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Novel Reactive PEG for Amino Group Conjugation

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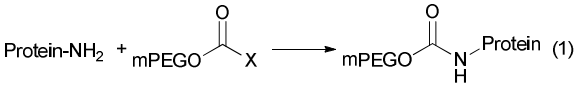
Activated mPEG carbonates are important reagents that have been widely used for the PEGylation of several peptides and proteins by means of stable urethane linkages. In fact, mPEG-*N*-hydroxysuccinimidyl carbonate and mPEG-*p*-nitrophenyl carbonate are among the most used reagents in PEGylation technology. However, the synthesis and storage of these reagents are not always easy to resolve. With the aim of surpassing some of the drawbacks associated with the use of activated mPEG-carbonates we have prepared and evaluated a new mPEG-carbonylimidazolium iodide, which can be used for the conjugation of NH_2 group by means of urethane linkages, as an interesting alternative to the known reagents. Noteworthy, the novel reagent is prepared by a simple two-steps procedure under mild experimental conditions. Moreover, we performed a detailed study of conjugation reaction of interferon-α2b with the carbonylimidazolium derivative, and evaluated the conjugate in *in vitro* and *in vivo* studies.

Introduction

In the last forty years the chemical conjugation with methoxy-poly(ethylene glycol) (mPEG) has emerged as a powerful tool for the improvement of the physicochemical and therapeutic properties of biopharmaceutical peptides and proteins. In this regard, the most traditional strategy for conjugation involves the reaction of electrophilic mPEG reagents with ubiquitously present nucleophilic groups of peptides and proteins, such as NH_2 and SH groups. The attachment of mPEG to the ε-NH_2 group of lysine proved to be particularly useful for conjugation using first and second generation acylation chemistries. From the eleven PEGylated products nowadays available in the market, seven are obtained using electrophilic-acylating mPEG reagents, which yield hydrolytically stable polymer-protein linkages.

Activated mPEGs **1**-**5** are all well-known acylating reagents, which react with nucleophilic NH_2 groups affording stable urethane bonds (equation 1, figure 1).



Remarkable examples of the use of reagents **1**-**5** include the conjugation of ribonuclease and superoxide dismutase (with mPEG-2,3,5-trichlorophenyl carbonate, reagent **1**);[1] recombinant mammalian urate oxidase, uricase and lysozyme (PEG-*p*-nitrophenyl carbonate, reagent **2**);[2] asparaginase, trypsin, chymotrypsin, bacteriorhodopsin, salmon calcitonin, interferon (IFN)-α2b, arginine deiminase and recombinant human arginase I, among other bio-macromolecules (PEG-succinimidyl carbonate, reagent **3**);[3] D-glucosamine, sodium heparin, human serum albumin, fibrin biomatrix, lysozyme and peptides, among other targets (PEG-benzotriazolyl carbonate, reagent **4**);[4] superoxide dismutase, α2-macroglobulin, lactoferrin, streptokinase, alkaline phosphatase, IgG and urokinase (PEG-carbonylimidazole, reagent **5**).[5]

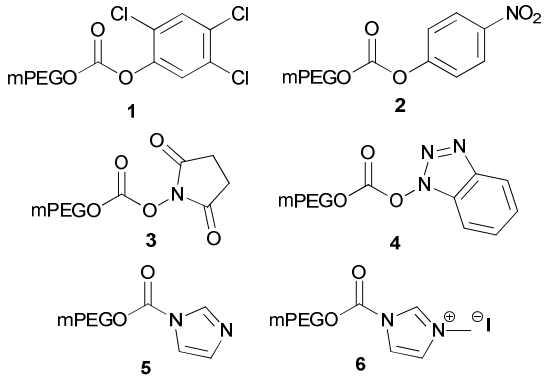


Figure 1: Common activated mPEG derivatives used for PEGylation of proteins by formation of urethane bonds. **1**: mPEG-trichlorophenyl carbonate (mPEG-TCP); **2**: mPEG-*p*-nitrophenyl carbonate (mPEG-pNPC); **3**: mPEG-succinimidyl carbonate (mPEG-SC); **4**: mPEG-benzotriazolyl carbonate (mPEG-BTC); **5**: mPEG-carbonylimidazole (mPEG-Im); **6**: mPEG-carbonylimidazolium iodide (mPEG-Im^+I^-).

mPEGs **2** and **3** are also used for the synthesis of branched $mPEG_2Lys$. mPEG2Lys consists of two linear polymeric chains linked together through a lysine spacer and is widely used in PEGylation technology.[6,7] The reactivity of compounds **1**-**5** towards NH_2 groups depends on nature of the leaving group. Thus, the reactivity of the mPEG reagent plays a key role in the outcome of the bioconjugation reaction, affecting not only the degree of PEGylation, but also the PEGylation site.

Kinetic data for hydrolysis rates of some of derivatives **1**-**5** have been published or patented. For example, the half-lives of compounds **3**-**5** are the following: **3**, 20.4 min (pH 8, 25ºC); **4**, 13.5 min (pH 8, 25ºC), and of around 10 h for **5** (pH 8, 25 ºC).

