Reversible PEGylation: A Novel Technology To Release Native Interferon α2 over a Prolonged Time Period

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Many peptide and protein drugs have a short circulatory half-life in vivo. The covalent attachment of polyethylene glycol (PEG) chains (PEGylation) can overcome this deficiency, but pegylated peptides and proteins are often inactive. In this study, we present a novel PEG−IFNα2 conjugate, PEG_{40}-FMS−IFNα2, capable of regenerating native interferon α2 (IFNα2) at a slow rate under physiological conditions. A 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) containing bifunctional reagent, MAL-FMS-NHS, has been synthesized, enabling the linkage of a 40 kDa PEG-SH to IFNα2 through a slowly hydrolyzable bond. By use of a BIAcore binding assay, the in vitro rate of regeneration of native interferon was estimated to have a half-life of 65 h. Following subcutaneous administration to rats and monitoring circulating antiviral activity, active IFNα2 levels peaked at 50 h, with substantial levels still being detected 200 h after administration. This value contrasts with a half-life of about 1 h measured for unmodified interferon. The concentration of active IFNα2 scaled linearly with the quantity injected. Comparing subcutaneous to intravenous administration of PEG_{40}-FMS−IFNα2, we found that the long circulatory lifetime of IFNα2 was affected both by the slow rate of absorption of the PEGylated protein from the subcutaneous volume and by the slow rate of discharge from the PEG in circulation. A numerical simulation of the results was in good agreement with the results observed in vivo. The pharmacokinetic profile of this novel IFNα2 conjugate combines a prolonged maintenance in vivo with the regeneration of active-native IFNα2, ensuring ready access to peripheral tissues and thus an overall advantage over currently used formulations.

Introduction

Type I interferons (IFNs) are proteins that initiate antiviral and antiproliferative responses. Interferons are clinically important, and several subtypes of IFNα2 have been approved as drugs for the treatment of hepatitis B and C, as well as for cancers such as chronic myelogenous leukemia and hairy cell leukemia.[1] Interferons regulate signals through the Janus tyrosine kinase (Jak/STAT proteins) and by reducing phosphorylation and activation of MEK1 and ERK1/2 through Ras/Raf independent pathways.[2] Human type I interferons induce differential cellular effects but act through a common cell surface receptor complex comprising the two subunits Ifnar1 and Ifnar2. Human Ifnar2 binds all type I IFNs but with a lower affinity and specificity than the Ifnar complex. Human Ifnar1 has a low intrinsic binding affinity toward human IFNs but modulates specificity and affinity of other ligands of the Ifnar complex.[3]

IFNα2 may be administered intramuscularly, subcutaneously, or intravenously, resulting in different pharmacokinetic profiles. In any mode, the administered cytokine is rapidly inactivated by body fluids and tissues[4] and cleared from the blood plasma several hours following administration.[5] The major routes of IFNα2 elimination from the circulatory system are through proteolysis, receptor-mediated endocytosis, and kidney filtration.[6]

Prolonging the maintenance dose of IFNα2 in circulation is a desirable clinical outcome. A nonreversible, 12 kDa PEG−IFNα2 was approved as a therapeutic conjugate in 2001. It is administered once a week to hepatitis C patients and facilitates a sustained antiviral response rate of 24%, as opposed to a 12% response rate obtained by the native cytokine[7] (Schering-Plough Corporation (2001), press release). However, while the covalent attachment of PEG chains to proteins prolongs their lifetime in vivo, it often results in a dramatic reduction or even loss of biological and pharmacological activities.[8−15] For example, 40 kDa PEG−IFNα2 has only 7% of the activity of the native cytokine, calling for higher doses to be administered.[16] Furthermore, PEG−IFN does not readily penetrate all tissues; while 12 kDa PEG−IFNα2b is widely distributed, 40 kDa PEG−IFNα2a is restricted to the blood and the interstitial fluid.[17,18] This major drawback can be overcome by designing a PEG−IFNα2 conjugate capable of generating native IFNα2 at a slow rate under physiological conditions.

In this study, we have designed, prepared, and tested such a PEG−IFNα2 conjugate. This was largely facili-

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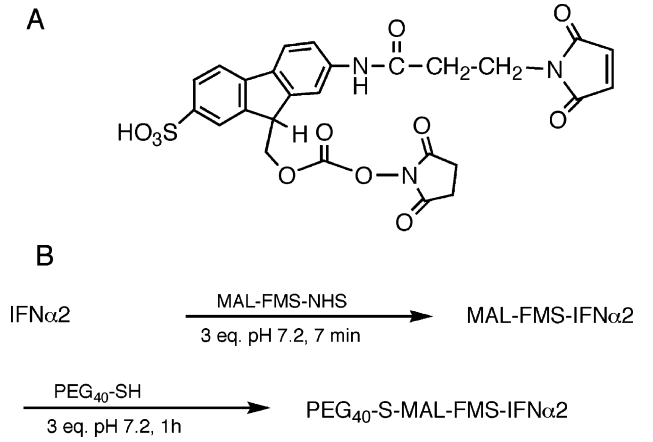


