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Pegylated Protein Encapsulated Multivesicular Liposomes: A Novel Approach for Sustained Release of Interferon α

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ABSTRACT Hepatitis C viral chemotherapy suffers from a relatively short half-life of the interferon α-2a (IFN α). To address this issue, we investigated the effects of polyethylene glycol modification and their subsequent encapsulation in multivesicular liposomes (MVLs), on the release properties of IFN α. In the present study, interferon-α was conjugated with methoxy-polyethylene glycol (mPEG, MW 5000). Prepared IFN α-$mPEG_{5000}$ conjugate (IFN α-$mPEG_{5000}$) was purified with size exclusion chromatography. The relative in vitro anti-viral activity of pegylated interferon α-2a was found to 87.9% of the unmodified IFN α. Pegylated IFN α encapsulated multivesicular liposomes were prepared by double emulsification technique followed by evaporation of organic solvents from chloroform ether spherules suspended in water. Prepared MVLs were then characterized for shape, size, vesicle count, encapsulation efficiency, and in vitro release rate. In process stability studies of pegylated IFN α protein exhibited better stability when exposed to chloroform: diethyl ether (1:1 ratio) mixture as well as variable vortexing time as compared to native IFN α. Relatively high percentage of encapsulation of protein (~75%) was achieved. In vitro release profile of pegylated IFN α-$mPEG_{5000}$ containing MVLs in the PBS showed lower initial burst release with sustained and incomplete release over a period of 1 week. In contrast, native IFN α entrapped MVLs were observed as higher initial burst release, i.e., nearly 35% followed by almost complete release. The results confirmed the possibility of multivesicular liposomes as a long-acting or sustained-release delivery system using a combination of pegylation and encapsulation technique for controlled delivery of interferon α.

KEYWORDS Sustained release, Multivesicular liposomes, Pegylation, Pegylated-protein encapsulation technology, Interferon α

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INTRODUCTION

The efficacy of present hepatitis C virus (HCV) chemotherapy is limited due to the multiple hosts (e.g., viral load) and viral factor (e.g., HCV genotype) as well as the inadequate pharmacokinetics of IFN α-2a (will be referred as IFN α). This limitation results from the fact that antiviral drugs presently used in chemotherapy, lack sustained effect due to the short half-life of IFN α, *i.e.*, 4–7 h (Liang et al., 2000). Thus, a longer acting IFN α formulation is highly desirable to enhance the efficacy as well as to improve the compliance of patients.

Previous studies indicate that pegylation is one of the most successful techniques in the recent years to prolong the residence time of proteineous drugs in plasma while retaining the actitvity of therapeutic proteins (Zalipsky & Harris 1997; Delgado et al., 1992; Bailon & Berthold 1998; Katre 1993; Vyas & Khar 2001). Polyethylene glycol (PEG) is advantageous as a protein-modifying agent because it is inert, water-soluble, nontoxic, and modular in size. Pegylation can offer several advantages, which include longer circulation times of the proteins, enhanced solubility and stability, and reduced immunogenicity of recombinant proteins (Katre 1993; Katre 1990). Methoxy-PEG (mPEG) derivatives of higher molecular weights that have been shown to increase the circulation half-life of IFN α up to 70 h are in the subsequent trial phases (Glue et al., 2000).

Liposomes as drug delivery systems have the potential for providing controlled release of administered drug and increasing stability of labile drugs (Vyas & Khar 2001; Gregoriadis 1991). Liposomal IFN α preparations can alter the pharmacokinetics, tissue distribution, and uptake of IFN α in comparison with free IFN α (Eppstein & Stewart 1981). Encapsulation of drugs into multivesicular liposomes (MVLs) offers a novel approach to sustained-release drug and protein delivery (Mantripragada, 2002). We hypothesized that a sustained release formulation of pegylated IFN α by formulating as MVLs could possibly be another way to increase the blood circulation time of parent drug. Double emulsification is a most common method of MVLs preparation method however, during encapsulation process, many factors, i.e., presence of organic solvents, sonication speed, could adversely affect the physical and biological properties of the protein drugs (Diwan & Park 2001; Johansen et al., 1998). Therefore, the preservation of protein stability during encapsulation and release is essential for the development of a successful controlled-release preparation of protein drugs. Our strategy was designed encompassing both approaches, *i.e.*, pegylation of IFN α and thereafter its entrapment in MVLs, to achieve enhanced stability during encapsulation as well as an improved release kinetic pattern of therapeutic protein IFN α.

