Bioconjugate Chemistry

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Conjugation of PolyPEG to Interferon Alpha Extends Serum Half-Life while Maintaining Low Viscosity of the Conjugate

B. Podobnik,*[,†] B. Helk,[‡] V. Smilović,[†] Š. Škrajnar,[†] K. Fidler,[†] S. Jevševar,[†] A. Godwin,[§] and P. Williams*[,∥]

[†]Sandoz Biopharmaceuticals, Mengeš, Lek Pharmaceuticals d.d., Kolodvorska 27, SI-1234 Mengeš, Slovenia

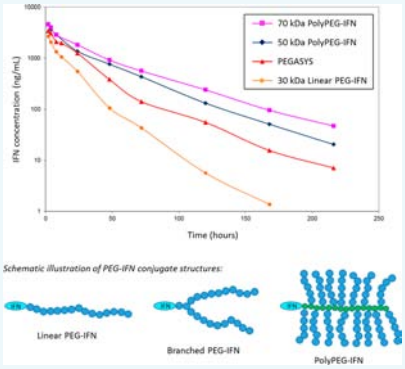
[‡]Novartis Pharma AG, CH-4056 Basel, Switzerland

[§]PolyTherics Ltd, Babraham Research Campus, Babraham, Cambridge CB22 3AT, United Kingdom

[∥]PolyTherics Ltd, Unit 4, Vanguard Centre, University of Warwick Science Park, Coventry, CV4 7EZ, United Kingdom

Supporting Information

ABSTRACT: The covalent attachment of poly(ethylene glycol) (PEG) to therapeutic proteins is a commonly used approach for extending *in vivo* half-lives. A potential limitation of this PEGylation strategy is the adverse effect of PEG on conjugate viscosity. Interferon-alpha (IFN) was conjugated via its N-terminal amino group by reductive amination to α-aldehyde functional comb-shaped PolyPEG polymers (50 and 70 kDa) and to linear PEG (30 kDa). *In vitro* potencies of the purified PEGylated IFN conjugates were measured by reporter gene assay using a HEK293P/ISRE-SEAP cell line. IFN levels were measured in rats following intravenous injection. Viscosities of various linear PEG and PolyPEG polymers along with the polymer−IFN conjugates were determined using a rotational rheometer with cone-and-plate geometry. *In vitro* potencies and half-lives of the PEGylated IFN conjugates were compared with those of the marketed branched PEG−IFN conjugate PEGASYS. Both PolyPEG−IFN conjugates retained a similar potency as that of the marketed comparator, whereas the linear PEG−IFN conjugate potency was greater. All conjugates showed extended half-lives compared to that of naked IFN, with the PolyPEG conjugates exhibiting the longest half-lives and the linear PEG conjugate, the shortest. Viscosity analysis showed that the linear PEG−IFN conjugate was over twice as viscous as both PolyPEG conjugates. Taken together, this work demonstrates the potential of PolyPEG conjugation to therapeutic proteins as a novel tool for optimizing pharmacokinetic profiles in a way that potentially allows administration of high-dose formulations because of lower conjugate viscosity.



INTRODUCTION

The covalent conjugation of poly(ethylene glycol), PEG, to therapeutic proteins is a clinically successful strategy for extending serum half-life to reduce administration frequency.[1−3] Currently, there are at least 10 PEGylated products with marketing approval by the FDA and numerous other conjugates entering clinical trials.[4] The optimization of pharmacokinetics is a key development requisite of biopharmaceuticals to maximize efficacy and safety. While there are other approaches to half-life extension, including Fc and albumin/albumin binding domain fusions,[5,6] altered glycosylation,[7] and nanoparticle platforms,[8] PEGylation remains a widely applicable, and arguably the most clinically validated, method for improving the efficacy of rapidly cleared protein-based medicines.

More recent concerns about the immunogenicity of PEG and the possibility of anti-PEG antibodies being generated, which would present safety and efficacy risks, have been challenged, and a consensus has yet to be reached.[9] As far as the authors are aware, no PEG−protein conjugates have failed due to the safety profile of the PEG component. Furthermore, the non-human-derived, highly immunogenic enzymes, uricase[10] and asparaginase,[11] are PEGylated due to the immune-masking effects of PEG.

