Synthesis and Bioactivity Study of 30KDa Linear PEG-Interferon and its Comparison with Tri-Branched PEG-Interferon

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Summary: Polyethylene glycol (PEG) is conjugated to the therapeutic proteins to enhance their circulating half life. PEGylation is a clinically proven strategy for increasing the therapeutic efficacy of protein-based medicines. PEG size and structure is very important in achieving specific properties in the conjugated protein. In this study we have compared the properties of newly synthesized linear PEG-IFN with a same sized (i.e., 30KDa) tri-branched PEG-IFN synthesized in the same lab earlier. For PEGylation reaction, interferon-α2 (IFNα2) concentration in sodium borate buffer pH 8.5 was optimized as 4.0mg/ml. The optimized molar ratio of PEG/IFNα2 was 3:1 instead of 5:1 in the case of tri-branched PEG published earlier. Other parameters of reaction were same as in our previous publication. Mono PEGylation degree reached to 21%. Cation exchange chromatography was used to separate and purify mono PEGylated IFN from the reaction mixture. The purity of mono PEGylated IFN was greater than 95%. It is noticed that PEGylation was more site specific in tri-branched than the linear conjugate. The *in vitro* bioactivity of linear mPEG-IFN is 10-fold lesser than as reported of tri-branched $mPEG_3L_2$-IFN. Thermal stability of linear mPEG-IFN is also smaller than $mPEG_3L_2$-IFN at 4°C.

Keywords**:** Bioactivity, Interferon, PEG, PEGylation, Peptide Digestion, HPLC.

Introduction:

Interferon-α has formed the basis of treatment regimens since the identification of HCV, either alone or in combination with the nucleoside analogue ribavirin [1]. A limitation to the therapeutic use of Interferon-α however, is its short serum half-life and rapid clearance. Polyethylene glycol (PEG) is conjugated to the therapeutic proteins to enhance their circulating half life. PEGylation is a clinically proven strategy for increasing the therapeutic efficacy of protein-based medicines [2]. The main advantage of PEG conjugated proteins is their markedly prolonged elimination half-life, although *in vitro* potency of the protein as another important property is usually reduced on conjugation [3]. PEGylation changes the physical and chemical properties of the biomedical molecule, such as its conformation, electrostatic binding, and hydrophobicity, and results in an improvement in the pharmacokinetic behavior of the drug [4]. In general, PEGylation improves drug solubility and decreases immunogenicity. PEGylation also increases drug stability and the retention time of the conjugates in blood, and reduces proteolysis and renal excretion, thereby allowing a reduced dosing frequency [5]. In order to benefit from these favorable pharmacokinetic consequences, a variety of therapeutic proteins, peptides, and antibody fragments, as well as small molecule drugs, have been PEGylated [4].

To date, at least eight such PEGylated peptide and protein conjugates have been approved as therapeutic agents and many more have undergone clinical trials [6]. Different sizes and structures of PEG molecules can be used to exhibit the required properties in the short lived therapeutic drugs. Schering-Plough developed a PEG conjugated form of interferon alpha-2b (PegintronA) by attaching a linear 12KDa mono-methoxy polyethylene glycol (mPEG) to it [7] which enhanced the half life to a substantial level (5-fold) but still not enough. Bailon et al., in 2001 conjugated interferon with 40KDa di-branched PEG molecule (PEGasys) which exhibited substantial prolonged serum half life (8.5-fold) although its *in vitro* bioactivity was reduced to very low level [3, 8]. In independent phase III trials, PEGasys showed sustained virologic response (SVR) rate as 68% (28% for native IFNα2a) compared to PegintronA as 49% (24% for native IFNα2b) [8]. This indicates that branched 40KDa mPEG-IFN may be better drug than native IFN and linear 12KDa mPEG-IFN in terms of long serum half life and sustained virologic response (SVR). Although there are several studies based on the comparison of linear and di-branched PEG-protein conjugates [9] but there is no study for the comparison of properties of linear and tri-branched PEG-IFN of same size.

So this study was conducted to compare the properties of linear and tri-branched PEG-IFNs. It is found that branching of PEG molecule makes the

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conjugate more acceptable than linear PEG interferon at least in terms of *in vitro* bioactivity.