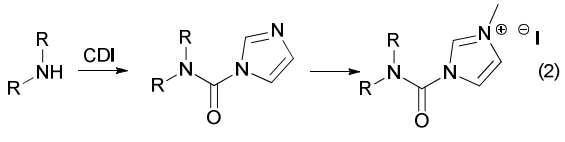
From a practical point of view, the reactivity order of derivatives **1**-**5** is **4**>**3**>**2**>**1**>**5**. Although all these mPEG reagents have been intensively used in early studies, the actual trend has moved towards derivatives **3** and **4**. These derivatives show a good balance between reactivity and selectivity, and does not present frequent toxicity problems, as found with reagents **1** and **2**. Even though different strategies for the synthesis of these reactive PEGs have been reported, their preparation usually involves the use of toxic and volatile reagents such as phosgene or triphosgene, and their supply and storage is not always easy to solve. In fact, a new mPEG reagent for conjugation by means of urethane linkages, which could be easily prepared using widely available and nontoxic starting materials, would represent a remarkable improvement in PEGylation technology. Thus, in this work we report the simple synthesis of acylating mPEG derivative **6**, which, as it will be shown later, can be used for PEGylation via urethane bond formation under slightly alkaline conditions. Noteworthy, reactive mPEG **6** is prepared in only two steps under mild experimental conditions, employing stable and easily available reagents. Furthermore, we employed this activated PEG derivative for the PEGylation of IFN-α2b. The mono-PEGylated protein was purified and showed a significantly improvement in pharmacokinetic parameters, thus demonstrating the usefulness of the novel mPEG reagent.

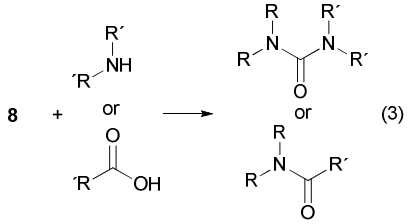
Results and Discussion

Chemistry

Urethane bond formation using acylation reactions is a well-documented method to achieve protein PEGylation at nucleophilic amino groups. These reactions are usually performed at neutral to alkaline pHs (7.0-9.0) using activated mPEG carbonates **1**-**5** as electrophilic reagents. The synthesis of the aforementioned activated polymers may involve the use of toxic reagents under strictly controlled conditions. Furthermore, the supply and storage of commercially available activated mPEG would be difficult to resolve.

mPEG-carbonyl imidazol **5** is a mild PEGylating reagent that has been used in the conjugation of several different proteins such as superoxide dismutase, α2-macroglobulin, lactoferrin, streptokinase, alkaline phosphatase, IgG and urokinase, among others. Due to its low reactivity, PEGylation reactions with reagent **5** should be performed using high molar excesses of the reagent (up to 10,000 fold-excess), and usually long reaction times are required to achieve acceptable yields of conjugates. In a series of investigations, Batey and co-workers showed that secondary amines react with 1,1'-carbonyldiimidazole (CDI) under mild conditions (acetonitrile, room temperature) yielding carbamoyl imidazoles **7** with excellent yields (equation 2).[8]

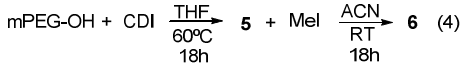




Compound **7** was treated with methyl iodide affording carbamoyl imidazolium salt **8** (equation 2), which by reaction with secondary amines and carboxylic acids in the presence of triethylamine, allowed the preparation of ureas and amides with good to excellent yields (equation 3). However, the usefulness of the reaction of imidazolium salts **8** with hydroxyl compounds, as a method for the preparation of carbamates, is only limited to reactive alcohols or alkoxides, such as benzyl and allyl alcohol, phenols and trifluoroethanol.

It has been reported that alkyl-carbamoylimidazolium iodides **8** are roughly 300 to 400 times more reactive than their parent alkyl-carbamoylimidazoles **7**. Thus, we assumed that this reactivity difference might also be maintained when moving from imidazole derivative **5** to imidazolium salt **6**. In fact, starting from from 20 kDa mPEG we prepared imidazolium salt **6** and used this activated mPEG derivative for the synthesis of branched $mPEG_2Ly$.[9] Challenged by the difficulties found in the methods currently available for the synthesis of compounds **1**-**5**, and stimulated by good results obtained in the synthesis of mPEG2Lys, we decided to study imidazolium salt **6** as an alternative reagent for the chemical conjugation of proteins.

Various methods for the synthesis of 5 have been reported.5[a,10]. Starting with mPEG of 12 kDa we prepared mPEG-carbonyl imidazole **5** by reaction with CDI in THF at 60ºC (equation 4).9



^{1}H NMR analysis of the crude product **5** clearly indicated the formation of the desired compound, and the ratio of OCH_3 to the 3 x 1 H imidazole signals suggested that the reaction proceeded to completion. Hence, the crude product was precipitated three times with diethyl ether from dichloromethane solutions and used in the next step without further purification.