Figure 1. (A) Structure of 9-hydroxymethyl-2-(amino-3-maleimidopropionate)-7-sulfoflourene-*N*-hydroxysuccinimide (MAL-FMS-NHS). (B) Schematic presentation of the steps involved in the preparation of PEG_{40}-FMS−IFNα2.

tated by our previous finding where 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), following linkage to the amino side chains of proteins, undergoes slow, spontaneous hydrolysis under physiological conditions, generating the native parent proteins.[19] The reversibility of the conjugated PEG−IFNα2 derivative is therefore based on the FMS principle. An FMS containing bifunctional reagent (MAL-FMS-NHS, Figure 1) has been synthesized, enabling us to link a 40 kDa PEG-SH to IFNα2 through a slowly hydrolyzable bond. This novel reversibly PEGylated conjugate and its prolonged antiviral activity in vivo are discussed here in detail.

Experimental Section

Materials. Dithiothreitol (DTT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), trinitrobenzenesulfonic acid, and all compounds used for the synthesis of MAL-FMS-NHS (see below) were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO).

Nonglycosylated human IFNα2 was prepared as described in detail by Piehler and Schreiber.[20]

Synthesis of 9-Hydroxymethyl-7-(amino-3-maleimidopropionate)-2-sulfofluorene-*N*-hydroxysuccinimide (MAL-FMS-NHS). The synthesis was initiated from 9-hydroxymethyl-2-aminofluorene.[21] Following three steps, the product was obtained in 48% overall yield.[22]

PEG_{40}-SH was prepared by dissolving PEG_{40}-OSu at a concentration of 40 mg/mL in an aqueous solution of cystamine−di-HCl (1 M) that was titrated to pH 8.5 with $NaHCO_3$. The reaction was carried out for 2 h at 25 °C. The product thus obtained was dialyzed overnight against 0.1 M $NaHCO_3$, treated with 30 mM dithiothreitol (25 °C, 1 h), and redialyzed against 0.01 M HCl containing 10 mM ascorbic acid. PEG_{40}-SH was obtained in 93% yield. It contained 1 mole of sulfhydryl moiety per mole of PEG_{40}, as determined with DTNB. PEG_{40}-SH was kept frozen until use.

Preparation of PEG_{40}-FMS-IFNα2. To a stirred solution of IFNα2 (1 mg/1.0 mL) in phosphate buffer, pH 7.2 (52 μM), 91 μg of MAL-FMS-NHS was added (9.1 μL from a fresh solution of MAL-FMS-NHS (10 mg/mL) in DMF (3.0 M excess over the protein). After 7 min, PEG_{40}-SH was added to obtain a final concentration of 156 μM (3 M excess over the protein). The reaction was carried out for 1 h and then dialyzed overnight against H_2O to remove residual DMF and phosphate buffer.

Receptor binding affinities were evaluated by BIAcore (SPR Detection) measurements. The BIAcore 3000 system, sensor chips CM5, HBS (10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.05% surfactant P20, pH 7.4), and the amine coupling kit were from BIAcore (Sweden). Chip immobilization by Ifnar2 and the BIAcore measurements were carried out according to Piehler and Schreiber.[23] In short, the extracellular (EC) portion of Ifnar2 was immobilized to the surface using the non-neutralizing anti-Ifnar2-EC mAb 46.10, followed by cross-linking with a second mAb (117.7) (gift from Daniela Novick, Weizmann Institute of Science). The binding curves were evaluated with the BIAevaluation software (Biacore AB, Sweden) using a simple one-to-one kinetic model. To estimate the increase in RU (Resonance Units) resulting from the nonspecific effect of the protein on the bulk refractive index, binding of the protein to a control surface with no immobilized ligand was also measured and subtracted. For the determination of the active interferon concentration, the equilibrium response was plotted against the estimated initial concentration. The data were fitted using KaleidaGraph (version 3.0.4, Abelbeck Software) using the equation

$$y = \frac{(1 \times 10^8) m_1 m_2}{(1 \times 10^8) m_1 m_0 + 1}$$

where a K_A of 1×10^8 was determined independently for IFNα2 binding and fixed for all samples, m_1 is R_u/R_{max} (the percent of active interferon measured in the sample), m_2 is R_{max}, and m_0 is the observed R_u.

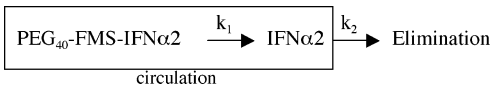
In vivo experiments were performed using male Wistar rats (150−170 g). Rats were injected either subcutaneously or intravenously (0.2 mL/rat). All animal experimentation was performed under approved institutional guidelines.

To validate our animal experiments, three rats were injected with 10 μg/rat of IFNα2. For PEG_{40}-FMS−IFNα2, 12, 60, 120 μg/rat were injected to individual rats in order to determine whether a dose dependency can be observed for the conjugate. Normalization of the various dose-dependent curves per microgram of interferon resulted in a clear overlay of the lines.