Results and Discussion

PEGylation is the technology of choice today to increase the serum half lives of recombinant proteins to enhance their efficacy. There are at least eight PEGylated recombinant products which have been approved as therapeutic agents and many more have undergone clinical trials [6]. There are two approved PEG-IFNs, one is conjugated with linear 12KDa PEG and the other with 40KDa di-branched PEG with approximately 20KDa each branch. In our previous study [10] we have showed that 30KDa tri-branched PEG-IFN exhibits better properties than native and 40KDa di-branched PEG-IFN but no study (to the best of our knowledge) have been undertaken to compare the synthesis of linear and tri-branched PEG-IFNs of same size.

In one of our previous studies [10], it has been shown that a tri-branched 30KDa $mPEG_3L_2$-IFN exhibited better some of the pharmacokinetic properties relative to native IFNa2 and 40KDa di-branched mPEG-IFN. Here we report a comparison of synthesis, purification, stability and bioactivity testing of 30KDa, linear PEG-IFN with tri-branched PEG-IFN. As it is the established fact that covalent attachment of PEG chains to therapeutic proteins prolong their half life *in vivo*, but at the same time they have the opposite effect of reducing *in vitro* bioactivity [3, 11] and the results of the present study also confirm the later fact.

In this paper we have discussed the effects and importance of structure of PEG molecule on the properties of PEGylated IFN. Results of this study show that although we yielded higher percentage of linear mPEG-IFN than tri-branched $mPEG_3L_2$-IFN, yet the later product showed more than ten times better *in vitro* bioactivity than the former. Both have the same size but $mPEG_3L_2$-IFN may exhibit better serum half life like other branched PEG-IFNs [3]. In stability studies it was seen that bioactivity of tri-branched $mPEG_3L_2$-IFN is more stable than native and linear PEG IFN at -20°, 4° and 25°C at least for one year. No any type of aggregation was seen in any product during the whole year of this study. It is further pointed out that while the bioactivity of the product retained at 4°C, additional data need to be collected to select the most appropriate storage conditions.

Same protocol for the synthesis of linear PEG-IFN was adapted as in the synthesis of tri-branched PEG-IFN [10] except that PEG/protein molar ratios of 1:3 was used for the highest yield as given in Table-1. SDS-PAGE of PK1 showed that it is di-PEGylated IFN (data not shown). PK2 from Fig. 1 was collected as mono-PEGylated product and undergone the SDS-PAGE analysis (Fig. 2), it is observed that electrophoretic mobility mPEG-IFN and $mPEG_3L_2$-IFN is considerably slower as compared to their actual molecular masses (i.e., 50KDa). mPEG-IFN gives ≈68KDa while $mPEG_3L_2$-IFN exhibits ≈90KDa apparent molecular mass in SDS-PAGE analysis (Fig. 2). SE-HPLC analysis (Fig. 3) of these products also confirms the fact (summarized in Table-2) that PEGylated IFNs have higher apparent masses than native IFN. This is attributed to PEG's large hydrodynamic volume as three moles of water form an adduct with each ethylene oxide subunit of PEG [12]. In addition, although both have same actual size, greater apparent molecular mass of tri-branched $mPEG_3L_2$-IFN (≈90KDa) than linear PEG-IFN (≈68KDa) in SDS-PAGE shows that branched PEGs could get trapped strongly into the polyacrylamide gel matrix, further slowing down the electrophoretic mobility [3, 10]. The slower mobility is also important in enhancing the serum half life of the PEGylated products (Bailon et al., 2001).

At pH 8.5, linear and tri-branched PEG-IFNs yielded 21 % and 17% (Table-1) respectively relative to native interferon. The purity level for both products was greater than 95% as indicated in SDS-PAGE (Fig. 2) and SE-HPLC (Fig. 3). *In vitro* studies revealed that bioactivity of linear and tri-branched products are 2.3×10^6 IU/mg and 2.38×10^7 IU/mg [10] respectively as indicated in Table-3. This shows that *in vitro* bioactivity of tri-branched $mPEG_3L_2$-IFN is 10-fold higher than the linear mPEG-IFN. Based on the results of this study, it is reported that tri-branched $mPEG_3L_2$-IFN can be considered superior to the linear mPEG-IFN with reference to *in vitro* bioactivity.