Looze and co-workers determined an ε value of 3590 $M^{-1}cm^{-1}$ for *t*-butyloxycarbonylimidazole (water, 231 nm, 25ºC), and it was indicated that this value can be used with confidence for compound **5** with a mPEG chain of 5 kDa. The UV-Vis spectrum of compound **5** with mPEG of 12 kDa showed a peak at λ_{max} 228 nm, and the ε value determined for compound **5** was 3760 $M^{-1}.cm^{-1}$ (water, 231 nm, 25ºC).

Compound **5** was reacted with methyl iodide under extremely mild conditions (acetonitrile, room temperature), affording the desired alkoxycarbonylimidazolium iodide **6** with excellent yield (98%). After standard work-up, the ^{1}H NMR analysis of the product indicated the formation of the desired compound. The ratio of Im-CH_3 to the OCH_3 and 3 x 1 H imidazole signals suggested that the akylation reaction was complete within 18 h.9

Imidazolium salt **6** was not stable in aqueous alkaline solutions. The UV-Vis spectrum of compound **6** presented peaks at λ_{max} at 360 and 291 nm (acetonitrile, 25ºC). The estimated ε values for **6** with mPEG of 12 kDa were 1907 and 3808 $M^{-1}cm^{-1}$, respectively.

Pegylation

IFNs belong to a well-known family of cytokines of documented therapeutic utility. Thus, IFN-α2b and IFN-α2a are commercially available proteins widely used for the treatment of viral infections, such as chronic hepatitis B and C and various cancers, including leukemia, melanoma and AIDS-related Kaposi´s sarcoma. However, it has been demonstrated that, due to their short plasma half-lives, frequent dosage of the proteins is required to achieve therapeutic effectiveness. With the aim of surpassing this drawback, IFN-α2b has been conjugated to mPEG of 12 kDa by means of urethane bond

formation using mPEG-succinimidyl carbonate **3** at pH 6.5. This PEGylated protein was approved by the FDA and successfully reached the market in 2000.[11]

Due to the intensive clinical application of PEG-IFN-α2b and given the increased reactivity observed for reactive mPEG **6**, we decided to evaluate this reagent in the conjugation of IFN-α2b.

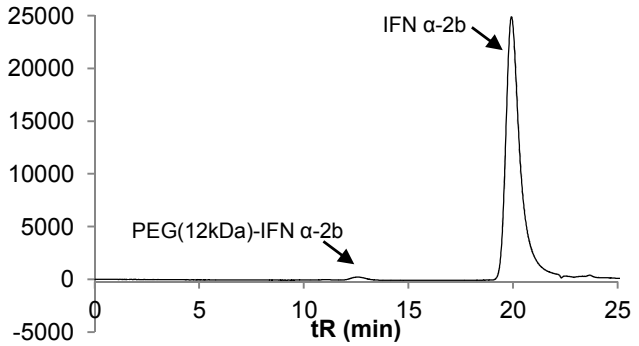


Figure 2: SE-HPLC analysis at 280 nm of PEGylation reaction mixture IFN-α2b with reagent **5**, after 48 h of reaction.

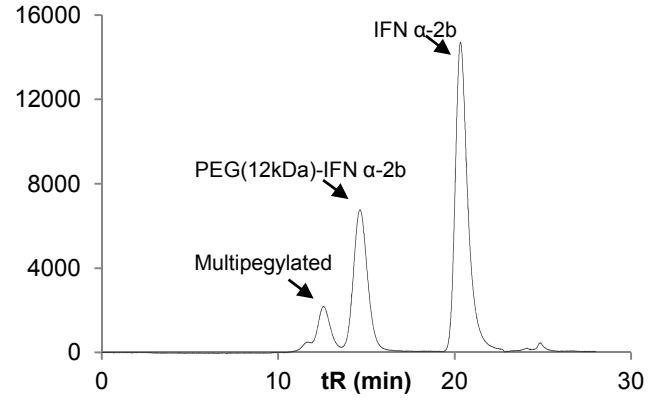


Figure 3: SE-HPLC analysis at 280 nm of PEGylation reaction mixture of IFN-α2b with reagent **6**, after 30 minutes of reaction.

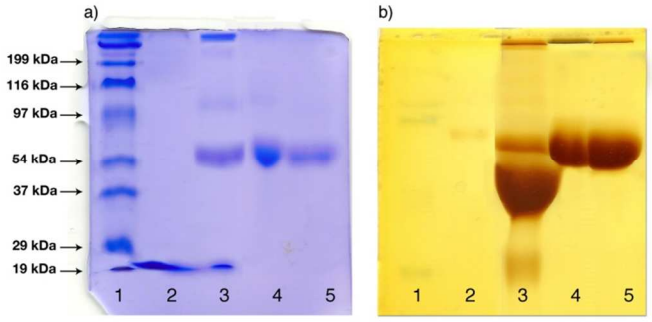


Figure 3: SDS-PAGE analysis of the PEGylation reaction with (**a**) Coomassie brilliant blue staining for protein detection and (b) $BaCl_2/I_3^-$ staining of PEG. Lanes: 1, molecular weight standards; 2, IFN-α2b; 3, PEGylation reaction mixture; 4) purified PEG-IFN-α2b; and 5) PEG (12kDa) – IFN-α2b reference product (PegIntron®).