Antiviral activity of IFNα2 and its derivatives was determined by the capacity of the cytokine to protect human amnion WISH cells against vesicular stomatitis virus (VSV) induced cytopathic effects.[24]

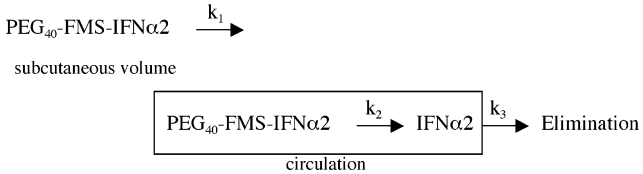
Simulations of experimental data were performed using Pro-Kineticist II, a second-order global kinetic analysis software (Applied Photophysics Ltd., England).

For the iv administration, the following model was considered:



where $k_1 = 0.01\ h^{-1}$ and $k_2 = 0.65\ h^{-1}$ (determined experimentally by antiviral assay).

For the sc administration, the following model was considered:



where $k_1 = 0.02\ h^{-1}$, $k_2 = 0.01\ h^{-1}$, and $k_3 = 0.65\ h^{-1}$.

k_1, which could not be determined directly, was estimated to be $0.02\ h^{-1}$ from the fit of the simulation to the experimental data.

The direct clearance of PEG_{40}-FMS−IFNα2 was assumed to be negligible. It has been established in the literature that

Table 1. Chemical Features of PEG_{40}-FMS_1−IFNα2

characteristic[a]	numerical value
absorbance at 280 nm[b,c]	$\epsilon_{280} = 39\,270 \pm 100$
mass spectra[d]	
PEG-FMS−IFNα2,[e] calculated	63 569 Da
PEG-FMS−IFNα2,[e] measured	63 540 Da
retention time (analytical HPLC)[f]	43 ± 0.5 min
solubility in aqueous buffer, pH 7.4	>20 mg/mL

[a] For characterization, IFNα2−FMS-MAL was dialyzed against H_2O prior to linking PEG_{40}-SH. The final product was filtered through a centricon having a cutoff value of 50 kDa. These procedures remove free MAL-FMS-NHS and any residual native IFNα2 or IFNα2-FMS-MAL that has not been linked to PEG_{40}-SH. [b] Determined by UV spectroscopy. Derivative concentration was determined by acid hydrolysis of a 20 μL aliquot followed by amino acid analysis, calculated according to aspartic acid (14 residues), alanine (9 residues), and isoleucine (8 residues). [c] Native IFNα2 absorbs at 280 nm with $\epsilon_{280} = 18\,070$.[20] [d] Mass spectra were determined by using MALDI-TOF mass spectrometry. [e] Calculated mass is obtained by the additive masses found for native IFNα2 (19 278 Da), for PEG_{40}-SH (43 818 Da), and for the spacer molecule following conjugation (473 Da). [f] Native IFNα2 elutes under an identical analytical HPLC procedure with a retention time of 33.9 min.

the urinary excretion of high molecular weight PEGs and PEG conjugates is extremely low and prolonged.[25]

Results

Preparation and Characterization of PEG_{40}-FMS−IFNα2. The attachment of a single PEG chain of 40 kDa to IFNα2 appears sufficient to grossly arrest kidney filtration of the conjugate.[16] We therefore envisioned that the linkage of a single PEG_{40}-FMS chain to IFNα2 would suffice to obtain a prolonged-acting conjugate that releases IFNα2, with a desirable pharmacokinetic profile. Figure 1A shows the structure of MAL-FMS-NHS synthesized by us, and Figure 1B shows the procedure found to be most optimal for introducing 1 mol of PEG_{40}-FMS/mol of protein. IFNα2 was allowed to react first with 3 equiv of MAL-FMS-NHS, followed by the addition of 3 equiv of PEG_{40}-SH. The NHS function of MAL-FMS-NHS is relatively unstable at prolonged aqueous neutral conditions, whereas the MAL function of the spacer preserves its alkylating capacity for several hours at pH 7.2 (not shown). We therefore preferred to react MAL-FMS-NHS first to IFNα2. PEG_{40}-SH can be subsequently linked to IFNα2-FMS-MAL at any time point afterward (within several hours; see Experimental Section and Figure 1B).

Table 1 summarizes several characteristic features of the conjugate thus obtained. MALDI-TOF mass spectral analysis shows a 1:1 PEG_{40}-FMS/IFNα2 stoichiometry. The experimental mass obtained, 63 540 Da, corresponds to the additive masses found for PEG_{40}-SH (43 818 Da), IFNα2 (19 278 Da), and the spacer molecule following conjugation (473 Da). PEG_{40}-FMS−IFNα2 migrates on analytical HPLC as a wide peak with t_R = 43 min. The conjugate is highly soluble in aqueous solutions. It has a molar extinction coefficient ϵ_{280} = 39 270 ± 100 corresponding to the absorption of the native cytokine ($\epsilon_{280} = 18\,070$ [20]) and of FMS (ϵ_{280} = 21 200[26]).