In addition to PEG, many other water-soluble polymers such as polysaccharides[12] and poly(methacrylates)[13] have also been shown to extend the serum half-life of biotherapeutics in preclinical and clinical studies. Polymer conjugation extends circulation times principally by reducing renal clearance rates, as the conjugates have larger hydrodynamic volumes that retard glomerular filtration. In addition to size, rigidity, charge, and architecture can all affect filtration rates of molecules eliminated through the kidney.[14] One limitation of conjugating synthetic polymers with large hydrodynamic volumes to proteins is their adverse effect on protein viscosity. Maintaining a low viscosity is important, as therapeutic proteins for systemic use tend to be given by injection and it becomes difficult to administer highly viscous solutions through needles of a suitable bore size that do

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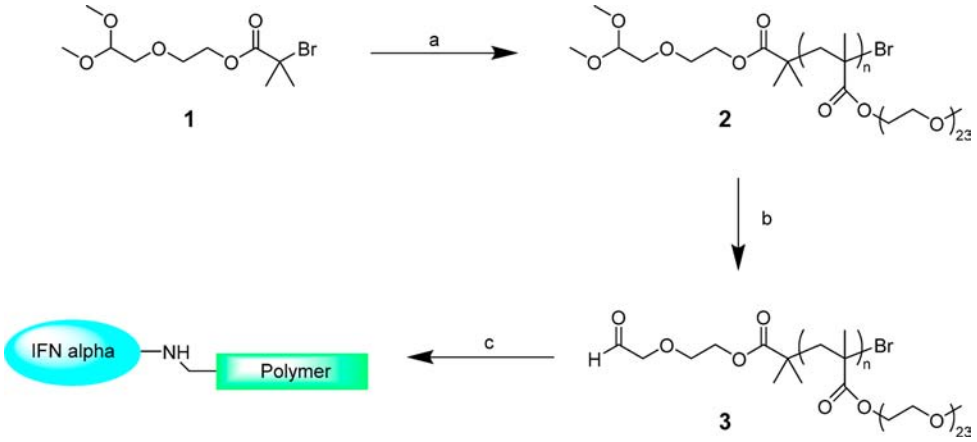
Scheme 1. Synthesis of α-Aldehyde Functional PolyPEGs and Conjugation to IFN-Alpha[a]



[a]Reagents and conditions: (a) $Cu^{I}Br$/*N*-(ethyl)-2-pyridylmethanimine/(methoxyPEG$_{1100}$)-methacrylate, toluene, 50 °C; (b) CF_3COOH/H_2O 1:1, 30 °C; (c) $NaCNBH_3$, sodium acetate buffer, pH 4.5, 22 °C.

not lead to unnecessary discomfort for the patient. Achieving a suitable viscosity post polymer conjugation is also important for fill and finish manufacturing, especially where the therapeutic dose is large.

The molecular size of PEG−protein conjugates is intrinsic to their observed *in vitro* potencies,[15] *in vivo* half-lives, and ultimate efficacy. Consequently, the hydrodynamic behavior of linear and branched PEG−protein conjugates has been well-studied.[16−19] It has also been proposed that the increased *in vivo* half-lives exhibited by branched PEG conjugates over those of linear PEG conjugates cannot be explained by a difference in size alone, as their respective hydrodynamic volumes can be very similar.[17] The observed longer circulation times of branched PEG conjugates may be due, in part, to differences in conformational flexibility and an umbrella-like shape of the polymer, which leads to greater masking of the protein surface relative to the linear PEG comparators.[20] While di- and tetra-branched PEG conjugation has been shown to lead to longer circulation half-lives, as well as affording improved proteolytic stability compared with linear PEG conjugation with the same total molecular weight of PEG conjugated,[21] little is described in the literature on the effect of conjugating highly branched, comb-shaped polymers on protein half-life and, more particularly, on the viscosity of the resultant protein−polymer conjugates.

Poly(poly(ethylene glycol)) or PolyPEG is a polymerized version of low MW linear PEG. The polymer possesses pendent PEG chains (or teeth) at every repeat unit of a poly(methacrylate) (or acrylate) backbone, giving a comb-like structure. PolyPEGs can be prepared by a variety of living radical polymerization techniques[22−26] using commercially available (meth)acrylic PEG-based monomers, including transition-metal-mediated living radical polymerization (TMM-LRP), often called atom transfer radical polymerization (ATRP).[22,23] This technique provides tailored, α-functional polymers of targeted molecular weight and low polydispersity.[27] Polymerizing PEG in this way affords a polymer suitable for protein conjugation that has the potential to limit the increase in viscosity of the protein−polymer conjugate while also extending the serum half-life.

