REPORT

PEG-interferon conjugates: Effects of length and structure of linker

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Abstract: Di-branched (Y-shaped) polyethylene glycols (PEGs) are considered more effective than linear molecules to enhance the efficacy of the conjugated drug. In the present study interferon α-2a was conjugated with three different 40 KDa di-branched PEGs. The results of this study show that length and/or the structure of linker between PEG and the protein is also involved in the synthesis, *in vitro* biological activity and stability of the conjugate.
Three conjugates i.e., $mPEG_2L$-IFN, $mPEG_2P$-IFN and $mPEG_2M$-IFN yielded 25%, 24% and 17%, with bioactivities 2.8 x 10^6 IU/mg, 3.95 x 10^6 IU/mg and 6.7 x 10^6 IU/mg, respectively. The order of bioactivity stability is $mPEG_2L$-IFN > $mPEG_2P$-IFN > $mPEG_2M$-IFN > IFN (native).
We report that although lengthy linkers are more reactive in terms of conjugation, they have opposite effect on the *in vitro* bioactivity of the conjugate. PEGylation as a whole increases the stability of the conjugate, and linkers also add in stability.

Keywords: PEG-interferon, Di-branched, polyethylene glycols, conjugation.

INTRODUCTION

PEGylation as a whole increases the stability of the conjugate, and linkers also add in stability. Despite the tremendous advances in recombinant biopharmaceuticals, the recombinant proteins possess several disadvantages including their proteolytic degradation, short *in vivo* circulating half life, rapid clearance through kidneys, low solubility and tendency to produce new trailing antibodies (Harris and Chess, 2003; Malik *et al*., 2007). These shortcomings limit the usefulness of these biological drugs. In the recent years an intensive research has been undertaken to overcome these problems. To improve the efficacy of these proteins, various strategies have been developed including the following:

a. Use of protein engineering techniques for the alterations in amino acid sequence of the proteins so that proteolytic degradation and antigenic side effects can be controlled.
b. Production of chimeric protein drugs fused to albumin for improving half life.
c. Incorporation of these proteins into some suitable drug-delivery vehicles such as liposome, (Mateo *et al*. 2000). Covalent attachment of polyethylene glycol (PEG) molecule to the protein is considered an extremely valuable technique to make the bio-drug more soluble, non-aggregating, non-immunogenic and more stable to proteolytic degradation (Harris and Chess 2003; Abuchouski *et al*. 1977).

PEGylation is a modern technique for improving the therapeutic and biotechnological potential of peptides and proteins. If PEG is properly attached to a polypeptide, it modifies many of its properties while the main biological functions, may remain the same. PEG conjugation masks the protein's surface and increases the apparent molecular size of the polypeptide, thus reducing its ultrafiltration through kidneys, preventing the antibodies or antigen processing cells to approach the polypeptide and reducing its degradation by proteolytic enzymes (Veronese *et al*., 2001). Therefore, PEGylation is a method of choice to enhance the serum half life of biological recombinant drugs like recombinant interferons (IFNs) as clinically, proven technology for increasing the therapeutic efficacy (Brocchini *et al*. 2008). Although the covalent attachment of PEG chains to therapeutic proteins can enhance their *in vivo* half life, at the same time they have the opposite effect of reducing pharmacological properties such as *in vitro* bioactivity (Sabar *et al*. 2010). This reduction in their biological activity depends upon three factors:

a. size of the PEG molecule attached,
b. structure of PEG molecule attached, and
c. attachment site of PEG on the protein molecule.

It is generally agreed that the properties of PEGs play a dominating role in the overall properties of the conjugates. In addition, the development of robust site specific PEGylation techniques and use of branched PEGs have expanded and perhaps will continue to expand the polymer options available for polypeptide PEGylation (Morar *et al*., 2006). For safe protein therapeutics more effective PEG structure and sizes are required (Jo *et al*. 2006). There are several reports on the comparison of effects of sizes of PEGs and position of attachments on

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the biological activity. Much attention has been given to the optimization of the degree of substitute with PEG chains, method of substitution and selection of the best PEG chain length. Often, little consideration has been given to the fact that structure or length of linker between PEG and protein can also be involved in degree of substitution and biological activity of the modified proteins (Jo *et al*. 2006).Here we report the effect of structure of linker between interferon protein and same sized Y-shaped 40KDa PEGs on the properties of the final conjugate.