Since most conjugations with reagents **1**-**5** are performed at neutral or slightly alkaline pH, we evaluated the PEGylation of IFN-α2b with activated mPEG in borate buffer pH 8.0, where mPEG reagent **6** should react with nucleophilic NH_2 groups yielding stable urethane bonds. In fact, we have found that low molecular weight alkoxy-carbonylimidazolium salts react with amines to afford carbamates with excellent yields.[12]

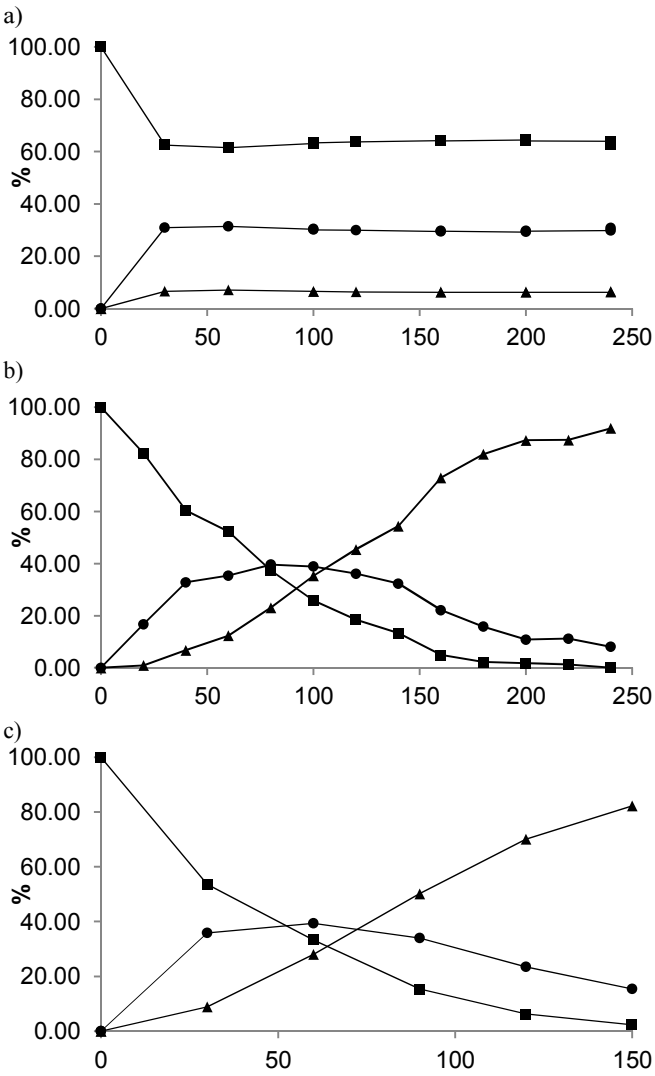


Figure 5: Plots of the reaction time (minutes) versus relative ratios of poly-PEGylated species (▲); mono-PEG-IFN-α2b (●) and unreacted IFN-α2b (■) obtained by (a) a single addition of 3 molar equivalents of PEG derivative **6**; (b) multiple additions of 1 molar equivalent of **6** every 20 min and (c) multiple additions of 3 molar equivalents of **6** every 30 min.

To evaluate the proposed different reactivity of **5** and **6**, IFN-α2b was PEGylated at pH 8.0 in 100 mM sodium borate buffer using 3 equivalents of both activated mPEGs at 4°C. Previous to SEC-HPLC and SDS-PAGE analyses, all reactions were quenched by addition of acetic acid to reach pH 4.5. Reagent **5** reacted slowly with the target protein, affording the conjugate with only 1.3% yield after 48 h (figure 2).[13] This result is in agreement with those previously published, where 10,000 molar excess of **5** and extremely long reaction times were needed to achieve acceptable yields of PEGylation. On the contrary, under identical reaction conditions, PEG derivative **6** gave the desired protein conjugate with 32% yield in only 30 min. A typical PEGylation reaction outcome by SE-HPLC and SDS-PAGE is shown in figures 3 and 4, respectively. As it can be seen, in SDS-PAGE gels, mono-PEGylated conjugate showed an

apparent molecular mass of around 53 kDa. Taking into account individual molecular masses of non-PEGylated IFN-α2b and mPEG, the molecular mass of the conjugate should be around 32 kDa. As usual, the increase in the hydrodynamic volume of the PEGylated product is assigned to PEG chain hydration.[14]

The results obtained in the conjugation of IFN-α2b with PEG imidazolium salt **6**, prompted us to further study the behavior of this new reagent. Thus, we analyzed the time-course of relative ratios of poly-PEGylated species (▲), mono-PEG – IFN-α2b (●) and unreacted protein (■), using a single addition of 3 equivalents of PEG derivative **6** (figure 5a), multiple additions of 1 equivalent of PEG derivative **6** every 20 min (figure 5b), and the sequential addition of 3 equivalents of activated PEG derivative **6** every 30 min. This last approach afforded complete protein conjugation, as is shown in figure 5c. In the case of multiple additions of reagent **6**, samples were taken immediately before the addition of a new aliquot and analyzed as indicated previously.