Many studies have shown the potential of PolyPEG as a covalent conjugating polymer for biological therapeutics, including siRNAs, and biological surfaces.[28−33] For example, maleimide[34−36] and aldehyde[37−40] end-functionalized PolyPEGs have previously been described for thiol and N-terminal amine conjugation, respectively, as well as NHS ester functionalized PolyPEG for nonspecific amine conjugation.[41,42] There are also accounts of the direct growth of PolyPEG from modified biological molecules, which represents an alternative approach to postpolymerization conjugation.[43−46]

In this study, two α-aldehyde-functionalized PolyPEGs of different molecular weight were conjugated to interferon-alpha (IFN), and the relative viscosities and *in vitro* and *in vivo* profiles of the conjugates were compared with those of a linear PEG−IFN conjugate and a branched PEG−IFN conjugate (PEGASYS).

RESULTS AND DISCUSSION

Synthesis of α-Aldehyde-Functionalized PolyPEGs. The two α-aldehyde-functionalized PolyPEGs (PolyPEG-CHO) used in this study were prepared by Cu^{I}/iminopyridine-catalyzed living radical polymerization. Polymerization of poly(ethylene glycol) methyl ether methacrylate using the acetal-protected aldehyde initiator **1** yielded polymer intermediate **2**, which, following deprotection by treatment with TFA/water, furnished aldehyde functional PolyPEG **3** (Scheme 1). Since PolyPEG is a comb-shaped polymer with a poly(methacrylate) backbone and pendent PEG chains, conventional narrow molecular weight linear PEGs are unsuitable calibration standards to enable molecular weight determination by size exclusion chromatography (SEC). Therefore, the molecular weights of the two PolyPEGs used in this study were determined by (i) end-group analysis using ^{1}H NMR spectroscopy by comparison of the integrals of the α-terminal aldehyde proton peak with the terminal methoxy proton peak of the pendant PEG teeth (^{1}H NMR spectra of products **2** and **3** can be found in the Supporting Information, S1 and S2, respectively) and (ii) by SEC-MALS (multi angle light scattering) analysis. The molecular weights measured by both characterization methods (i) and (ii) were in broad agreement (Table 1), with the SEC-MALS values being slightly lower but within 15% difference. Analysis of an additional series of five α-functional PolyPEG polymers with molecular weights ranging from 28 to 88 kDa by methods (i) and (ii) also gave values within ±15% (data not shown), indicating that the NMR method was reliable despite the large size of the PolyPEGs.

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Table 1. PolyPEG Molecular Weight Determination by ^{1}H NMR Spectroscopy and SEC-MALS Analysis

PolyPEG-CHO	M_n by ^{1}H NMR (kDa)	M_w by SEC-MALS (kDa)
50 kDa PolyPEG	48.6	42.4
70 kDa PolyPEG	74.7	72.6

The aqueous SEC elution profiles of the two PolyPEGs (50 kDa and 70 kDa) were compared with that of 30 kDa linear PEG (Figure 1). Both PolyPEG samples eluted later than the linear PEG despite their larger masses, thereby demonstrating smaller hydrodynamic volumes. Highly branched comb-shaped polymers, sometimes referred to as molecular bottle brushes, typically have densely packed, stiff, cylindrical structures due to intramolecular excluded-volume interactions between side chains.[47,48] Solution conformation can also depend on the solvation of the comb polymer backbone, which, if hydrophobic, may, for example, adopt a collapsed form in poor solvents such as water, whereas hydrophilic side chains remain solubilized. Such differences in physical properties between polymer architectures could potentially lead to differentiated *in vitro* and *in vivo* behavior of the respective protein conjugates. Consequently, PolyPEGs of molecular weights 50 and 70 kDa were considered interesting for comparative IFN conjugation studies against 30 kDa linear PEG and 40 kDa branched PEG (PEGASYS) IFN conjugates both *in vitro* and *in vivo*.

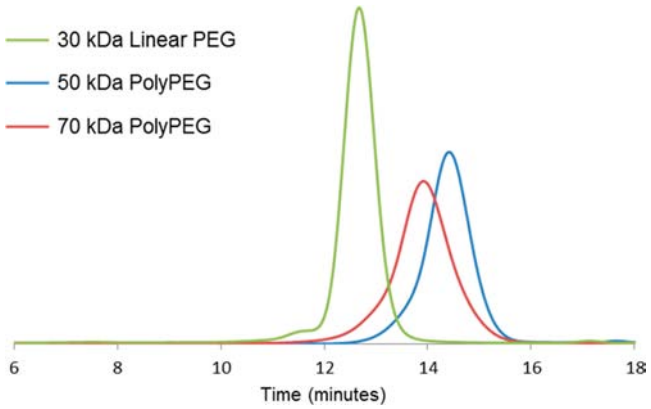

Figure 1. Overlaid aqueous SEC chromatograms of 30 kDa linear PEG and the later eluting 50 and 70 kDa PolyPEGs (differential refractive index detection).