As 40 KDa di-branched (Y-shaped) PEGs are considered more effective than linear molecules to enhance the efficacy of the conjugated drug (Bailon *et al*., 2001; Harris and Chess 2003), we used three different 40 KDa di-branched PEGs for the conjugation of interferon α-2a. The difference was the length of the linker between PEG and the protein. The results of this study show that length and/or the structure of the linker is also involved in the synthesis, *in vitro* biological activity and stability of the conjugated protein.

MATERIALS AND METHODS

Materials

Recombinant IFNα2a was produced and kindly provided in sodium acetate solution by the Biopharmaceutical Labs at Centre for Applied Molecular Biology (CAMB), Lahore. Di-branched 40KDa N-hydroxy succinimidyl polyethylene glycol reagents (i.e., $mPEG_2L$-NHS and $mPEG_2P$-NHS) were purchased from NEKTAR Therapeutics, USA while $mPEG_2P$-NHS was purchased from Jenkem Company, USA. PEGasys injection was purchased from local market. SP HP Sepharose 5 ml column was purchased from GE Healthcare, USA and TSK gel column used was from Agilent Technologies Inc. USA. Fractogel weak cation exchanger resin was purchased from Merck which was packed in 16/26 XK column from GE Healthcare. Sodium dodecyl (lauryl) sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Hoefer Inc. USA systems. Solution of polyacrylamide was from Sigma-Aldrich, USA. Barium chloride and iodine, for PEG specific staining of the gel, was purchased from Merck. Protein Low Molecular Weight marker was purchased from GE Healthcare, USA.

Pre-reaction IFNα2a solution processing

Buffer (sodium acetate) containing IFN protein was exchanged by 100 mM sodium borate buffer pH 8.0 by using gel filtration with SP HP Sepharose 5 ml column on AKTA-10 FPLC system from GE Healthcare. $A_{280nm}1.05$ = 1 mg/ml was used for the quantification of protein. Characterization of IFNa2 was done by SDS-PAGE and size exclusion (SE) chromatography using 50 mM Na-acetate, pH 5.2 + 0.2 M NaCl + 10% ETOH as mobile phase on TSK gel with isocratic gradient on Shimadzu HPLC system.

PEGylation reactions

40KDa $mPEG_2L$-NHS, $mPEG_2P$-NHS and $mPEG_2M$-NHS reagents were placed at room temperature for half an hour before the reaction. 2.5 molar excess PEG reagents or 5 times of protein quantity was used. Before use, the reagents were stored in an inert atmosphere of argon at -20˚C. The tube in which the required amount of the reagent was to be weighed was wrapped with aluminum foil to protect it from light. Required amount of the reagent was dissolved quickly in freshly prepared 1mM HCl in a concentration of 100 mg/ml (2.5mM). The HCl solution was placed at 4˚C or in ice before and at the time of dissolving the reagent. After dissolving for few seconds, the reagent was quickly transferred to the concentrated IFN solution in 100mM Sodium borate solution pH 8.0 which was also placed in ice. PEG reagent was added by continuous stirring of IFN solution at 0°C. The mixture was placed at a slow magnetic stirring (150 RPM). During the reaction, progress of conjugation was monitored by RPC by using Source 5RPC ST 4.6/150 column using the "method RPC" mentioned earlier (section 3.5.1). After two and half hours the reaction was stopped by adjusting the pH to 4.5 by adding glacial acetic acid in the reaction mix and verifying the pH by pH strips. The conductivity of the reaction mixture was adjusted to 2.5mS/cm by diluting it in 10mM Sodium acetate pH 4.6. After this the next purification step was followed within two hours as delay in the purification process caused the poor purification as well as reduction in the percentage yield of the required product.

Purification of PEG-IFN conjugates

For purification of the desired products, Fractogel (a weak cation exchanger) column (16/26 XK) was used. 10mM sodium acetate buffer pH 4.5 was used as equilibration buffer (i.e., buffer A). 1 M sodium chloride in 50 mM sodium acetate (buffer B) was used with a gradient of 7% in 60 min at a rate of 2.5 ml/min. Due to its strong binding capacity, un-reacted IFN was eluted at a gradient of 100% buffer B in 5 minutes.

