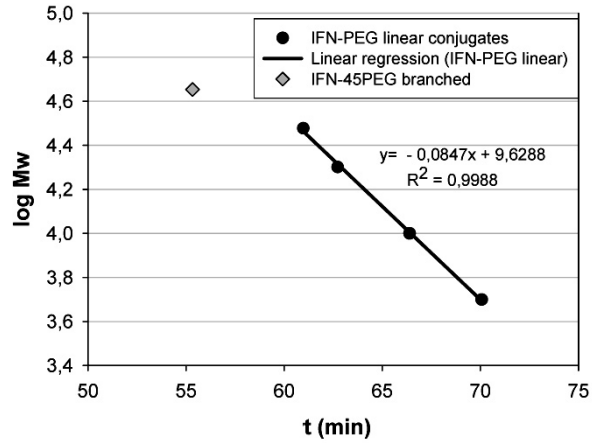

Figure 6: Calibration curves for CEC analysis of N terminally pegylated IFN conjugates shown in Fig 5.

Reversed phase chromatography (RP-HPLC) also gave some unexpected results (Fig 7). It has long been known that PEG is capable of binding several water molecules per each ethylene oxide unit. PEG is also generally anticipated to possess hydrophilic properties and consequentially pegylated proteins should exhibit shorter retention times on reversed phase chromatography than their un-pegylated counterparts. On the contrary, on reversed phase chromatography columns, distinctly longer retention times are usually observed for pegylated proteins. Retention is also increased with increasing molecular mass of PEG (Fig 7), which is consistent with its somehow hydrophobic nature. This is also the reason why PEGs themselves are retained and can be separated on RP matrix.[14] As seen from Fig 7, PEG-to protein mass ratio is again the main factor ruling the elution order. Although

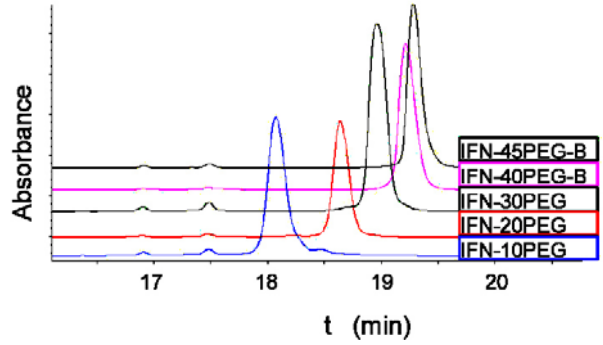

Figure 7: Reversed phase chromatography of PEG-IFN conjugates: IFN-10PEG, IFN-20PEG, IFN-30PEG, IFN-40PEG branched, IFN-45PEG branched.

RP- HPLC, in contrast to CEX, is incapable of separating positional isomers, it possesses an unexpected ability to separate pegylated proteins on the basis of the PEG length and total weight of the grafted PEG, respectively. Interesting is also RP-HPLC calibration curve of conjugates. Which gives us similar picture to CEX calibration curve, with excellent logarithmic agreement (Fig 8).

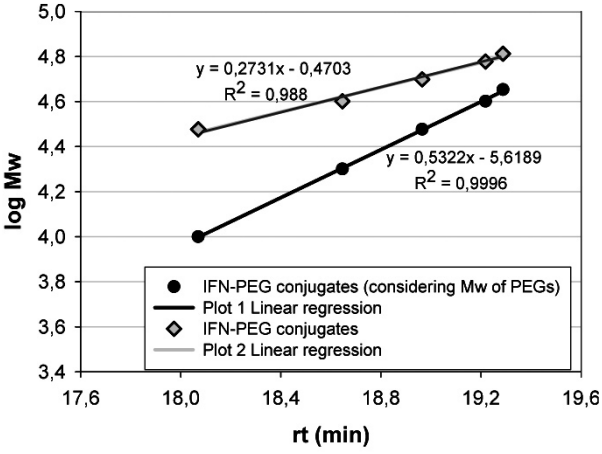

Figure 8: Calibration curves for RP-HPLC analysis of PEG-IFN conjugates shown in Fig 7.

4. Conclusion

We have prepared a series of N-terminally pegylated conjugates of interferon alpha 2b, bearing PEGs of different lengths and shapes. For characterization, various methods have been investigated with intention to obtain relevant data about the size of PEG-protein conjugates. Additionally to methods commonly used for estimating molecular mass and size, such as SEC, SDS-PAGE and DLS, chromatographic methods based on ion exchange and hydrophobicity were also applied. The last two methods are not related to the size of molecules, but their use resulted in very interesting correlations between elution times and molecular mass of the PEG-IFN conjugates.

Although biological and pharmacological properties of pegylated proteins were usually connected with their hydrodynamic radii and superior pharmacological properties of branched PEG-protein conjugates were mostly explained on the assumption of increased hydrodynamic radii (by comparison with conjugates bearing linear PEGs of the same nominal weight),[20 21] our studies do not confirm these hypotheses. Similarly to some recent literature data[22] our SEC experiments have not revealed any size differences among linear and branched PEGs of the same nominal weight. Our DLS experiments (Fig 4) have shown even smaller hydrodynamic radii for branched conjugates, which are indicative of more compact structure. Increased half-life of branched PEG-protein conjugates could be probably ascribed to more pronounced charge shielding effects of the branched structured PEGs. However, establishing the real cause of increased half-life of branched PEG-protein conjugates is beyond the scope of this article.

5. Acknowledgements

This study was initiated by our dear colleague and inspiring leader Dr. Viktor Menart who unexpectedly passed away on Feb. 1st, 2007 leaving behind a lot of unfinished work. We are grateful for the opportunity of having worked with him and we will do our best to continue his work.

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Povzetek

Pegilacija kot danes najbolj razširjena metodologija za izboljšanje bioloških zdravil povzroča precejšnje spremembe fizikalno-kemijskih in bioloških lastnosti proteinov. Pri pegiliranih proteinih je večina njihovih pozitivnih farmakoloških učinkov povezana s povečanjem hidrodinamskega volumna oziroma velikosti molekule. Za študij vpliva polietilenglikola (PEG) na velikost molekule smo pripravili serijo dobro definiranih konjugatov interferona alfa 2b, ki imajo PEGe različnih dolžin in oblik vezane specifično na N-konec proteina. Za karakterizacijo smo uporabili različne metode, ne le gelsko izključitveno kromatografijo, elektroforezo na poliakrilamidnih gelih in dinamično sipanje svetlobe, ki so običajne za določanje molekulske mase in velikosti molekul, ampak tudi ionsko izmenjevalno kromatografijo in kromatografijo na reverzni fazi. Čeprav separacije na osnovi naboja in hidrofobnosti niso direktno povezane z velikostjo molekul, smo ugotovili zelo zanimive korelacije med molekulsko maso pegiliranih konjugatov interferona in elucijskim profilom pri omenjenih vrstah kromatografije.