PEG_{40}-FMS-IFNα2 Releases Native-Active IFNα2 upon Incubation, at a Rate Constant of 0.01 h^{-1}. In the set of experiments summarized in Figure 2, PEG_{40}-FMS−IFNα2 was incubated either in 0.1 M phosphate buffer in the presence or absence of 0.6% BSA and 2 mM sodium azide (NaN_3) (pH 8.5, 37 °C) or in normal human serum (37 °C). At this pH, the rate of FMS hydrolysis from FMS proteins is nearly identical to that obtained in normal human serum, in vitro, or in the circulatory system in vivo.[26−28] Aliquots were drawn at different time points and analyzed for the release of IFNα2 from the conjugate by SDS−PAGE, by Western blot (using the mAb (23.4), gift from Daniela Novick, Weizmann Institute of Science) (Figure 2A), and by BIAcore, measuring the active concentration of IFNα2 according to the law of mass action (Figure 2B). The interferon binding curve on the Ifnar2 surface resembles that of a homogeneous population of native interferon, suggesting that PEG_{40}-FMS−IFNα2 does not bind

Ifnar2. For the SDS−PAGE and Western blot analysis, the amounts of IFNα2 discharged were quantified relative to an IFNα2 reference of known concentration and intensity. The discharge profiles are all in good agreement. Regarding the Western blot, no interferon was detected in untreated normal human serum control. The active interferon observed at time zero in the BIAcore profile (∼10%) is due to native interferon present in the sample. The rate of discharge was determined by fitting the quantity of active interferon to a single-exponential equation (Figure 2C). Accordingly, IFNα2 is released from the conjugate with a rate constant of 0.01 h^{-1} (Figure 2). Upon 66 h of incubation, 50% of the IFNα2 in the conjugate is discharged and is fully active. From the extrapolation of the curve fit obtained, it is assumed that nearly all of the interferon will eventually be released and regain full activity. Extended periods of time at 37 °C may result in protein degradation that can compromise the analysis of the BIAcore data.

Subcutaneous Administration of PEG_{40}-FMS−IFNα2 Dramatically Increased Its Half-Life in Vivo. Initially, to validate our animal experiments, three rats were subcutaneously injected with 10 μg/rat of IFNα2 (Figure 3). Next, we determined the half-life and activity of PEG_{40}-FMS−IFNα2 in rats. Human IFNα2 is not active in rats, although its concentration can be determined from the antiviral activity in rat serum by measuring the VSV-induced cytopathic effects in WISH cells (Experimental Section, antiviral activity assay). Native IFNα2 or PEG_{40}-FMS−IFNα2 was administered subcutaneously to rats. Blood aliquots were drawn at various time points and analyzed for their antiviral activity. Following the administration of the native unmodified IFNα2 (100 μg/rat), the circulating antiviral activity declined with a $t_{1/2}$ value of ∼1 h, reaching a level lower than 20 pM IFNα2, 12 h after administration (Figure 4).

The circulatory behavior of PEG_{40}-FMS−IFNα2 following subcutaneous administration to rats shows a clearly visible dose-dependent behavior (Figure 4). Administration of 12 μg/rat of the conjugated IFNα2 yielded maintenance levels of 70 ± 10 pM IFNα2 that were maintained 56 h following administration. When a 10-fold increase in PEG_{40}-FMS−IFNα2 was administered, IFNα2 was continuously present in the serum for 56 h at 450 pM. Administration of 60 μg/rat of the conjugate resulted in interferon levels of 225 pM at 56 h and of 25 pM at 200 h. Native IFNα2 present in the administered sample (approximately 10% as determined