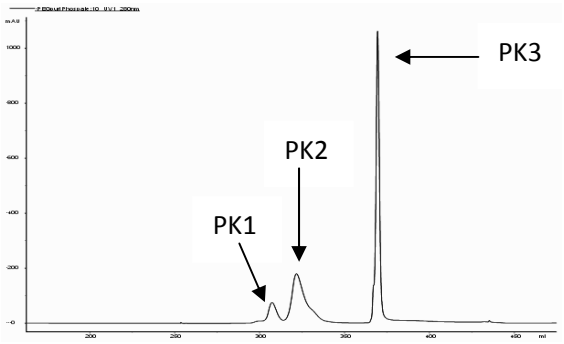


Fig. 1: Purification Profile of mPEG-IFN using Fractogel, a weak cation exchanger on AKTA-10 system. PK1-di-mPEG-IFN; PK2- mono-PEG-IFN; PK3- Unreacted IFN.

Table-1: Reaction Conditions and final yield of mPEG-IFN products.

PEG Reagent	IFN Conc.	Reaction Buffer	Protein:PEG molar ratio	(%Yield) PEG-IFN	(%Yield) DiPEG-IFN	(%Yield) Unreacted-IFN	%age of Protein Loss During Purification
Linear mPEG-NHS	4.0mg/ml	100mM Borate pH 8.5	1:3	21.11	7.29	56.00	15.60
Tri-branched $mPEG_3L_2$-NHS (synthesized earlier)	4.0 mg/ml	100mM Borate pH 8.5	1:5	17.00	7.00	58.00	18.00

Table-2**:** Relative Retention Time of PEG-IFNs in SE_HPLC.

S.No.	Product Name	Actual Molecular Mass(KDa)	Retention Time (minutes)
1	**Native IFN**	**20**	**19.349**
2	**$mPEG_3L_2$-IFN**	**50**	**11.123**
3	**mPEG-IFN**	**50**	**11.263**

Table 3: Bioassay results of mPEG-IFN products.

Product	Bioactivity (IU/mg)	% Bioactivity relative to native IFN
Native IFN	**2.5×10^8**	**100**
Linear mPEG-IFN	**2.3×10^6**	**0.92**
Tri-branched $mPEG_3L_2$-IFN	**2.38×10^7**	**9.52**

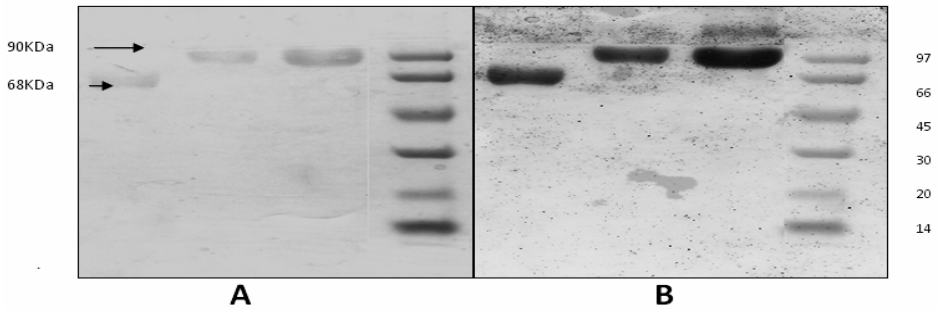


Fig. 2: SDS-PAGE analysis of purified linear and tri-branched PEG-IFNs. (A) Coomassie Staining (B) PEG specific (barium iodide) staining. Lane 1: Linear mPEG-IFN, Lane 2: Tri-branched mPEG3L2-IFN (3µg), Lane 3: Tri-branched $mPEG_3L_2$-IFN (5µg) and Lane4: LMW Protein Marker.