Preparation of IFN Conjugates. The N-terminal amino group of the IFN was targeted for conjugation via reductive amination using aldehyde end-functionalized versions of the 50 and 70 kDa PolyPEG and 30 kDa linear PEG. Although reductive amination is considered to be broadly a site-specific method of conjugation, no optimization of the conditions were attempted. Exploiting the difference in the pK_a of a single N-terminal amino acid to achieve high-yielding and homogeneous imine formation in an aqueous medium is not generally efficient in terms of reagent stoichiometry, so an excess of both the PolyPEG-CHO and linear PEG reagents (8 and 6 mol equiv, respectively, to protein) was used to promote reaction without further optimization.

Each reaction mixture was purified in a single step by cation exchange chromatography (CEX) to isolate mono-PEGylated protein. The fractions obtained from the CEX were analyzed by SDS-PAGE, and the purest fractions were pooled (Figure 2). Similar to conventional linear PEG conjugates, protein species conjugated with PolyPEG migrate with a larger apparent MW

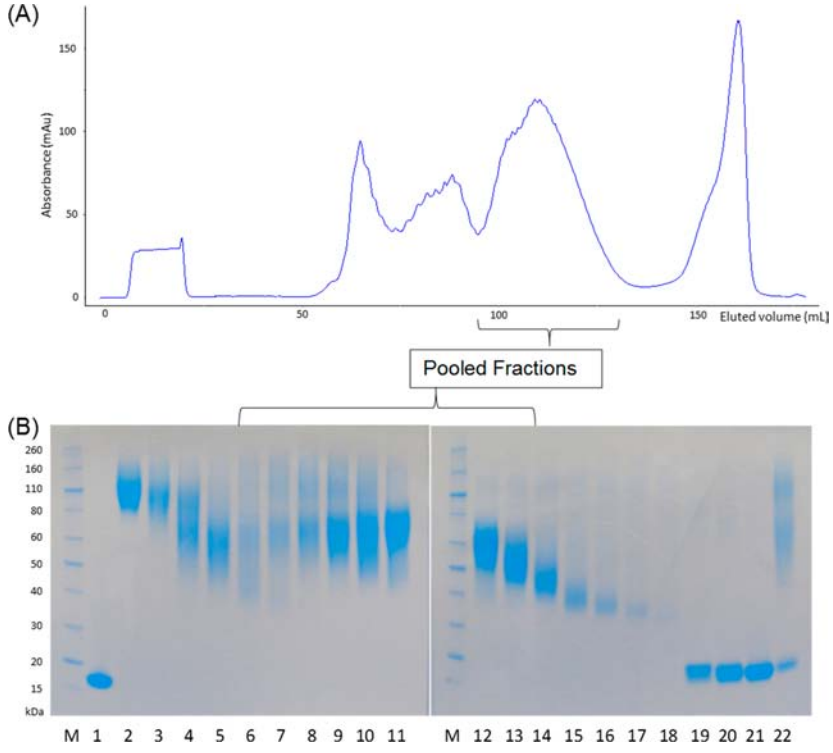

Figure 2. Isolation of 50 kDa PolyPEG–IFN conjugate by cationic exchange chromatography: (A) CEX chromatogram from purification of 50 kDa PolyPEG–IFN conjugate. (B) SDS-PAGE analysis of CEX fractions. Lanes: M, molecular weight marker proteins; 1, native IFN; 2–5, nonpooled early eluting fractions; 6–13, pooled 50 kDa PolyPEG–IFN conjugate fractions; 14–21 nonpooled later eluting fractions; 22, reaction mixture.

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than the theoretical combined MW of the protein and polymer by SDS-PAGE (when compared to protein standards). Unlike conventional PEG conjugates, PolyPEG conjugates do not migrate as tight bands by SDS-PAGE.[34,36] Instead, the bands were more diffuse, both for the PolyPEG polymer itself and for the resultant conjugates. The smearing pattern is believed to be a consequence of electrostatic repulsions between the poly(methacrylate) backbone of PolyPEG and SDS, which inhibit association of the PEG teeth with the surfactant[49] and are also observed with other water-soluble poly(methacrylate) polymers.[13] In addition, we have observed that the mobility of PolyPEG on SDS-PAGE can differ from that of linear PEG. Whereas conventional linear and branched PEGs migrate at approximately twice their apparent MW against protein standards, PolyPEGs with 1 kDa PEG teeth migrate at approximately 1.5 times their true MW against protein standards, e.g., a 50 kDa PolyPEG runs like a 75 kDa protein.