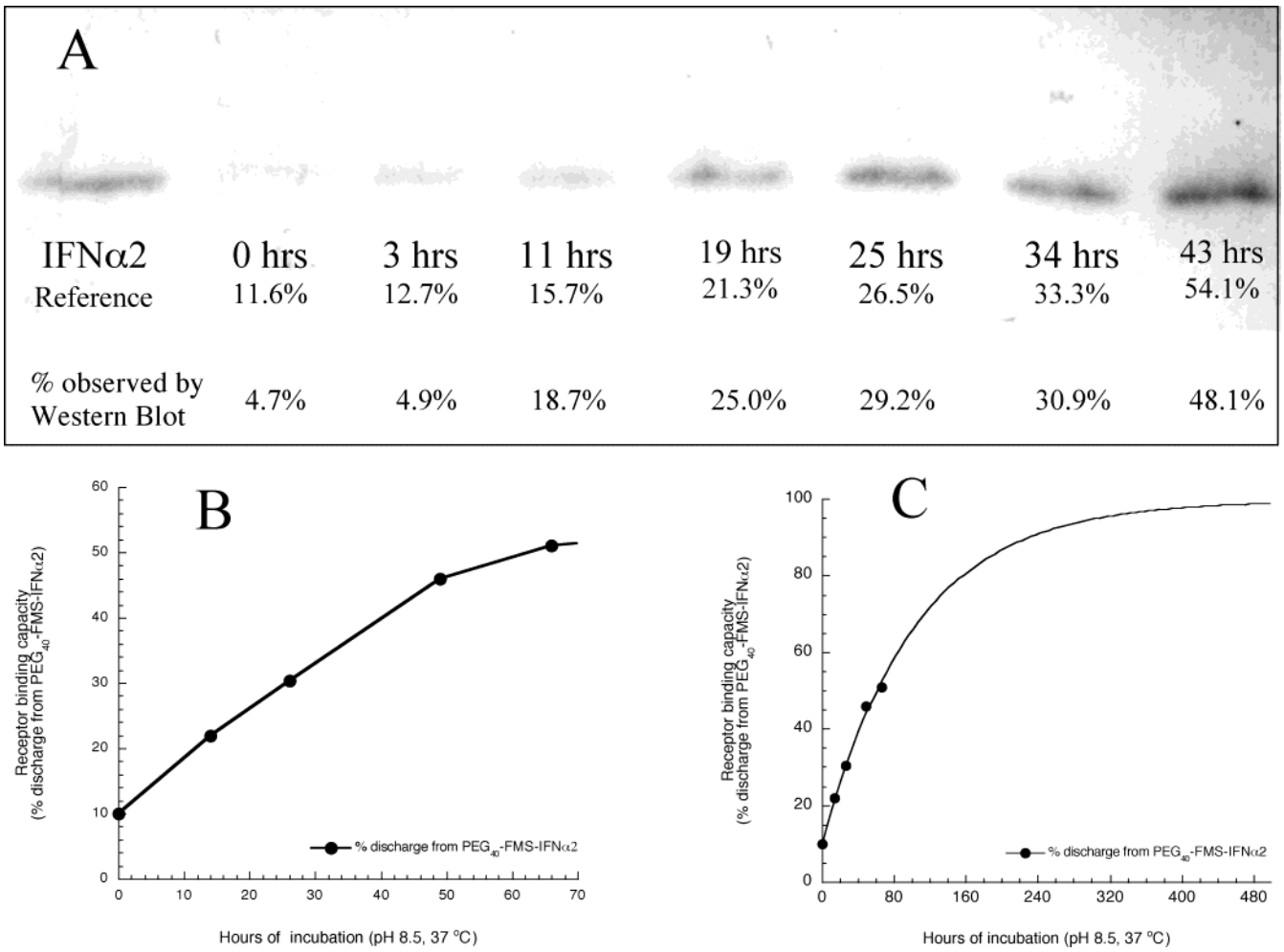


Figure 2. Release of active IFNα2 upon incubation of PEG_{40}-FMS−IFNα2 at pH 8.5, 37 °C, and in normal human serum, 37 °C. PEG_{40}-FMS−IFNα2 (0.3 mg of protein/mL) was incubated in 0.1 M phosphate buffer with 2 mM NaN_3 and 6 mg/mL BSA (pH 8.5, 37 °C) or in normal human serum. At the indicated time points, aliquots were withdrawn. (A) Analysis of % IFNα2 recovery, according to the molecular weight of IFNα2, following discharge from the conjugate by coomassie blue stained SDS−PAGE and by Western blotting using an anti-IFNα2 mAb, 23.4; the amounts of IFNα2 discharge were quantified relative to an IFNα2 reference of known concentration and intensity (the time increments and the percentages are indicated. (B) Aliquots withdrawn at the indicated time points were analyzed for their Ifnar2 binding capacity on BIAcore. (C) Fitted BIAcore profile of native IFNα2 discharge from PEG_{40}-FMS−IFNα2.

by BIAcore) contributed to the initially high levels of IFNα2 observed in the rats' serum. These values display a clearance curve similar to that of native IFNα2. The remaining 90% of the IFNα2 was slowly discharged from the conjugate.

Intravenous Administration of PEG_{40}-FMS−IFNα2 to Rats. To eliminate the contribution of the subcutaneous exchange, both the conjugate and the native cytokine were administered to rats intravenously. For native interferon, the same half-life of ∼1 h was measured, indicating that it readily penetrates the circulatory system following subcutaneous administration (Figure 5). For PEG_{40}-FMS−IFNα2, antiviral activity was still detected 150 h following intravenous administration, demonstrating the prolonged effects of the PEGylated cytokine. A discharged IFNα2 level of 10 pM still remained 150 h after administration, while native IFNα2 was eliminated within 30 h after administration. It is noted that the large shoulder observed following subcutaneous administration of the conjugate (Figure 4) is not observed when PEG_{40}-FMS−IFNα2 was administered intravenously (Figure 5).