Figure 5a shows that the relative ratios of mono-PEGylated protein (32%), as well as oligomeric conjugates (7%) and unmodified protein (61%), remained almost constant after 30 min of reaction. This result suggests that the conjugation reaction may compete with the hydrolysis of activated mPEG reagent **6**. It has been reported that carbamoylimidazolium iodides are roughly 300 to 400 times more reactive than the related carbamoyl imidazoles. On the other hand, it has been demonstrated that the half-life of mPEG carbonyl imidazole **5** is around 10 h at pH 8 (25°C). If the reactivity difference between carbamoyl imidazolium iodides and carbamoyl imidazoles is conserved when moving from **5** to **6**, the half-life of **6** should be around 6 min, suggesting that the reagent should be hydrolyzed rapidly in aqueous basic conditions.

The sequential addition of 1 equivalent of **6** every 20 min afforded the highest yield of mono-PEGylated product (37%) at 80 min of reaction (*i.e.* using 4 equivalents of **6,** figure 5b). Unfortunately, a high yield of oligomeric conjugates was also obtained at this point (19%). Following this approach, complete protein consumption was observed at 220 min of reaction using 11 equivalents of **6** (figure 5b).

Upon addition of 3 equivalents of **6** every 30 min (figure 5c) a maximum yield of mono-PEGylated product was reached at around 60 min (39%), while the yield of oligomeric conjugates was around 28%. From this point, the yield of mono-PEGylated product decreased as the yield of poly-PEGylated products increased. Noteworthy, complete native protein consumption was reached using 15 equivalents of **6** at approximately 150 min of reaction.

PEG-IFN-α2b was successfully purified from the PEGylation reaction mixture (quenched at 30 min) using ionic exchange chromatography. As can be seen in figure 6, purified monoconjugate contained only 2% of oligomers and non-conjugated IFN was not detected.

Antiviral and antiproliferative *in vitro* activities

Both IFN-α2b and PEG-IFN-α2b were analyzed for biological *in vitro* activity. The antiviral activity of rhIFN–α2b and PEGylated IFN-α2b was determined by its ability to inhibit the cytopathic effect caused by vesicular stomatitis virus (VSV) on MDBK cells, while the antiproliferative activity of both molecules was measured as the capacity to inhibit the growth of the human Daudi cell line. As can be seen in table 1, both biological activities decreased after the PEGylation process. As a consequence, PEG-IFN-α2b preserved 37% of antiviral activity and 18% of antiproliferative activity in comparison with the non-conjugated cytokine. This fact is commonly found when studying the effect of PEGylation in proteins, since PEG chain may interfere with specific amino acid residues that are important for the recognition or effector functions. Moreover, the results obtained are in agreement with what it was expected after perturbation of the interaction of rhIFN-α2b with its receptor; that is, conjugation with PEG affected the growth-inhibitory activity of rhIFN-α2b to a greater extent than the antiviral activity. This effect has been described by many authors who have demonstrated that the formation of a high affinity complex between the two receptor subunits and the cytokine is required for eliciting an antiproliferative transcriptional cascade, while the stability of the ternary complex is not so critical for triggering the antiviral response.[15]

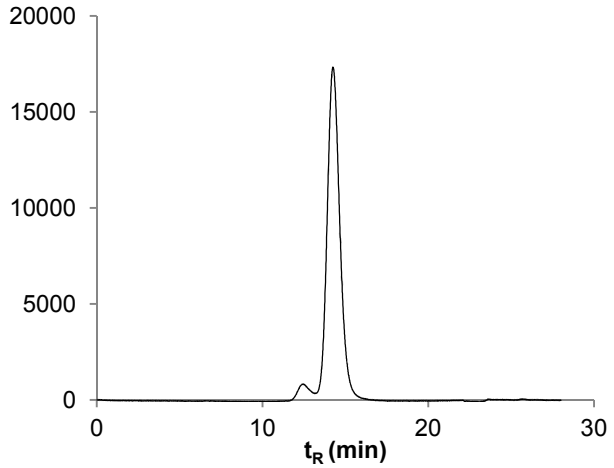


Figure 6: SE-HPLC chromatogram of purified fraction of PEG-IFN-α2b.

Reduced *in vitro* activity associated with increased *in vivo* biological activity has been observed with other PEGylated proteins like IL-2[16], IL-6[17], TNF-α [18] and G-CSF[19], among others. In all cases, the diminished biological activity of the PEGylated derivatives was compensated by the improvements in their pharmacokinetics properties, making the PEGylated proteins promising drugs for clinical use which combine less frequent dosing with enhanced therapeutic efficacy.

Table 1: *In vitro* specific antiviral and antiproliferative activities of IFN-α2b and PEG-IFN-α2b.