With the aim to develop controlled-release preparation of therapeutic protein, i.e., IFN α in the present study, IFN α was conjugated with methoxy polyethylene glycol of molecular weight 5000 using N-hydroxy succinimide ester of mPEG ($mPEG_{5000}$-NHS). Prepared pegylated interferons (IFN α-$mPEG_{5000}$) were purified with size exclusion chromatography and further characterized for SDS-PAGE, matrix-assisted laser desorption and ionization time of flight (MALDI-TOF) mass spectroscopy analysis, *in process* protein stability and antiviral activity. Then IFN α-$mPEG_{5000}$ conjugates were encapsulated in multivesicular liposomes using water-in-oil-in-water double emulsification method. The prepared MVLs were characterized for particle size, shape, percent encapsulation efficiency, storage stability, and in vitro release and results were compared with plain or unmodified IFN α.

MATERIALS AND METHODS

Materials

Interferon α-2a was obtained from Shantha Biotech (Hyderabad, India) as a kind gift. N-hydroxy succinimide derivative of monomethoxy polyethylene glycol ($mPEG_{5000}$-NHS, MW 5000 Da) was obtained from shearwater polymers (Huntsville, AL). Soya phophatidylcholine (soyaPC), cholesterol (Chol), phosphatidylglycerol (PG), tristearin (TS), and Triton X-100 were purchased from Sigma Chemicals (Sigma, St. Louis, USA). SDS-PAGE gel electrophoresis kit (KT-31) and microBCA protein assay kit was purchased from Genei (Genei Ltd. Bangalore, India). All other chemicals and reagents used were of analytical grade.

Preparation and Characterization of mPEG Derivative of IFN α

IFN α was derivatized using N-hydroxy succinimidyl ester of mPEG of molecular weight 5000 as reported previously with few modifications (Bailon et al., 2001).

The reaction conditions were optimized to obtain high conjugation yield in a reproducible manner. Briefly IFN α was dissolved in 50 mM borate buffer pH 9.0 in a final protein concentration of 10 mg/mL and filtered through a 0.45 μm pore size syringe filter. $mPEG_{5000}$-NHS (10–30 molar times) was dissolved in DMSO and was added dropwise to 2.0 mL of cold solution of IFN α. The conjugation was allowed to proceed overnight at 4°C with an end-to-end rotation and quenched with addition of glycine (15 mM). Finally, the reaction product was purified by size exclusion chromatography, lyophilized (Hetodrywinner Lyophilizer, Germany), and stored at 4°C until use.

Size Exclusion HPLC (SE-HPLC)

The composition of IFN α-$mPEG_{5000}$ conjugates was analyzed on a HPLC system consisting of Waters 660E pump and UV detector equipped with an autochrodata module (Shimadzu, Japan). The sample was applied to 8 mm × 300 mm Shodex protein column KW-802.5 (Showa Denko KK, Japan) through a guard column and eluted with phosphate buffered saline (PBS, 50 mM, pH 7.4) as a mobile phase. The flow rate was 0.8 mL/min and elutes were monitored at 280 nm using UV detector.