It is noted that only a minute percentage of the injected interferon is detected in the serum following administration. We were able to detect ∼1.5% of the total injected amount of interferon to the rats. In the work of Chiang et al., interferon β has been injected to human subjects. At 1 and 2 h following administration, 4−5% and 1.5% of the interferon injected were detectable, respectively.[29] In a recent study, we have also found that only 5% of the active IFNβ reached the circulatory system following sc administration to rats.[30]

Simulations of Experimental Data. By use of the rate constants obtained from both BIAcore data (k = 0.01 h^{-1} for the discharge of IFNα2 from the PEG conjugate) and the antiviral activity assay of native IFNα2 (k = 0.65 h^{-1} for the elimination of interferon), both the subcutaneous and intravenous administration modes of PEG_{40}-FMS−IFNα2 to rats were simulated (parts A and B of Figure 6, respectively). In both cases, the simulated data and the experimental results were in good agreement. As became evident from the simulated data, the passage of the conjugate, but not of the native interferon, from the subcutaneous volume to the

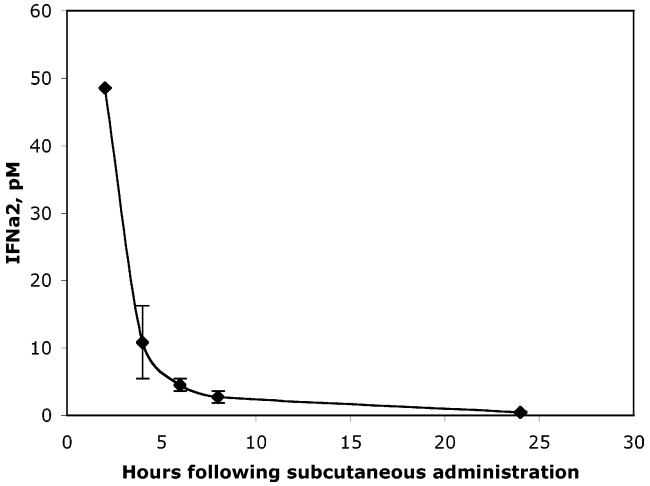


Figure 3. Subcutaneous administration of native IFNα2. A group of three rats received IFNα2 (10 μg/rat in 0.2 mL of PBS). Blood aliquots were withdrawn at the indicated time points. Circulating antiviral activities were determined using human WISH cells with 3-fold serial dilutions of each aliquot (Experimental Section). Endogenous IFNα2 concentrations in the control (untreated rats) did not exceed 5 pM in this assay.

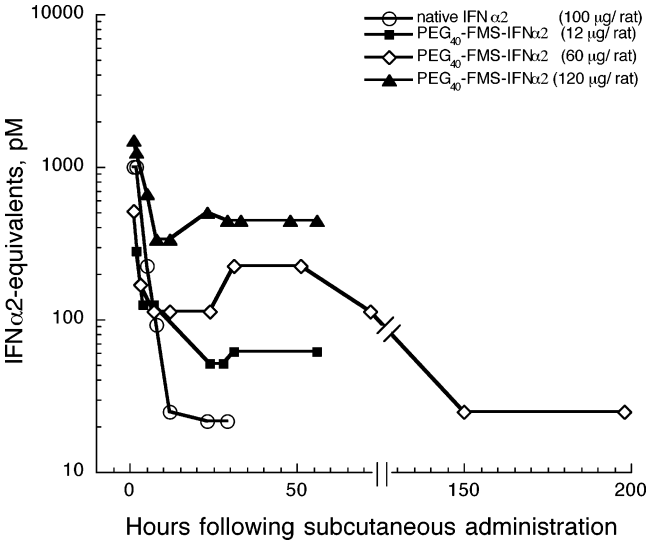


Figure 4. Subcutaneous administration of native IFNα2 and PEG_{40}-FMS−IFNα2. Rats were subcutaneously injected with the indicated concentrations of native IFNα2 or the conjugates (0.2 mL/rat, dissolved in PBS). Blood aliquots were withdrawn at the indicated time points. Circulating antiviral activities were determined using human WISH cells with 3-fold serial dilutions of each aliquot (Experimental Section). Endogenous IFNα2 concentrations in the control (untreated rats) did not exceed 5 pM in this assay.

bloodstream proceeds at a slow rate and is on the order of the discharge of interferon from the conjugate. This explains the shoulder observed between 10 and 70 h in the active protein concentration. As expected, this shoulder is not found when PEG_{40}-FMS−IFNα2 is administered intravenously.