Protein	Specific antiviral activity (IU/ng)	Specific antiproliferative activity (IU/ng)	Residual activity
IFN-α2b	230 ± 16	300 ± 84	100%
PEG-IFN-α2b	85 ± 14	55 ± 6	37%/18%

Wang and coworkers reported that PEGylation of IFN-α2b with mPEG-SC resulted in a mixture of 95% mono-PEGylated species which retained 28% of antiviral activity.3[f] The authors found that this effect was due to the amino acid residue to which PEG was attached. Since PEGylation was carried out at pH 6.5, histidine residues were the preferred site for conjugation, being PEG-IFN (His34) the major positional isomer (47% of total PEGylated species).[20] Moreover, after studying the antiviral activity of the different positional isomers, the authors discovered that the major isomer also exhibited the highest antiviral biological activity (37% residual activity compared to non-conjugated protein).

Increasing the pH of the PEGylation reaction medium would increase the relative amount of nucleophilic deprotonated form of

lysine residues, which hence would become the predominant reactive sites for PEGylation. In our study, PEGylation was carried out at pH 8, so that a different distribution of preferred conjugation sites conducing to a different degree of antiviral activity retention could be expected. However, the residual activity of the PEGylated species obtained in our study was that of the most active isomer (37%), indicating that our method can favour the retention of high specific antiviral activity of rhIFN-α2b.

Pharmacokinetics

The effect of PEGylation on the pharmacokinetic properties of PEG-IFN-α2b in comparison with non-PEGylated IFN-α2b was evaluated in rats following subcutaneous administration of a single dose of 2.10^5 IU per animal of each molecule. Plasma samples were obtained at different times and assayed for IFN antiviral activity. Pharmacokinetic profiles were plotted (figure 7) and pharmacokinetic parameters were calculated by the method of residuals (Table 2). IFN-α2b peaked 1.5 ± 0.2 h post-injection and began to decrease after 7 h since administration. PEG-IFN-α2b reached a 6.4-fold higher maximum plasma concentration compared to IFN-α2b at a later time (7.5 ± 2.0) and its plasma levels remained considerably high until 24 h post-administration. Thus, significant differences between pharmacokinetic parameters for both molecules were found, showing PEG-IFN-α2b a 6.5 increase in elimination half-life ($t_{1/2}$) and a 43-fold decrease in apparent plasma clearance (CL_{app}). The reduction of the elimination mechanisms of the PEGylated derivative is achieved through the prevention of glomerular filtration by increasing the molecular mass and hydrodynamic volume of the cytokine to a value near the cut off of the kidney filtration mechanism.

Table 2: Pharmacokinetic parameters of IFN-α2b and PEG-IFN-α2b following subcutaneous administration in rats.

	IFN-α2b	PEG-IFN-α2b
t_{max} (h)	1.5 ± 0.3	7.5 ± 2.0
C_{max} (IU/mL)	982 ± 672	6238 ± 3712
$t_{1/2elim}$ (h)	1.2 ± 0.3	7.8 ± 0.9
AUC (IU.h/mL)	1103 ± 102	47208 ± 3013
CL_{app} (mL/h)	182 ± 17	4.2 ± 0.3
Vd (mL)	318 ± 119	47.8 ± 8.5

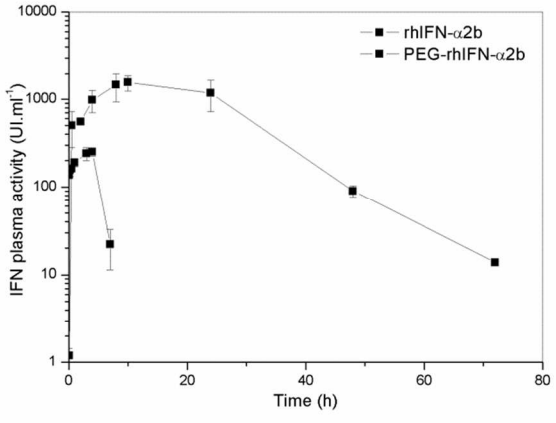


Figure 7: Pharmacokinetic profiles of IFN-α2b and PEG-IFNα2b.

In this way, these results demonstrate that the new PEGylating reagent obtained in this work can be successfully employed to generate a PEGylated IFN-α2b derivative that, like many other PEGylated proteins, exhibits an enhanced pharmacokinetic profile in comparison with the unmodified molecule.

Experimental

Materials

mPEG-OH 12 kDa was purchased to JenKem Technology (Allem, USA). *N,N´*-carbonyl*bis*imidazole (CDI) was supplied by Sigma Aldrich (St. Louis, MO). Acetonitrile was distilled from anhydrous $MgSO_4$ and stored over molecular sieves (4 Å). THF and diethyl ether were freshly distilled from sodium benzophenone ketyl. Dry dichloromethane was obtained by distillation from P_2O_5. Methyl iodide was prepared according to a reported method.[21] All other reagents and solvents were used as received from the suppliers. IFN-α2b was obtained from Zelltek (Santa Fe, Argentina). Fractogel EMD COO^- (M) was supplied by Merck Chemicals (Darmstadt, Germany).