Following purification, SEC-HPLC analysis of the various conjugates using a Tosoh TSKgel column, suited to the analysis of biopolymers due to its low protein adsorption characteristics, showed that the two PolyPEG–IFN conjugates eluted later than the linear PEG–IFN conjugate, despite having higher molecular weights (Figure 3). This trend is consistent with the

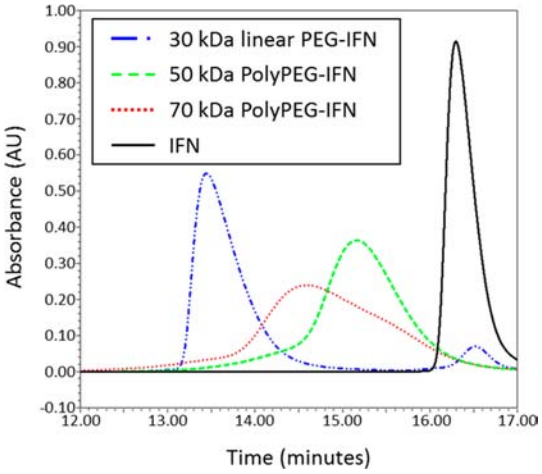

Figure 3. Overlaid SEC-HPLC chromatograms of the 30 kDa linear PEG–IFN conjugate and later eluting 50 and 70 kDa PolyPEG–IFN conjugates (UV detection, 215 nm).

aqueous SEC analysis of the preconjugated polymers of Figure 1 (obtained using PL aquagel-OH columns suited to the analysis of water-soluble polymers over a wide MW range and organic buffer constituents) and demonstrated that the SEC behavior was dominated by the polymer component of the conjugates.

Viscosity Measurements of Polymers and Polymer–IFN Conjugates. The viscosities of each of the conjugates prepared were measured by rotational viscometry and all at a protein concentration, i.e., not including the polymer component, of approximately 0.7 mmol/L. Rotational rheometers measure the torque required to rotate a disk in a fluid, which is a function of the viscosity of that fluid. Zero shear stress viscosity values were determined by plotting shear stress against shear rate diagrams. The results, summarized in Table 2, show that the 30 kDa linear PEG sample was over twice as viscous as both the 50 kDa and 70 kDa PolyPEG–IFN conjugates (8.4 versus 3.8 and 2.9 mPas, respectively).

Table 2. Viscosity Analysis of Linear PEG and PolyPEG–IFN Conjugates Measured at 21 °C

compound	viscosity (mPas) (IFN conjugates at ~c = 0.7 mmol/L)
IFN	not measured
30 kDa linear PEG–IFN	8.4
50 kDa PolyPEG–IFN	3.8
70 kDa PolyPEG–IFN	2.9

Similarly, the solution viscosities of a range of linear PEG and PolyPEG polymers of differing molecular weight and at various concentrations were measured (Figure 4). The 40 kDa linear PEG displayed the highest viscosity, and the 30 kDa PolyPEG had the lowest viscosity.

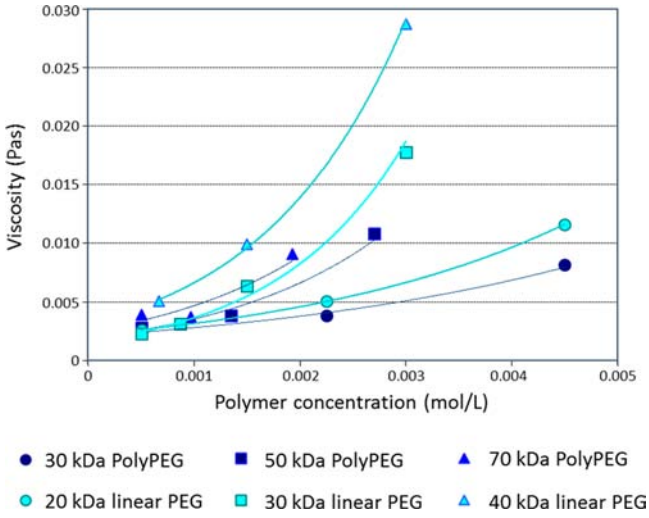

Figure 4. Viscosities of a range of linear PEG and PolyPEG polymers at 21 °C in aqueous solution at various concentrations.