Discussion

Despite the profound advantages often gained by PEGylating therapeutic proteins, this technology suffers from a principal drawback. On one hand, covalently attaching PEG chains to proteins prolongs lifetime in vivo, protecting the conjugates from proteolysis and

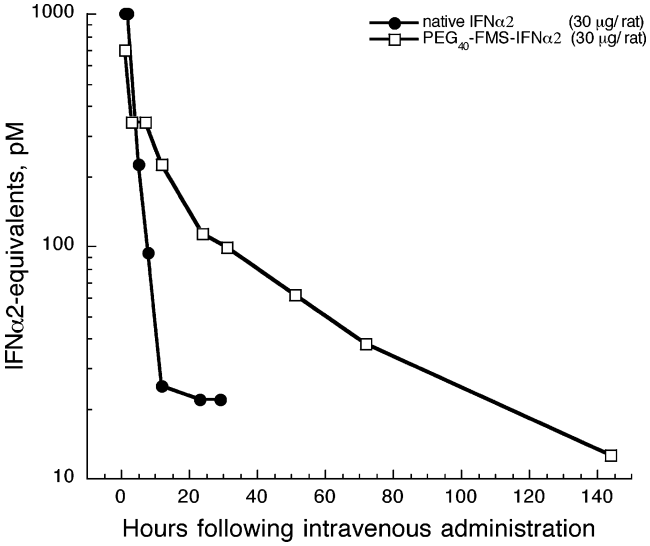


Figure 5. Intravenous administration of PEG_{40}-FMS−IFNα2 to rats. Rats were intravenously injected with the indicated concentrations of native IFNα2 or the conjugate (0.2 mL/rat, dissolved in PBS). Blood aliquots were withdrawn at the indicated time points. Circulating antiviral activities were determined using human WISH cells with 3-fold serial dilutions of each aliquot (Experimental Section). Endogenous IFNα2 concentrations in the control (untreated rats) did not exceed 5 pM in this assay.

shielding them from the immune system. On the other hand, the steric interference of the PEG often leads to a drastic loss in the biological and the pharmacological potencies of the conjugates.[8−15] In principle, this deficiency can be overcome by introducing the PEG chains via a chemical bond that is sensitive to hydrolysis or can be cleaved enzymatically by serum proteases or esterases. Clearly, a consistent rate of hydrolysis is crucial. A prerequisite condition is therefore that the hydrolysis of the PEG chains from the conjugate is to take place at a slow rate and in a homogeneous fashion in vivo.

Two basic irreversible PEG−IFNα2 conjugates are in therapeutic use at present. The first, a 12 kDa PEG−IFNα2b, satisfactorily permeates into tissues. This preparation, however, is relatively short-lived in vivo, since its low molecular mass (calculated mass of 32 kDa) is insufficient to markedly arrest kidney filtration. The second formulation, a 40 kDa PEG−IFNα2a, is an extremely long-lived species in vivo. This conjugate, however, has poor permeability into tissues. Following administration, the conjugate circulates for long periods of time in the blood.[16] We have therefore anticipated that the two prerequisite features for an optimal PEG−IFNα2 conjugate, namely, a prolonged maintenance in vivo combined with free access to peripheral tissues, can be obtained by linking a slowly hydrolyzable PEG_{40} chain to IFNα2.

We have previously found that upon linkage of FMS to proteins it undergoes hydrolysis at physiological conditions with a desirable pharmacokinetic pattern.[26−28,31] The rate of FMS hydrolysis is dictated exclusively by the pH and the nucleophilicity of the serum, both of which are maintained in mammals under strict homeostasis.[32] We therefore based our new development on the FMS principle. In neutral, aqueous solutions, FMS moieties undergo slow, spontaneous