Column was washed with the equilibration buffer to remove excess PEG reagent, reaction byproducts, and IFN α-$mPEG_{5000}$ oligomers. The desired monopegylated IFN α-$mPEG_{5000}$ was then eluted with 200 mM sodium chloride in the equilibration buffer. Unmodified interferon α-2a still adsorbed onto the column was removed by washing with 750 mM sodium chloride in the equilibration buffer. Monopegylated IFN α-$mPEG_{5000}$ elute was further concentrated to approximately 1 mg/mL and diafiltered into the final storage buffer, 20 mM sodium acetate, pH 5.0, containing 150 mM sodium chloride. Concentrated IFN α-$mPEG_{5000}$ was sterile filtered with a 0.2 μm filter and stored at 4°C.

In Vitro Antiviral Activity

The antiviral activities of interferon α-2a and IFN α-$mPEG_{5000}$ were determined in vitro in a cell culture bioassay using HepG2 cells received from National Center for Cell Science (Pune, India) challenged with vesicular stomatitis virus as described previously with slight modifications (Rubinstein et al., 1981). Serially diluted interferon samples were incubated with HepG2 cells followed by a challenge with virus. The endpoint of the assay is approximately a 50% protection of HepG2 cells. The quantity of interferon present is determined relative to a reference preparation of interferon. The relative antiviral activity is calculated as

$$\text{Relative antiviral activity} = \frac{\text{Activity of IFN } \alpha\text{-mPEG}_{5000}}{\text{Activity of Standard IFN } \alpha}$$

Preparation of Multivesicular Liposomes (MVLs)

The multivesicular liposomes were prepared by water-in-oil-in-water double emulsification process with few modifications (Kim et al., 1983; New, 1990). Briefly in the first step, water-in-oil emulsion was perpared. Five milliliters of a lipid solution consisting of 19.8 mM soyaPC, 30 mM cholesterol, 4.2 mM phosphatidylglycerol, and 3.75 mM triglyceride in chloroform were mixed with 5 mL of an aqueous buffered solution of pegylated IFN α (5 mg/mL IFN α-$mPEG_{5000}$ in 25 mM acetic acid, pH 4.5, and 4% w/v sucrose) and emulsified to produce a water-in-oil emulsion. The emulsification conditions were 10,000 rpm for 12 min with a mechanical stirrer. The first emulsion was further mixed and emulsified (4500 rpm for 2 min using mechanical stirrer) with a second aqueous solution containing 3.2% glucose (w/v), and 40 mM lysine resulted in a water-in-oil-in-water double emulsion. Chloroform was removed by flushing nitrogen over the surface of the second emulsion at 37°C. The resulting MVL particles were harvested by centrifugation for 10 min at 600 × *g*, washed and resuspended in isotonic buffer solution. Plain IFN α MVLs were also prepared similarly as mentioned above, except the IFN α was included in the first aqueous solution.

Vesicle Size and Shape

Particle size distribution and the median diameter were determined by the method of laser light diffraction using a CILAS-1604 particle size analyzer (Cilas Inc. France).

In Process Protein Stability Assay

The formulation steps involved in the preparation of multivesicular liposomes were simulated to assess the stability of IFN α and IFN α-$mPEG_{5000}$ conjugate. Duplicate samples were employed for each of the following experiments. Protein adsorption to the sample tubes was found to be insignificantly low and hence neglected. Native IFN α or IFN α-$mPEG_{5000}$ aqueous solution (0.05% w/v, 50 µl) was emulsified with chloroform:diethyl ether mixture (1:1 v/v) (500 µl) using a vortex mixer for different time intervals. The duration of vortexing was varied from 1 min to 30 min. After emulsification, the samples were kept at room temperature for solvent evaporation. The samples were extracted for soluble protein fraction with PBS similarly as described above.