SDS-PAGE analysis

Purified products were analyzed on 8% SDS-PAGE (Laemmli, 1970). Coomassie brilliant blue was used to reveal the protein bands. PEG-conjugates were stained using barium chloride and iodine solution according to a modified procedure of Kurfurst (Kurfurst 1992). Detailed procedure described in our other article (Sabar *et al*., 2010).

Size exclusion high performance liquid **Chromatograpghy (SE-HPLC)**

SE-HPLC was performed with the Shimazu LC solution HPLC System using TSK gel 3000SW 7,5×600mm,

(Tosoh Bioscience, Japan, P/N 05103). The mobile phase was 50mM Na-acetate, pH 5.2, 0.2M NaCl, 10% ethanol (pH 5.2). The flow rate was 1.0 ml/min. Detection was performed at 280 nm

In vitro anti-viral bioactivity

Antiviral bioactivities of both purified products were determined using the method of Rubinstein *et al*. (1981). Reduction in cytopathic effect (CPE), which determines the ability of IFN or PEG-IFN to protect Mardin-Darby bovine kidney (MDBK) cells challenged with vesicular stomatitis virus (VSV), was measured. Reference IFN-α2b (Catalog No.82/576) was used from National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK. Units of bioactivity were measured in international units (IU). One unit is defined as the quantity of IFN or PEG-IFN required per milliliter to reduce the cytopathic effect of viral infection by 50%.

Site Specificity of PEGylation

mPEG-IFN and $mPEG_3L_2$-IFN were digested using Trypsin Lys-C from Sigma, USA and then products were analyzed by reverse phase HPLC (RP-HPLC) on ZORBAX 300SB-CN 4.6 × 150mm, 5µm column from Agilent Technologies. 0.1% trifloroacetic acid in water and 0.1% triflouroacetic acid in acetonitrile were used as mobile phase A&B respectively with a flow rate of 1 ml/min. Products were eluted using a linear gradient of 60% mobile phase B in 1 h.

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 and reduction in bioactivity.

RESULTS

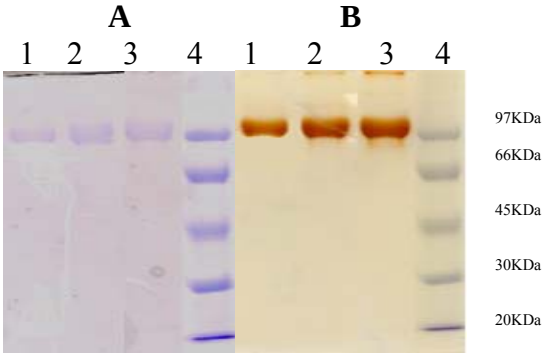


Fig. 1: SDS-PAGE analysis of PEGylated interferons A) Coomassie Staining B) PEG Staining. Lane 1- $mPEG_2L$-IFN, Lane 2- $mPEG_2P$-IFN, Lane 3- $mPEG_2M$-IFN Lane 4- Protein Marker.

Fig. 1 shows that in SDS-PAGE analysis bands of all PEGylated IFNs appearing almost at the same molecular weight i.e., 100KDa.

Figs. 2 and 4 indicate that there is no significant degradation of PEGylated and Un-PEGylated IFNs after one year storage at 4˚C