***In Vitro* Potencies of PEG–IFN and PolyPEG–IFN.** The *in vitro* potencies of the (PolyPEG, linear PEG, and branched PEG) IFN conjugates were compared using a HEK293P/ISRE-SEAP reporter cell line assay (Table 3). As expected, all IFN

Table 3. *In Vitro* Potency of IFN, 30 kDa Linear PEG–IFN, 40 kDa Branched PEG–IFN (PEGASYS), 50 kDa PolyPEG–IFN, and 70 kDa PolyPEG–IFN

compound	IFN potency (%)
IFN	100
PEGASYS	1.1
30 kDa linear PEG–IFN	6.3
50 kDa PolyPEG–-IFN	0.8
70 kDa PolyPEG–IFN	0.5

conjugates exhibited a significant reduction in potency compared to that of native IFN, as it is well-established that PEGylation often decreases measured *in vitro* potencies, especially for cytokines, due to reduced on-rates caused by steric shielding effects on the conjugated protein to its receptor or ligand.[50,51] These steric effects are normally adequately compensated by the prolonged *in vivo* residence that the PEG affords to the biotherapeutic, so even small retained potencies, as measured *in vitro*, can still lead to efficacious and effective medicines *in vivo*.

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The marketed branched PEG−IFN conjugate PEGASYS (entry 2 in Table 3) retained only 1.1% of the native IFN potency in the assay. The 50 and 70 kDa PolyPEG conjugates retained a similar level at 0.8 and 0.5%, respectively, whereas the 30 kDa linear PEG conjugate retained most potency (6.3%). While PEGASYS is known to be a mixture of 9 PEG positional isomers, all with different potencies,[52] the potencies of the linear and PolyPEG conjugates can be more directly compared because they were prepared by site-specific conjugation and therefore are expected to be predominately composed of one PEG positional isomer. As the linear PEG conjugate retained the greatest potency, it can be speculated that the degree of steric hindrance on IFN receptor binding by linear PEG and PolyPEG differed and that the more compact solution structure of PolyPEG, as indicated by the SEC analysis, is more sterically hindering.

However, the comparable levels of retained potency for the PolyPEG conjugates against the marketed branched PEG conjugate does not preclude the development of an effective PolyPEG medicine if the PolyPEG conjugates demonstrate sufficiently long serum half-lives *in vivo*. In fact, achieving an optimal balance between pharmacodynamic and pharmacokinetic properties is an advantage of PEGylation, where PEG size and architecture can be matched to the application. It was therefore necessary to next compare the *in vivo* half-lives of the prepared conjugates.

Pharmacokinetics of PEG−IFN and PolyPEG−IFN Conjugates. The pharmacokinetic properties of the prepared linear PEG and PolyPEG−IFN conjugates were evaluated in a rat model along with 40 kDa branched PEG−IFN (PEGASYS). The representative PK profiles are shown in Figure 5, and the

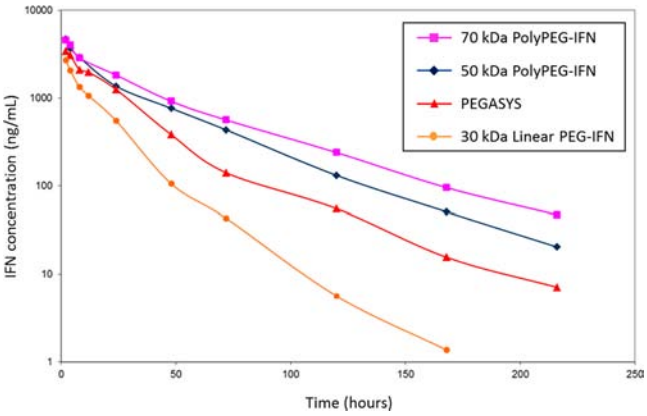

Figure 5. Pharmacokinetic profiles of the 50 and 70 kDa PolyPEG−IFN conjugates and the 30 kDa linear and 40 kDa branched PEG−IFN conjugate (PEGASYS).

elimination half-lives are presented in Table 4. While all conjugates demonstrated a significantly extended half-life over that of naked IFN, there were differences among the conjugates. The two PolyPEG−IFN conjugates (50 and 70 kDa) exhibited the longest half-lives, and the 30 kDa linear PEG−IFN conjugate, the shortest. The order of conjugate half-lives was PolyPEG > branched PEG > linear PEG ≫ free IFN. This observation is despite the smaller solution sizes of the 50 and 70 kDa PolyPEG conjugates compared with that of the 30 kDa linear PEG conjugate.