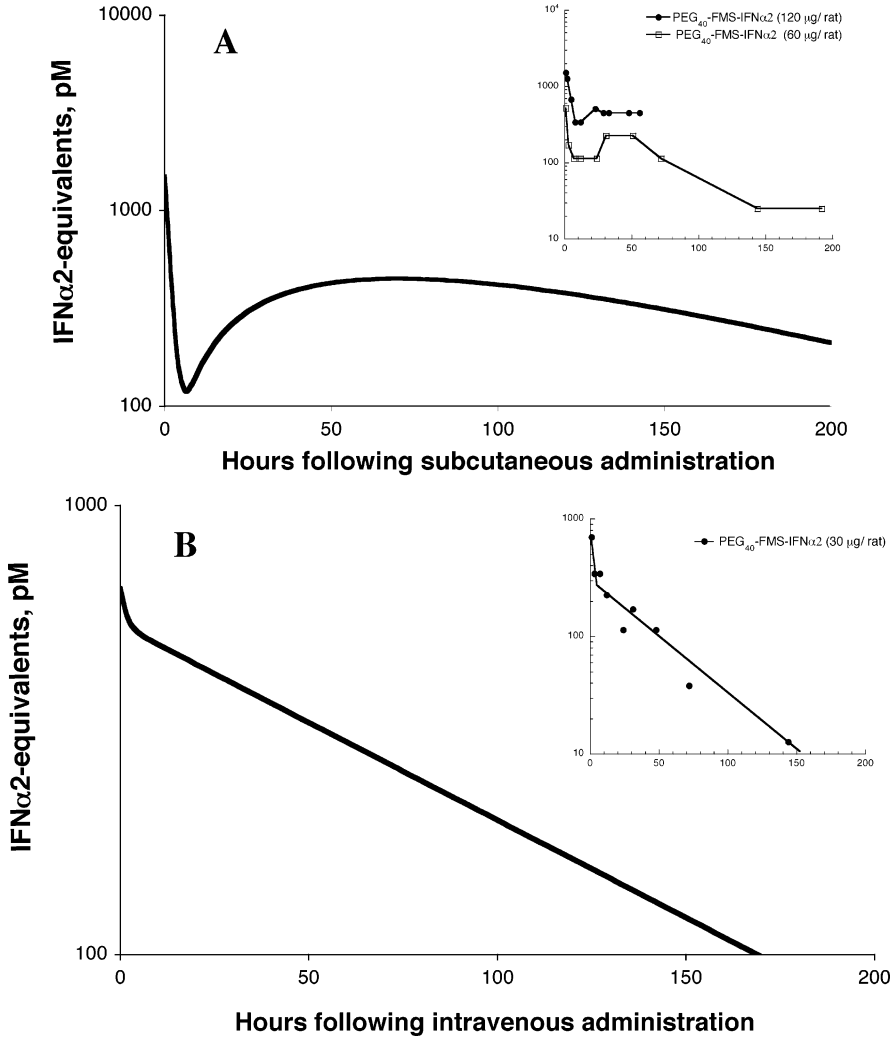


Figure 6. Experimental vs simulated behavior of IFNα2: (A) following subcutaneous administration, with initial concentrations of 60 and 1.5 nM of PEG_{40}-FMS−IFNα2 and native IFNα2, respectively; (B) following intravenous administration to rats with initial concentrations of 20 nM of PEG_{40}-FMS−IFNα2, 1.5 nM of native IFNα2 in the subcutaneous volume, and no conjugate in circulation. The insets are the experimental curves.

hydrolysis, resulting in the regeneration of the native proteins.[27] For this purpose, MAL-FMS-NHS was synthesized, enabling us to link sulfhydryl-containing PEG chains to the amino groups of peptides and proteins via the hydrolyzable FMS function. The working hypothesis was that an inactive PEG−interferon conjugate could regenerate the native protein in its active form in a continuous fashion over a long period of time. The principal monomodified PEG_{40}-FMS−IFNα2 conjugate obtained (Table 1) is devoid of the cytokine binding activity and can therefore be referred to as a "prodrug". Upon incubation, the native cytokine is released by hydrolysis, and the binding activity of IFNα2 to Ifnar2 is regenerated with a rate constant of 0.01 h^{-1}.

A single subcutaneous administration of PEG_{40}-FMS−IFNα2 significantly prolonged the levels of IFNα2 in the serum of rats. While IFNα2 was short-lived in vivo, having a half-life of ∼1 h, the native IFNα2 liberated from the PEG_{40}-FMS−IFNα2 conjugate exerted its antiviral activity over a period of 200 h. Furthermore, there is a dose-dependent ratio between the quantity administered and the active interferon levels over a prolonged duration in vivo. This observation is beneficial for optimization of dosing regimens in future clinical use.

It is noted that the IFNα2 molecule contains 13 amino functions theoretically available for PEG_{40}-FMS attachment. The exact site of PEGylation was not determined. However, in view of its reversibility and the generation of the native protein, it seems that this point deserves a rather minor consideration.

Several methods for reversible pegylation were proposed.[33−37] They suffer, however, from major potential drawbacks. For example, reliance on enzymatic detachment as a rate-determining step[33,34] of PEGs from conjugates by serum proteases and/or esterases might not yield desirable pharmacokinetic profiles in situ. Moreover, it is dependent on the availability of enzymes. Disulfide-bonded conjugates are unlikely to be cleaved in the nonreducing environment of the body fluids.[36] A reversibly PEGylated conjugate that still retains an active moiety capable of reacting with free SH functions may result in complex undesired cross-linking.[37] These deficiencies prompted us to design the version of reversible PEGylation described here.

In summary, we present here a new conceptual approach for reversible PEGylation and applied it to IFNα2. Accordingly, a pharmacologically "silent" conjugate is "trapped" in the circulatory system and re-

leases the parent protein with a desirable pharmacokinetic profile. With regard to IFNα2, we have combined prolonged maintenance in vivo with the release of active-native IFNα2 to ensure access to peripheral tissues. Our new approach might have potential use regarding other short-lived therapeutic peptides and proteins that undergo inactivation by conventional PEGylation.

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Appendix

Abbreviations. FMS, 2 sulfo-9-fluorenylmethoxycarbonyl; IFN, interferon; IFNα2, interferon α2; MAL-FMS-NHS, 9-hydroxymethyl-2-(amino-3-maleimidopropionate)-7-sulfofluorene-*N*-hydroxysuccinimide; PEG, poly(ethylene glycol); PEG_{40}-FMS−IFNα2, an IFNα2 conjugate containing 1 mol of PEG_{40}-FMS/mol of IFNα2; PEG_{40}-SH, a 40 kDa branched poly(ethylene glycol) containing a sulfhydryl moiety; PEG_{40}-Osu, 40 kDa branched poly(ethylene glycol)-*N*-hydroxysuccinimide.

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