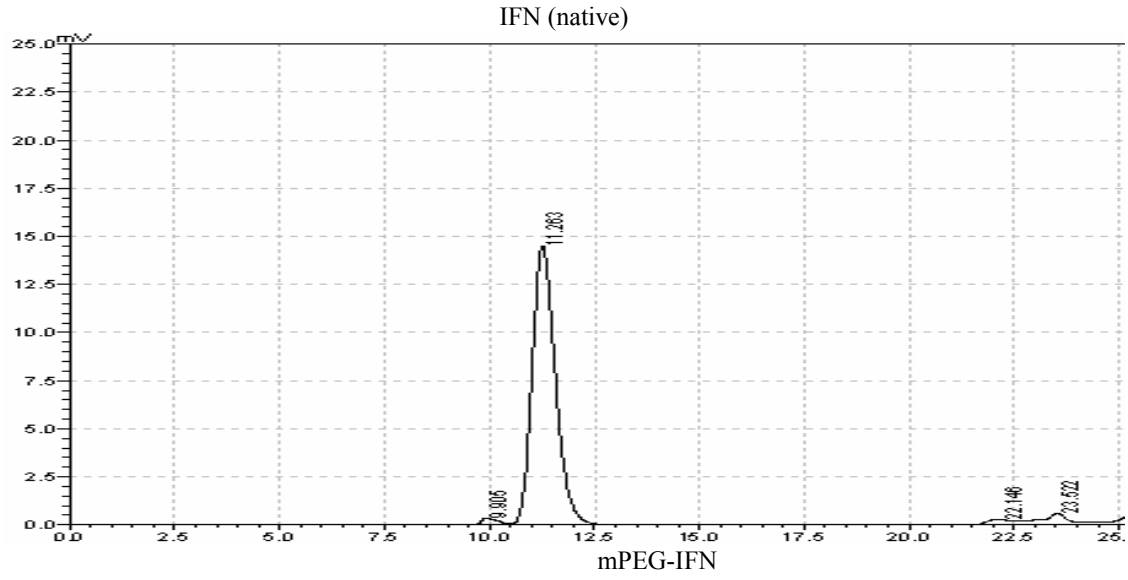


Fig. 3**:** SE-HPLC analysis of purified native and PEGylated IFN.

It has already been reported [3] that PEG conjugation with PEG-NHS reagents may be specific on one of the four major PEGylation sites, Lys31, Lys121, Lys131, or Lys134 of IFN [13], peptide map of mPEG-IFN after Trypsin Lys-C digestion experiment shows that PEGylation for this product may also be lysine specific (Fig. 5) as in the case of $mPEG_3L_2$-IFN [10]. In RP-HPLC analysis (Fig. 5) mPEG-IFN showed the same pattern as $mPEG_3L_2$-IFN giving only a single additional broad peak appeared at the retention time of 33.1 ± 0.1 minutes while the reference peak at 41.7 ± 0.1 minutes reduced in height indicating the site specific PEGylation [14]. The smaller area of linear PEGylated peptide peak than tri-branched PEGylated peak at retention time 33.1 ± 0.1 minutes in the two products confirms the fact that there is more site specificity in tri-branched product than the linear one. Greater site specificity of attachment of tri-branched PEG molecule to the protein may be due to its specific structure which is not readily accessible to all lysine sites in IFN protein [3, 10]. The broadness of the PEGylated peptide peak (33.1 min) is due to broad mass distribution (polydispersity) induced by mPEG moieties.

There was a no significant degradation pattern seen in any PEGylated product in SDS-PAGE analysis after one year storage at 4°C (Fig. 4). Similarly there was no significant loss in their bioactivities and no aggregation was seen after one year storage at 4°C (Table-4). It should be pointed out that while bioactivity was retained at 4°C, additional data need to be collected to select the most appropriate storage conditions.

Experimental

Materials

Recombinant IFNa2 was produced and kindly provided in sodium acetate solution by the Biopharmaceutical Labs at Centre for Applied Molecular Biology (CAMB), Lahore. 30KDa N-hydroxy succinimidyl polyethylene glycol reagents were purchased from Jenkem Company, USA. SP HP Sepharose 5 ml column was purchased from GE Healthcare, USA and TSK gel column was purchased from Agilent Technologies Inc. USA. Fractogel from Merck was packed in 16/26 XK column from GE Healthcare. Sodium dodecyl (lauryl) sulfate polyacrylamide gel electrophoresis (SDS-PAGE) units used were from Hoefer Inc. USA and polyacrylamide solution from Sigma-Aldrich, USA. Barium chloride and iodine were purchased from Merck. Protein Low Molecular Weight marker was purchased from GE Healthcare, USA.

Pre-reaction and post-reaction protocols like, purification, SDS-PAGE, HPLC, bioactivity testing etc. were used as mentioned in our other publication [10]

Site Specificity of PEGylation

mPEG-IFN and $mPEG_3L_2$-IFN were subjected to peptide digestion using Trypsin Lys-C from Sigma, USA and then reaction mixes were analyzed by reverse phase HPLC (RP-HPLC) using ZORBAX 300SB-CN 4.6 x 150mm, 5µm column from Agilent Technologies on Shimadzu LC system. 0.1% trifloroacetic acid in water was used as mobile phase A and 0.1% triflouroacetic acid in acetonitrile as mobile phase B with a flow rate of 1 ml/min. Elution was performed with a linear gradient of 60% mobile phase B in 1 h.