Determination of Percent Encapsulation, Lipocrit, Free Drug and Particle Size

Drug loading, percent encapsulation, and lipocrit were determined as described earlier (Ye et al., 2000). Isopropanol: 2N HCl (90:10) solvent was used to extract the protein from the formulations (10-fold dilution in solvent), and this extract was used to determine the amount of encapsulated protein by microBCA assay. Percent recovery of IFN α-$mPEG_{5000}$ is the percent ratio of the amount of drug in the final liposome suspension to the total amount of IFN α-$mPEG_{5000}$ used in the first aqueous solution. Lipocrit is analogous to hematocrit, the percent ratio of the pellet volume of MVLs particles to the suspension volume. Percent free IFN α-$mPEG_{5000}$ is the percent ratio of the amount of IFN α-$mPEG_{5000}$ in the supernatant to the amount of IFN α-$mPEG_{5000}$ in the final suspension multiplied by (1-lipocrit). IFN α-$mPEG_{5000}$ loading is the amount of IFN α-$mPEG_{5000}$ encapsulated in the particle fraction of the suspension.

In Vitro Release Studies

In vitro release rate of prepared formulations was determined by method described by Maghraby et al. with slight modifications (Maghraby et al., 1999). Briefly, 0.2 mL MVLs suspensions were taken in a tube and mixed with 1 mL of human plasma containing 0.01% NaN_3. The mixture was stirred on a mechanical shaker for an appropriate time. The IFN α released during this time interval was separated by minicolumn centrifugation. The amount of protein released was then calculated indirectly from total amount of protein entrapped at that time. The process was repeated in triplicate and the samples were assayed by microBCA method.

Storage Stability Studies

The prepared formulations were tested for stability at 4 ± 0.5°C and 25 ± 0.2°C temperatures. Formulations were stored in amber-colored glass vials for 6 months at controlled conditions and then were evaluated for changes in size, shape, number of vesicles per cubic mm, and residual drug content.

Statistical Analysis

The results were expressed as mean ± standard deviation. Statistical analysis was carried out by student t-test, and statistical significance was designated as $p < 0.05$.

RESULTS AND DISCUSSION

The present study was performed to enhance the release of therapeutic protein interferon α by employing two different approaches, i.e., PEGylation and their subsequent encapsulation in multivesicular liposomes. PEGylation is a well-known method for modifying the pharmacokinetic properties of therapeutic proteins while retaining the therapeutic efficacy. The encapsulation of proteins in the controlled-release delivery vehicles possess many challenges, namely, the maintenance of structural integrity of the encapsulated protein to preserve its bioactivity and stability, and encapsulation of sufficient amount in order to provide sustained and reproducible pharmacological effects with a single injection. Pegylated interferon α-2a was prepared by simple end-to-end mixing and rotation method. IFN α-$mPEG_{5000}$ was prepared by using N-hydroxysuccimide ester of $mPEG_{5000}$. Fifteen percent SDS-PAGE was successfully performed for plain IFN α and IFN α-$mPEG_{5000}$ using coomassie blue staining in locus of molecular weight of 20 kD-40 kD depending upon the molar ratio of IFN α and $mPEG_{5000}$NHS ester used for the conjugation. It was observed that by increasing the molar ratio from 1:10 to 1:30 the extent of pegylation also increases (Fig. 1).

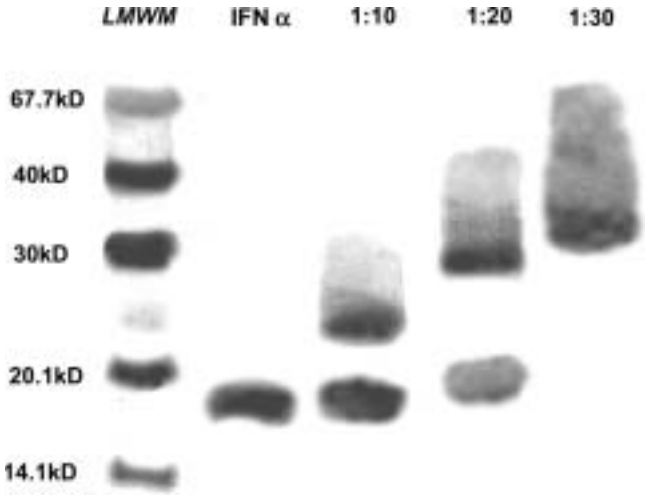

FIGURE 1 SDS–PAGE Analysis of IFN α–mPEG$_{5000}$ Conjugates and Optimization of Conjugation Process Using Low Molecular Weight Marker (LMWM; First Lane from the Left). Individual Lane Indicates the Molar Ratio of IFN α-mPEG$_{5000}$ Derivative.