Physico-chemical characterization and analyses

^{1}H NMR experiments were performed in $CDCl_3$ in a Bruker Avance 300 MHz spectrometer and referenced to the residual solvent signal. UV-Vis analyses were performed on a Shimadzu UV-Vis spectrophotometer. Size exclusion-HPLC (SE-HPLC) analyses were performed in a Shimadzu apparatus equipped with TSK Gel 3000SW, 7.5 x 600 mm column (Tosoh) with UV detection at 280 nm. The mobile phase for the analyses of different species in the conjugation reaction and the chromatographic purification of the conjugate was 50 mM sodium acetate buffer pH 5.2, 0.2 M NaCl, 10% (v/v) ethanol.

Polyacrilamide gel electrophoresis (SDS-PAGE) was performed according to the methods of Laemmli[22], using 15% (w/v) polyacrylamide resolving gels. After running, gels were rinsed with distilled water and placed in a 5% barium chloride solution. The gels were maintained for 10 min with gentle mixing, and then they were rinsed again, and placed in a 0.1 N I_3^- solution for 5 to 10 min. In the case of proteins or PEG-protein conjugates, 15% polyacrilamide gels were run, and standard staining procedures with Coomasie Brilliant Blue were followed.

Synthesis and purification

*Synthesis of mPEG-carbonylimidazole (12 kDa) (mPEG-Im **5**):* 1.200 g of mPEG-OH (12 kDa, 0.1 mmol) were dissolved in dry THF (6 mL) at 60°C. CDI (49 mg, 0.3 mmol) was added and the solution was stirred at 60°C for 18 h. The solvent was removed under vacuum. The residue was dissolved in water and then 5-fold extracted with chloroform (25 mL each). The organic phase was evaporated at reduced pressure and dried under vacuum until constant weight. The solvent was removed under reduced pressure and the resulting solid was dissolved in dicholoromethane and precipitated using ethyl ether. This process was repeated two more times and compound **6** was dried under vacuum. A white solid was obtained (1.188 g, 99%). ^{1}H NMR (300 MHz – Cl_3CD), δ: 3.35 (s, 3H, OMe); 3.60 (brs, mPEG chain); 4.43–4.52 [m, superimposed with mPEG chain, $CH_2OC(O)$]; 7.04 (s, 1H, Im-H); 7.40 (s, 1H, Im-H); 8.11 (s, 1H, Im-H).

*Synthesis of mPEG-carbamoylimidazolium salt (12 kDa) (mPEG-Im^+I^- **6**):* 1.188 g (0.099 mmol) of mPEG-carbonylimidazole **5** were dissolved in 4.0 mL of acetonitrile at room temperature. Freshly prepared methyl iodide was added (62 µL, 1 mmol), and the solution was stirred at room temperature for 18 h. The solvent was removed under reduced pressure and the resulting solid was dissolved in dicholoromethane and precipitated using ethyl ether. This process was repeated two more times and compound **6** was dried under vacuum. A yellow solid was obtained (1.16 g, 98%). ^{1}H NMR (300 MHz – Cl_3CD), δ: 3.36 (s, 3H, OMe); 3.63 (brs, mPEG chain); 3.86 (m, superimposed with mPEG chain peak, $CH_2OC(O)$); 4.06 (s, 3H, CH_3); 7.51 (s, 2H, 2 x Im-H); 9,96 (s, 1H, Im-H).

*Conjugation of mPEG-carbamoylimidazolium salt **6** with IFN-α2b:* mPEG-Im^+I^- **6** (12 kDa) was added to IFN-α2b solution (3,2 mg/mL, 50 mM sodium-borate buffer pH 8.0). The reaction proceeded for 3 h at 4°C with gentle stirring. Different molar ratios and strategies were evaluated in the PEGylation reaction: a) 3 molar equivalents of **6** were added at once; b) ten independent aliquots of 1 molar equivalent of **6** were added every 20 min; c) six additions of 3 molar equivalents of **6** were performed every 20 min. Samples were taken before the addition of a new aliquot of **6** and SE-HPLC analysis allowed the determination of different species (multi-PEGylated, mono-conjugate and unreacted protein). The final solution was quenched with acetic acid to pH 4.5, then diluted eight times with 10 mM ammonium acetate pH 4.5 and loaded on an ion exchange chromatography column (matrix: Fractogel EMD COO^- (M); column: XK 26/20, GE Healthcare; CV=60 mL; 4°C). The purification process was carried out under conditions of stepwise gradient elution with the following buffers: 40 mM ammonium acetate pH 4.5; 0.12 M NaCl in 40 mM ammonium acetate pH 4.5; 0.5 M NaCl in 40 mM ammonium acetate pH 4.5 and 1 M NaCl in 40 mM ammonium acetate pH 4.5. Eluting samples were monitored by UV absorbance at 280 nm. Fractions containing the conjugate IFN-α2b–PEG were pooled and analyzed. SE-HPLC and SDS-PAGE gels (stained with Coomasie brilliant blue, and $BaCl_2/I_3^-$; using a commercially available conjugate as reference, Peg-Intron Redipen; Schering Plough; Singapore) were used in different stages of the process to confirm the presence of the conjugate. Concentration of proteins was determined by UV absorbance at 280 nm and SE-HPLC. Final yield of conjugation and purification procedure was 26%.