Fig. 2A: IFN

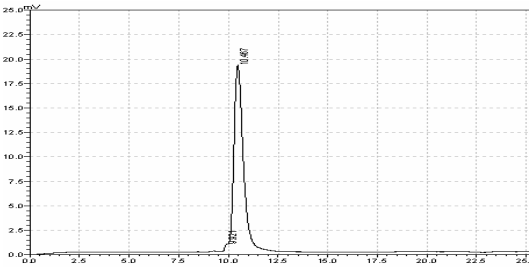


Fig. 2B: mPEG2L-IFN

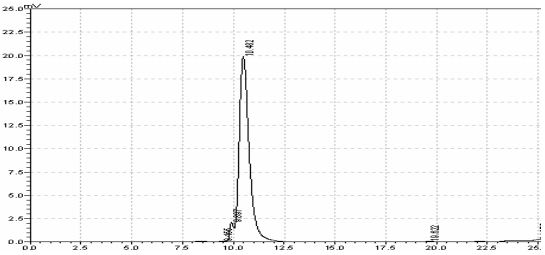


Fig. 2C: mPEG2P-IFN

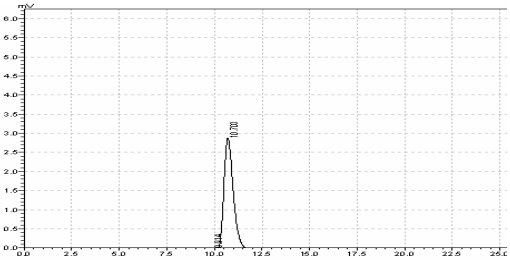


Fig. 2D: mPEG2M-IFN

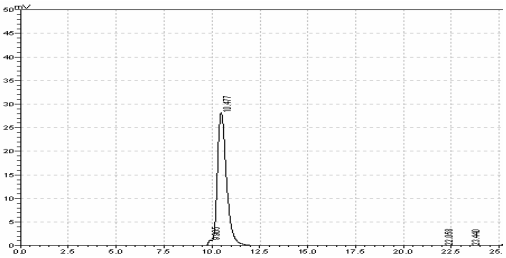


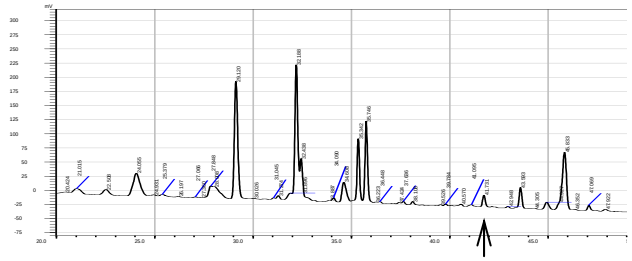
Fig. 2E: PEGasys

Fig. 2: SE-HPLC analysis of all products after one year storage at 4˚C.

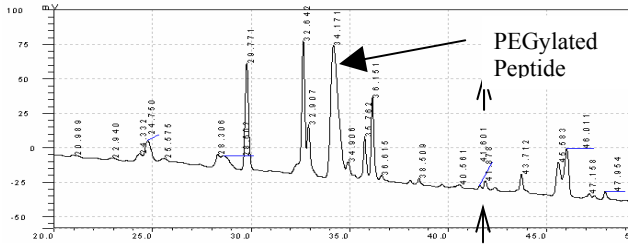
In this study $mPEG_2L$-IFN, $mPEG_2P$-IFN and $mPEG_2M$-IFN yielded 25%, 24% and 17% respectively of the total IFN protein used.

Similarly bioactivities calculated for $mPEG_2L$-IFN $mPEG_2P$-IFN and $mPEG_2M$-IFN are 2.8×10^6 IU/mg, 3.95×10^6 IU/mg and 6.7×10^6 IU/mg, respectively.

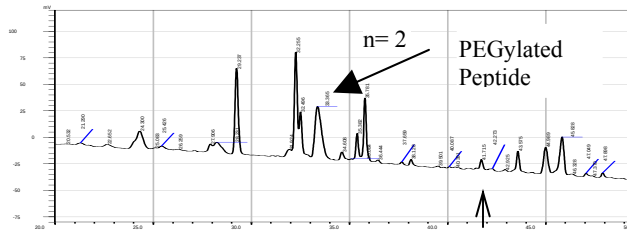
Fig. 3 shows RP-HPLC analysis data generated after peptide digestion with Trypsin Lys-C. The peak of reference IFN peptide at ≈41.7±0.1 minutes retention time (indicated by narrow arrow) is reduced in height and an additional peak at ≈33.1-34.1±0.1 minutes (indicated by broad arrow) appeared in all mono-PEGylated IFNs of this study (including PEGasys from Roche).



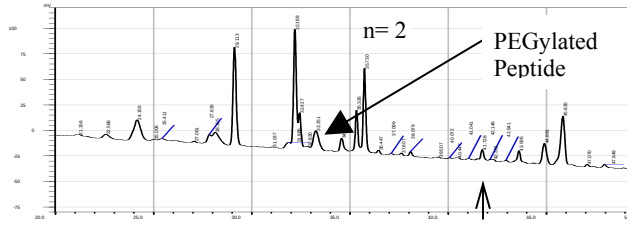
IFN (Ref)