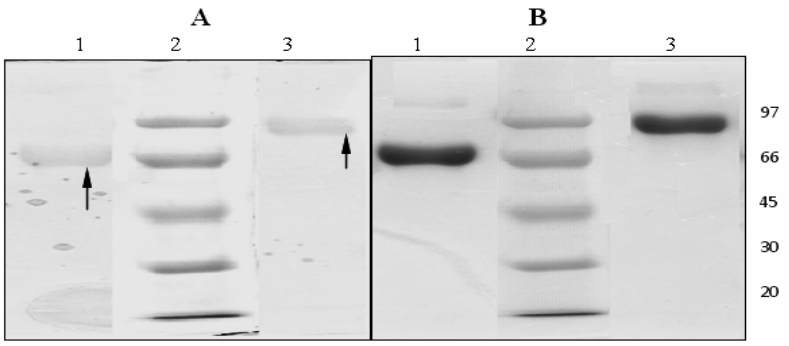
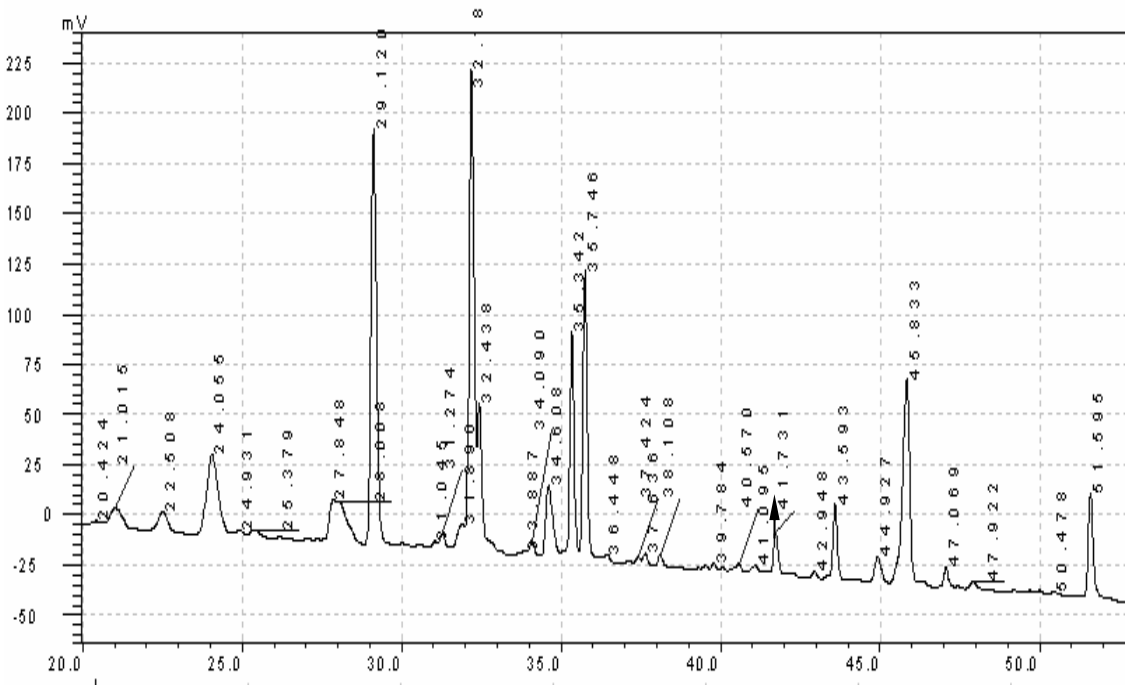


Fig. 4: SDS-PAGE analysis of PEGylated interferons after one year storage at 4˚C.

A) Coomassie Staining B) PEG Staining. Lane 1- mPEG-IFN 30KDa (Linear) Lane 2- Protein Marker, Lane 3- $mPEG_3L_2$-IFN 30KDa.