Table 4. Comparison of Elimination Half-Lives for PEG and PolyPEG−IFN Conjugates Measured in Rats

compound	elimination $t_{1/2}$ (h)
PEGASYS	23.9 ± 6.5
30 kDa linear PEG−IFN	19.9 ± 4.9
50 kDa PolyPEG−IFN	32.0 ± 1.3
70 kDa PolyPEG−IFN	39.8 ± 3.2

Clinically, the efficacy of PEGylated IFN has been shown to be primarily the result of reduced renal clearance and increased blood circulation due to the increased hydrodynamic volume afforded by PEG. PEGylated IFN studies have shown that the size, architecture, and site of attachment of the PEG affects the pharmacokinetics and pharmacodynamics of IFN.[53] Other branched PEG−protein conjugates have also been shown to be more resistant to proteolytic degradation than their linear PEG counterparts[21,54,55] as well as being less antigenic due to increased shielding of the conjugated protein from the immune system. In a separate study, salmon calcitonin (sCT)−PolyPEG conjugates have also demonstrated improved proteolytic resistance *in vitro* when compared to that of sCT−linear PEG conjugates of a similar size.[38] In this study, if *in vivo* clearance is assumed to be governed by the reduction in renal clearance of the conjugates, then the compact and potentially more rigid solution conformation of PolyPEG may be reducing the glomerular filtration rate further compared with that for standard PEGs to improve the pharmacokinetic profile of IFN. Additionally, the steric hindrance of intermolecular interactions *in vivo* may also decrease rates of clearance by reduction of proteolysis and opsonization, resulting in extended *in vivo* half-lives. In turn, the lower *in vitro* potencies of the PolyPEG conjugates relative to those of the linear PEG conjugates may be due to greater steric shielding of the protein by the PolyPEG polymer with its comb-like structure and differentiated solution conformation.[56]

In summary, these results clearly indicate the potential of PolyPEG to improve the pharmacokinetics of protein-based medicines with a lower increase in viscosity post conjugation compared with those of conventional linear PEG reagents, which may aid manufacturing and administration to patients by syringe.

EXPERIMENTAL PROCEDURES

Materials and Methods. All polymerizations were carried out using standard Schlenk techniques under an inert atmosphere of dry nitrogen. *N*-(Ethyl)-2-pyridylmethanimine and 2-bromo-2-methyl-propionic acid 2-(2,2-dimethoxy-ethoxy)-ethyl ester **1** were synthesized as previously reported[57,37] and stored at 4 °C under a nitrogen atmosphere. Copper(I) bromide (Aldrich, 98%) was purified as described elsewhere.[58] Hydrosart membrane, MWCO 10 kDa, filtration area 0.1 m^2, was purchased from Sartorius AG (Goettingen, Germany). Aldehyde-functionalized linear PEG was purchased from NOF (Japan). IFN was produced at Sandoz Biopharmaceuticals using recombinant biotechnology in a microbial expression system. All other reagents and solvents were purchased from Sigma-Aldrich, Fisher Scientific, or Acros and used without any further purification unless otherwise stated.

Preparation of α-Aldehyde-Functionalized PolyPEGs. In a typical polymerization, a mixture of **1** (0.385 mL, 1.85 mmol), poly(ethylene glycol) methyl ether methacrylate ($mPEG_{1100}$) (50 g, 46.3 mmol), and copper(I) bromide (1.50 g, 10.5 mmol) in toluene (100 mL) was deoxygenated by purging with nitrogen for 1 h. Degassed *N*-(ethyl)-2-

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pyridylmethanimine (6.25 mL, 45.7 mmol) was added, and the resultant solution was stirred at 50 °C. At the end of the polymerization, the solution was diluted with toluene (200 mL) and bubbled with air for 7 h. The solution was then filtered through a basic alumina column, and the solvent was removed under reduced pressure to yield an off-white solid, which was dissolved in water and purified by dialysis using a Hydrosart membrane (MWCO 10 kDa). The solution was freeze-dried, and product **2** was obtained as a white powder.

A solution of polymer **2** (5 g, 0.098 mmol) was dissolved in a 1:1 trifluoroacetic acid/water mixture, and the solution was stirred at 30 °C for 16 h. The acid was removed under reduced pressure, and the crude reaction mixture was purified by dialysis as described above. The solution was freeze-dried, and product **3** was obtained as a white powder.