***In vitro* biological activity**

Cell culture. MDBK cells (Madin-Darby bovine kidney, ATCC, Manassas, VA, CCL-22) were grown in Minimum Essential Medium, MEM (Gibco, Rockville, MD) supplemented with 10% (v/v) FCS (Gibco) (growth medium). For bioassays, MEM supplemented with 2% (v/v) FCS (assay medium) was employed. Daudi cells (human Burkitt´s lymphoma, DSMZ, Braunschweig, Germany, ACC-78) were maintained in RPMI 1640 medium (Gibco) plus 10% (v/v) FCS.

Determination of antiviral activity. The antiviral activity of IFN-α2b and PEGylated IFN-α2b was determined by its ability to inhibit the cytopathic effect caused by vesicular stomatitis virus (VSV, Indiana strain) on MDBK cells.[23] For this, MDBK cells were seeded into culture microtiter plates in growth medium ($2.5x10^4$ cells per well) and incubated at 37ºC overnight. After removing culture supernatants, 1:2 serial dilutions of IFN-α2b WHO international standard (NIBSC 95/566) from 20 $IU.ml^{-1}$ to 0,16 $IU.ml^{-1}$ or 1:2 serial dilutions of IFN-α2b test samples in assay medium were added. Plates were incubated for 6 h at 37°C and, after removal of supernatants, an appropriate dilution of VSV virus was added. Virus replication was allowed to proceed until the cytopathic effect was clearly observable in control wells (no IFN). The medium was discarded and cells were fixed and stained simultaneously with a solution of 0.75% (w/v) crystal violet in 40% (v/v) methanol. After 10 min, plates were washed with water and the remaining dye was solubilized in 20% (v/v) acetic acid. The plates were read at 540 nm with a microtiter plate reader and the signal intensity of each dilution was reported as the mean of the absorbance measured in five wells.

Determination of antiproliferative activity. The antiproliferative activity of IFN-α2b and its PEGylated derivative were measured as the ability to inhibit the growth of the human Daudi cell line.[24] Serial 1:2 dilutions of IFN-α2b WHO international standard from 100 $IU.ml^{-1}$ to 0.78 $IU.ml^{-1}$ or IFN-α2b test samples were placed into microtiter plate wells. Then, previously washed Daudi cells were added ($5x10^3$ cells per well) and plates were incubated at 37ºC for 96 h. Cell proliferation was determined using CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay (Promega). Absorbance was read at 492 nm using a microplate reader. The assay was reproduced by triplicates.

Pharmacokinetic studies.

Two-month-old female Wistar rats (Comisión Nacional de Energía Atómica, Argentina) with an average body weight of 200 g were divided into four groups of three animals each. They were housed in a temperature-controlled room at 23 ºC, with a 12 h light/dark cycle and free access to food and water. Groups 1 and 2 were injected subcutaneously (s.c.) with 2×10^5 IU in a total volume of 250 µl of non-glycosylated IFN-α2b. Blood samples were taken at 5 min, 30 min, 2 h, 4 h and 24 h (group 1) and 15 min, 1 h, 3 h and 7 h (group 2) post-injection. Groups 3 and 4 were injected in the same way with PEGylated IFN-α2b and blood samples were collected at 5 min, 2 h, 8 h, 24 h and 72 h (group 3) and 30 min, 4 h, 10 h, 48 h and 96 h post-injection. Blood collected on heparin was centrifuged and plasma was stored at -20 ºC. Samples were assayed for IFN antiviral activity. Plots of IFN-α2b and PEGylated IFN-α2b concentration vs. time were constructed and pharmacokinetic parameters were calculated. Results were expressed as mean ± SD. Maximum plasma concentration (C_{max}), time to reach the C_{max} (t_{max}), terminal half-life ($t_{1/2elim}$), area under curve (AUC), apparent plasma clearance (CL_{app}) and volume of distribution (V_d) were calculated by the method of residuals, using Microcal Origin software version 5.0 (Microcal Software, USA).

All animal experimental protocols were in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, USA, 1996), and efforts were made to minimize the number of animals used and their suffering.

Conclusions

In this work, we have developed a new PEGylating derivative than can be easily prepared in only two simple synthetic steps with excellent yields, avoiding the use of toxic and volatile reagents, such as phosgene or triphosgene. We have shown that mPEG-cabonyl imidazolium **6** can be used for protein conjugation under standard PEGylation experimental conditions within short reaction times. Furthermore, reagent **6** reacts with IFN-α2b giving 32% of the desired mono-PEGylated protein, with only 7% of multi-PEGylated species, in only 30 min. This yield is comparable with those obtained with other acylating PEGylation reagents, but with shorter reaction times. Moreover, we also demonstrated that PEGylation of IFN-α2b with reagent **6** produces conjugates with improved pharmacokinetic properties when compared with non-PEGylated molecules, showing a 43-fold decrease in CL_{app}, which, in turn, yields an enhanced *in vivo* activity.

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Notes and references

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