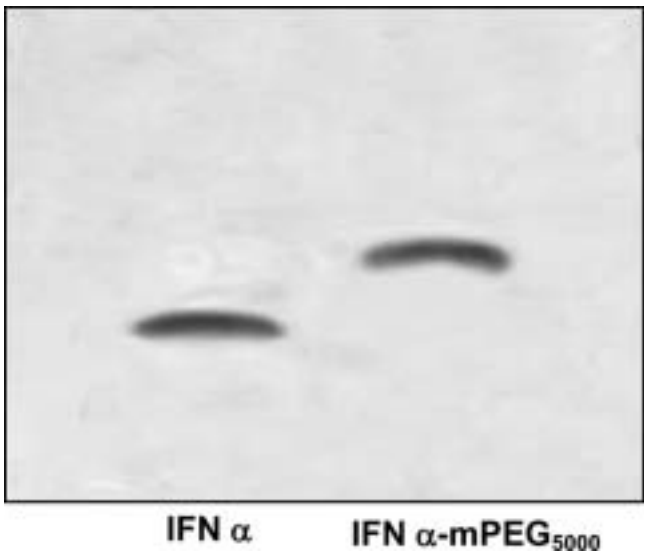

FIGURE 2 SDS-PAGE Analysis of Purified Monopegylated IFN α and Unmodified Native IFN α.

Enhanced pegylation degree may be accomplished by a rise in the formation of multipegylated over monopegylated derivative of IFN α. The dependence of mono and multipegylated IFN α bands formation on the molar ratio was also found in accordance with Diwan and Park (2003). The optimized IFN α-mPEG$_{5000}$ conjugate preparation was further confirmed by MALDI-TOF mass spectroscopy and the results were also found similar as reported previously by Diwan and Park (2003). IFN α-mPEG$_{5000}$ conjugate mainly consisted of, di, tripegylated, tetrapegylated IFN α along with a small fraction of native IFN α. A broad range of multipegylated IFN α conjugates was also detected along with a small amount of fragment of IFN α-mPEG$_{5000}$ (mass spectra not shown).

Optimized IFN–mPEG conjugates were analyzed and purified by size-exclusion HPLC. mPEG derivative of MW 5000, mono-, di-, and multi-pegylated IFN α conjugates were separated due to the larger MW difference between IFN α-mPEG species. A similar chromatogram was observed as for conjugates as reported previously (Diwan & Park 2001). In a different SDS-PAGE experiment, native and purified monopegylated IFN α protein bands were observed after coomassie blue staining in different loci just above the position of native IFN α near the molecular weight region of IFN α (i.e. ~24 kD) due to monopegylated IFN α (Fig. 2).

The inhibitory effect of interferon on virus-induced cell lysis (cytopathic effect) was determined in this assay. The antiviral activities of interferon α-2a and IFN α-mPEG$_{5000}$ are compared utilizing HepG2 cells challenged with vesicular stomatitis virus. IFN α-mPEG$_{5000}$ retains 87.90% relative antiviral activity of that of unmodified interferon α-2a (Table 1).

TABLE 1 In Vitro Antiviral Activities of Interferon α and IFN α-PEG$_{5000}$

S. No.	Proteins	Antiviral activity (IU/mg)*	Relative activity (%)
1.	IFN α	1.58×10^6	100
2.	IFN α-PEG$_{5000}$	1.39×10^6	87.90

*Anti-viral activity determined in HepG2 cells infected with vesicular stomatitis virus. Values are shown as mean of three independent determinations.