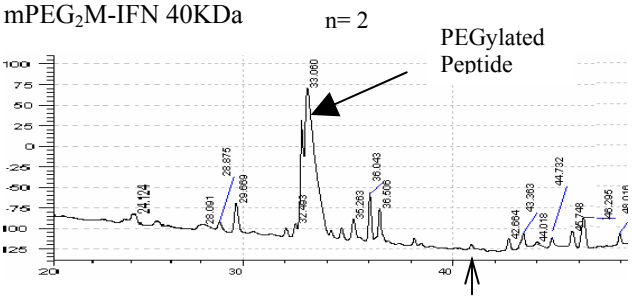
$mPEG_2L$-IFN 40KDa



$mPEG_2P$-IFN 40KDa



$mPEG_2M$-IFN 40KDa



PEGasys (Roche-40KDa PEG_2-IFN

Fig. 3: Trypsin Lys-C Peptide Maps of Un-PEGylated IFN and PEGylated IFNs. Peak indicated by narrow arrow is reduced in hight as compared to reference peak and broad arrow indicates the additional peak appeared in PEGylated product

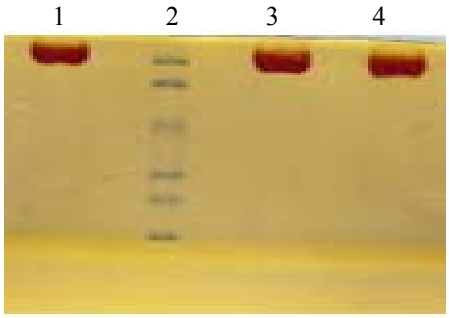


Fig 4: SDS-PAGE analysis of PEGylated interferons after one year storage at 4˚C (PEG Staining). Lane 1- $mPEG_2L$-IFN, Lane 2- Protein Marker, Lane 3- $mPEG_2P$-IFN, Lane 4- $mPEG_2M$-IFN

DISCUSSION

Understanding, of how specific characteristics of PEG-IFN influence their *in vitro* bioactivity that may be translated to their *in vivo* efficacy is very important. Although extensive studies concerning the effects of size, structure, nature of active groups on PEG, synthesis reactions and site of PEGylation on the protein have been done, little information is available on the effects of structure and length of linker between PEG and protein on the over all properties of the conjugates. Here in this manuscript the effects of structure and length of linkers are discussed.

PEG can accumulate in muscles, skin, bone, and liver to higher extent than other organs irrespective of the molecular weights and its eliminations occurs either in kidneys for PEGs smaller than 30KDa and or faeces for PEGs greater than 20KDa (Yamaoka *et al.*, 1994; Harris & Chess 2003). The clearance rate of PEG from the body after being injected to the blood stream is inversely proportional to the molecular weight of the PEG (Yamaoka *et al.*, 1994).

Although the length and structure of the linker used in this study to join PEG and protein in the three conjugates were different, for site specific PEGylation, N-hydroxy succinimidyl (NHS) functional group was used with all three PEG molecules. We used the reported branched Y-type design of 40KDa PEG ($mPEG_2L$-NHS) with lysine linker and N-hydroxy succinimidyl (NHS) active group (Bailon *et al.*, 2001; Greenwald *et al.*, 2004), along with

Table 1: Bioactivity analysis before and after one year storage at different temperatures

S. No.	Product Name	Actual Bioactivity IU/mg	Bioactivity After one Year (IU/mg)			Change in Bioactivity			% Change in Bioactivity		
			-20˚C	4˚C	25˚C	-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	6.00×10^7	1.50×10^7	2.20×10^7	24.00	6.00	8.80
2	$mPEG_2L$-IFN	2.8×10^6	2.50×10^6	2.70×10^6	2.65×10^6	3.00×10^5	1.00×10^5	1.50×10^5	10.70	3.57	5.36
3	$mPEG_2P$-IFN	3.95×10^6	3.10×10^6	3.80×10^6	3.55×10^6	8.50×10^5	1.50×10^5	4.00×10^6	21.50	3.70	10.13
4	$mPEG_2M$-IFN	6.7×10^6	5.60×10^6	6.30×10^6	5.90×10^6	1.10×10^6	4.00×10^5	8.00×10^5	16.42	5.97	11.94
5	PEGasys	3.0×10^6	2.60×10^6	2.90×10^6	2.90×10^6	4.00×10^5	1.00×10^5	1.00×10^5	13.33	3.33	3.33

two new Y-type structures without lysine linker ($mPEG_2P$-NHS with propylene linker and $mPEG_2M$-NHS with methylene linker). Due to hydrophilicity of PEG molecules, apparent size of all 40KDa branched PEG-IFNs in SDS-PAGE was estimated to approximately 100 KDa (fig. 1), which is in concordance with the reported size (Bailon *et al*., 2001), and exceeding the renal threshold (~65KDa) of filtration (Maack, 1992). SE-HPLC indicated that linker length also had some impact on the retention time of the conjugate (fig. 2) i.e., lengthy linker makes the conjugate to elute faster than the shorter linker.