Preparation of PEGylated IFN Conjugates. In a typical PEGylation reaction using PolyPEG, to a solution of IFN (1.7 mg/mL, 20 mM sodium acetate, pH 4.5) were added PolyPEG-CHO (8 mol equiv) and $NaCNBH_3$ (40 mM). The resulting mixture was allowed to incubate at 22 °C for 16 h. Purification was carried out by cationic exchange chromatography on a Toyopearl SP650S column with a linear salt gradient in 20 mM aqueous sodium acetate (pH 4.0). To prepare the corresponding linear PEG conjugate, 6 mol equiv of 30 kDa linear PEG was used.

SEC and SEC-MALS Analysis of PolyPEG Polymers. Aqueous SEC analysis of the PolyPEG-CHO polymers was performed using a high-performance liquid chromatography (HPLC) system fitted with one PL-Aquagel-OH 30, one PL-Aquagel-OH 40 (both 8 μm, 300 × 7.5 mm), and a PL-Aquagel-OH guard column (8 μm, 50 × 7.5 mm) (Agilent Technologies) with differential refractive index detection using a 200 mM $NaNO_3$, 10 mM $NaH_2PO_4{\cdot}2H_2O$ aqueous buffer (pH 7.0) at 1.0 mL/min as the eluent.

SEC-MALS analysis of the PolyPEG-CHO polymers was performed on an Agilent Technologies 1200 series analytical HPLC system fitted with a TSK gel column (G4000SWxl, 8 μm, 300 × 7.8 mm, Tosoh Bioscience, Japan). The mobile phase was 20 mM Na_2HPO_4, 150 mM NaCl, 1% diethylene glycol, and 10% ethanol (pH 6.8) at 0.4 mL/min. Detection was performed at 215 nm. The PolyPEG-CHO samples were assessed by analyzing multi angle light scattering (MALS) signals of the main chromatographic peak eluting by SEC using a miniDAWN TREOS instrument (Wyatt Technology Europe GmbH).

SEC-HPLC Analysis of Polymer−IFN Conjugates. SEC-HPLC analysis of the polymer−IFN conjugates utilized the same HPLC system as that described for the SEC-MALS analysis of the PolyPEG polymers.

Viscosity Measurements of Polymer Solutions and Polymer−IFN Conjugates. Viscosity measurements were performed with the use of a rotational rheometer (Physica MCR 301, Anton Paar) with cone-and-plate geometry using an Anton Paar CP25-1 measuring cell (25 mm diameter, 1° angle). Measurements were recorded at an instrument-controlled temperature of 21 ± 0.5 °C. Different concentrations of PolyPEG-CHO and linear PEG polymers in aqueous solution and the IFN conjugates in 20 mM sodium acetate solution (150−200 mM NaCl) were measured, and zero shear stress viscosity was determined from shear stress against shear rate plots.

Potency of PEGylated IFN Conjugates. The *in vitro* potency of the conjugates was determined using a HEK293P/ISRE-SEAP reporter cell line (Human Genome Sciences, Rockville, MD, USA) containing a transfected ISRE-SEAP cassette.[59] After stimulation with interferon (standard or sample), SEAP (secretory alkaline phosphatase) was secreted into the cell culture medium, and the colorimetric substrate was added. Potency was calculated using PLA (parallel line assay). Results are stated as a percentage of the potency of the starting material (interferon alpha 2b).

Pharmacokinetic Study of PEGylated IFN Conjugates in Rats. The PK study was conducted in young female Wistar rats. PolyPEG−IFN conjugates (50 and 70 kDa PolyPEG), the 30 kDa linear PEG−IFN, and the 40 kDa branched PEG−IFN conjugate (PEGASYS) were administered intravenously via the tail vein at a dose of 50 μg per animal (based on IFN). Each sample was dosed to four animals, and blood samples were collected at predetermined time points: before administration and then 2, 4, 8, 24, 48, 72, 120, 168, and 216 h after application. Blood samples (200−300 μL) were collected via a jugular catheter and centrifuged prior to analysis. For the determination of IFN content, serum samples were analyzed by anti-IFN sandwich ELISA assay using anti-IFN coating antibody (Abcam, ab9386) and anti-IFN−HRP detection conjugate (Abcam, ab5258). The quantification limit of the method was ∼5 ng/mL in serum samples.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Representative ^{1}H NMR spectra of PolyPEG polymers and methods for aqueous SEC characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*(B.P.) E-mail: barbara.podobnik@sandoz.com.

*(P.W.) E-mail: paul.williams@polytherics.com.

Notes

The authors declare no competing financial interest.

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