The multivesicular liposomes containing IFN α-mPEG$_{5000}$ were prepared by a modified reverse-phase evaporation method (w/o/w double emulsification technique). Double emulsification technique requires an exposure of protein to an aqueous/organic interface that is continuously being generated during emulsification. The shear-induced stress of homogenization further adds up to the unfavorable conditions for physical stability of the protein. Proteins exhibit greater resistance towards aggregation induced under the harsh conditions of w/o/w double emulsification. Figure 3 depicts a phase contrast micrograph of a representative MVLs formulation containing the IFN α-mPEG$_{5000}$. The photomicrograph reflects the smooth, spherical, and multivesicular nature of particles.

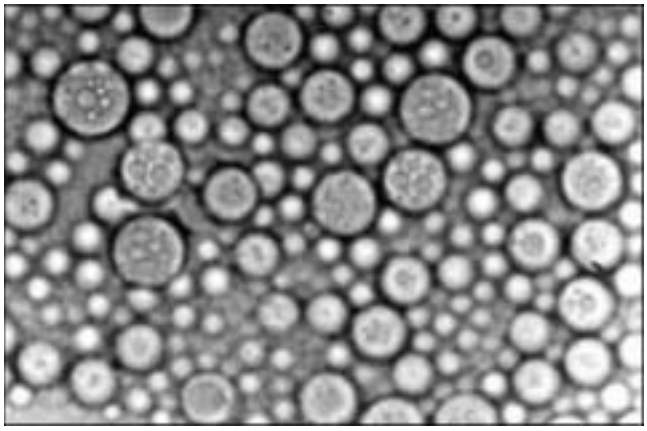

FIGURE 3 **Photomicrograph Showing Phase Contrast Image of IFN α–mPEG$_{5000}$ Encapsulated MVLs at 400X Magnification.**

Table 2 presents a summary of some major characteristics of pegylated and nonpegylated IFN α entrapped MVLs formulations shows with good encapsulation efficiency (~65–75%). The higher percent entrapment may be attributed to their unique structure of vesicles, as these MVLs contain numerous nonconcentric aqueous chambers. Conceivably it would be possible to achieve 100% encapsulation by this method of liposome preparation, since all the initial aqueous volume is trapped within the lipid phase, when the w/o emulsion is first prepared. The losses in percent encapsulation occurred at several points in the preparation. The first major loss occurs when the w/o emulsion is placed in the 0.2 M sucrose and mechanically shaken to make the chloroform-ether spherules. Some of the internal aqueous compartments burst through the lipid phase and join the external aqueous phase; carrying with them the solute intended to stay within the liposomes. A more significant reduction ($P < 0.05$) in percent encapsulation occurs during evaporation of solvents when the lipid phases separating the internal aqueous from external aqueous compartment thins to a bilayer membrane, thus the percent encapsulation decreased to a final value of ~75%. Structural studies on multivesicular liposomes (depofoam particles) using transmission electron microscopy (Kim et al., 1983) confocal microscopy utilizing fluorescence-labeled lipids, and ^{13}C nuclear magnetic resonance study (Ellena et al., 1999), have indicated that the internal chambers of the particles meet at phospholipid bilayer junctions that are filled with bulk triglyceride molecules, which impart the unique multivesicular structure.

Particle size distributions of a pegylated and nonpegylated IFN α entrapped MVL formulation, with volume-weighted median diameters of 17.83 ± 1.04 and 19.47 ± 1.20 μm, respectively, and 92% of the particles were found in size ranging between 8 and 25 μm for pegylated and 95% of the particles ranging from 5 to 40 μm for nonpegylated formulations. In general, monomodal and relatively narrow particle size distributions were achieved for MVLs encapsulated protein and peptide formulations, with no particles smaller than 1 μm or greater than 100 μm. Particle size can be modulated by the process parameters, mostly the emulsification conditions of the second emulsification, which results in the water-in-oil-in-water emulsion, and this size is not dependent on the first aqueous conditions of sucrose or protein concentrations. The vesicle count for MVLs was determined by haemocytometer using an optical microscope. It was observed that the number of liposomes was in the range of ~51–56 particles per mm^3 for pegylated as well as nonpegylated IFN α entrapped multivesicular liposomes.