Results of this study show that in PEGylation reactions, $mPEG_2L$-IFN and $mPEG_2P$-IFN yielded almost 25% (25% and 24%, respectively) of the total IFN protein used, while $mPEG_2M$-IFN yielded 17%. Lower yield of $mPEG_2M$-IFN can be explained according to Robert *et al*. (2002) who have reported that reducing the length of linker between PEG and protein by reducing the methylene units can reduce the reactivity of the PEG reagent. As $mPEG_2M$-IFN has reduced length of the linker so its yield is lesser than the other two PEGs.

In fig. 3, the decrease in peak height at ≈41.7±0.1 minutes is considered to be due to the PEGylation of the respective peptide. The peptide which is PEGylated appears at lower retention time in RP-HPLC at 33.1-34.1±0.1 minutes (Baker *et al*., 2006; Sabar *et al*., 2010). The presence of a broad peak at 33.1-34.1±0.1 minutes also indicates a PEGylated peptide due to broad mass distribution (polydispersity) induced by PEG moiety as mentioned by Foser (Foser *et al*., 2003). Zeuzem (2000) also quoted that the lysine residue which is attached to PEG polymer is not recognized by the Lys-C proteinase (Baker *et al*., 2006), therefore the polypeptide chain is not cleaved at that particular position and its peak will be missing from the map (Zeuzem, 2000). It should be noted that in the present study the peak is not completely missing, and it may be due to partial PEGylation of IFN or there may have some rare isomers (Bailon *et al*., 2002). The peptide map of PEGasys in the same conditions generated the same pattern of peaks (fig. 3) indicating that PEGylated interferon products synthesized in this study may have the same site of conjugation on the protein as PEGasys. The N-hydroxy succinimidyl (-NHS) ester derivative forms stable amide bonds with primary amino groups of IFN-α2a, (Zhi *et al* 1995). This fact is quite evident from the stability study experiments of all PEGylated products synthesized in this study. SDS-PAGE and SE-HPLC results show that all products are intact and stable at 4˚C studied for one year as there was no detectable degradation of the products (fig. 4). This shows that the amide bonds between PEGs and IFN protein are stable at these storage conditions. Bioactivities results of PEGylated and unPEGylated interferon summarized in table 1 also confirm that all studied products are stable in their *in vitro* bioactivities at 4˚C storage conditions (with insignificant variability in bioactivities) at least for one year.

The results in this table also indicate that PEGylation can make the protein more stable than the native protein at these storage temperatures. Furthermore, lysine linker between PEG and protein adds in the stability of the conjugate. Samples stored at -20˚C exhibited reduced bioactivity to a significant level. This may be due to the changes occurred during "freeze thaw" process of the samples. So the order of stability in bioactivities at 4˚C is PEGasys > $mPEG_2L$-IFN > $mPEG_2P$-IFN > $mPEG_2M$-IFN > IFN (native). Although results of this study showing inverse relationship of bioactivities with the length of linker i.e., greater the length lower is the bioactivity but at the same time stability in bioactivity is opposite in order. It indicates that length of linker makes the conjugate more stable.

CONCLUSION

This study demonstrates that linkers between PEG and protein and their lengths affect the properties of the PEG-protein conjugates. Although lengthy linkers have more reactivity toward the conjugation but they have opposite effect on the *in vitro* bioactivity of the conjugate i.e.,

lengthy linkers have lower bioactivities. PEGylation as a whole increases the stability of the conjugate and linkers also add in stability. PEG-protein conjugates with lengthy linkers are more stable than others with short linkers.

Authors' contributions

This study was conducted during 2008-2011. MFS conceptualized and designed the study. MFS also performed all the bench work on synthesis, purification and analysis of conjugates. MFS and SK drafted the manuscript. SK and MS carried out the SDS-PAGE analysis. MS also helped in preparing the manuscript. AUZ performed the bioactivity testing. All authors read and approved the final manuscript.

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