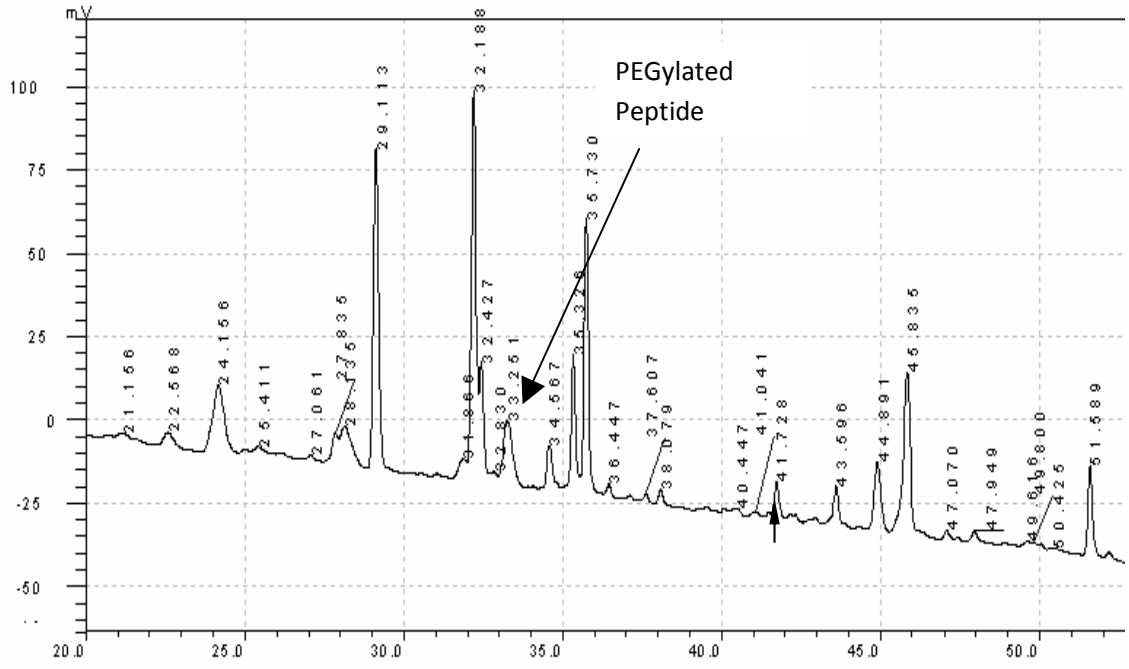


Fig. 5: SE-HPLC analysis of Lys-C endoproteinase digested PEGylated and un-PEGylated IFN samples. Lys-C generated peptide maps of IFNα2 (reference) and mPEG-IFN. Reduction in the ‘‘small arrowed’’ peak in mPEG-IFN map indicates that this particular peptide is being PEGylated during the PEGylation reaction and an additional broad ‘‘long arrowed’’ peak (PEGylated peptide) appears as a result.

Table-4: Stability in Bioactivities after One Year Storage at Different Temperatures.

S.N.	Product Name	Actual Bioactivity IU/mg	Bioactivity After one Year (IU/mg)			% Decrease in Bioactivity		
			-20°C	4°C	25°C	-20°C	4°C	25°C
1	IFN	2.50×10^8	1.90×10^8	2.35×10^8	2.28×10^8	24.00	6.00	8.80
2	mPEG-IFN	2.36×10^6	2.10×10^6	2.26×10^6	2.17×10^6	11.02	4.20	8.05
3	$mPEG_3L_2$-IFN	2.38×10^7	1.97×10^7	2.37×10^7	2.35×10^7	17.22	0.4	1.26

Stability Studies

Filter sterilized (with 0.45µm filter) aliquots of native and PEGylated IFN solutions were stored at -20°C, 4°C and 25°C for one year and at the end of the year these were subjected to SDS-PAGE and bioactivity analysis to check any degradation.

Conclusion

Results of this comparison study confirm that although degree of PEGylation is slightly higher with linear PEG reagent but higher *in vitro* bioactivity of tri-branched $mPEG_3L_2$-IFN makes it better therapeutic conjugate. As serum circulating half lives of bio molecules are proportional to their mobility and tri-branched $mPEG_3L_2$-IFN shows slower mobility and higher apparent molecular mass, so it is predicted that its serum half life will be greater than both of native IFN and linear PEG-IFN as reported by Bailon [3]. PEGylation in tri-branched $mPEG_3L_2$-IFN seems to be more specific than the linear mPEG-IFN and the former is also more stable than the later and native IFN. So over all tri-branched $mPEG_3L_2$-IFN is reported to be having better therapeutic efficacy than native IFN and linear mPEG-IFN.

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