Proteins are highly sensitive macromolecules, hence pegylation appears to be particularly useful in improvement of physical and thermal stability, protection against susceptibility to enzymatic degradation. The effect of exposure of organic solvent and shear-induced stress of homogenization were individually studied and results were compared against native IFN α. Organic solvent system chloroform: diethyl ether in (1:1 v/v ratio) was used for preparation of MVLs, hence the effect of exposure of this organic solvent system on the solubility of IFN α in PBS (pH-7.4) was observed (Table 3). After treatment, soluble protein

TABLE 2 **General Characteristics of Multivesicular Liposomes**

MVLs formulations	% Encapsulation	Volume weight median diameter (μm)	% Free drug	Vesicle count (No. of vesicles / mm^3) × 1000
IFN α MVLs	75.38 ± 4.24	19.47 ± 1.20	3.4 ± 0.16	51.14 ± 2.82
IFN α-mPEG$_{5000}$ MVLs	64.28 ± 3.81	17.83 ± 1.04	3.1 ± 0.13	56.87 ± 2.67

All the values are representative of mean ± S.D. for three independent determinations ($P < 0.05$).

TABLE 3 **Recovery of Soluble IFN α Fraction After Exposure to Chloroform: Diethyl Ether (1:1 v/v)**

Proteins	Percent recovery
IFN α	52.64 ± 2.51
IFN α-mPEG$_{5000}$	72.81 ± 3.37

Values are shown as representative of mean ± S.D. for three independent determinations ($P < 0.05$).

fraction recovered in aqueous media was considered as stable form i.e. "soluble protein fraction," while the insoluble fraction that comprises aggregated species was regarded as an unstable form. A substantial loss of ~48% of soluble fraction of native IFN α was observed. On the other hand, pegylated IFN α-mPEG$_{5000}$ showed considerably higher recovery IFN α as soluble protein fraction (72.81 ± 3.37%).

The effect of agitation duration on the recovery of soluble protein fraction was observed (Fig. 4). Proteins appeared as aggregated form in greater amount and hence, a decline in the percent soluble recovered fraction that reduced down to merely 16% upon increment of duration of vortexing up to 15 min, respectively. In contrast, IFN α-mPEG$_{5000}$ largely retained their soluble fraction up to ~58% even after undergoing the agitation stress for the same duration.

In-vitro release profile of various formulations has been shown graphically in Fig. 5. The slow release pattern of the MVLs formulations may be attributed to the fact that there are more barriers to the diffusion of protein from the depofoam particles. In case of MVL formulations, as these are multiple nonconcentric compartments, drug will pass through these compartments and then will ultimately diffuse into the external medium. It is clear from the above data that optimized formulation exhibits a better, sustained and more controlled in vitro protein release profile. Furthermore, in vitro release profile of pegylated IFN α and native IFN α carrying MVLs were studied and results were compared (Fig. 5). It is evident from the graph that all the stable fraction of IFN α from the MVLs was released as a burst within 1 h of incubation. Initial burst release was lower in case of IFN α-mPEG$_{5000}$ entrapped MVLs as compared to its counterpart, i.e., native IFN α entrapped MVLs. Pegylated IFN α carrying MVLs continued the release of stable protein fraction in a sustained manner up to 6 days. The hydrophilic polymer chain of mPEG, when conjugated to IFN α might help the retention of its native conformation under detrimental conditions. A possible mechanism of its protective action could be that mPEG acts as a barrier onto the interface between aqueous and polymer phases. Initial burst release might be due to an uneven distribution of pegylated IFN α. The preferential surface localization of proteins in the vicinity of the surface of vesicles could be an

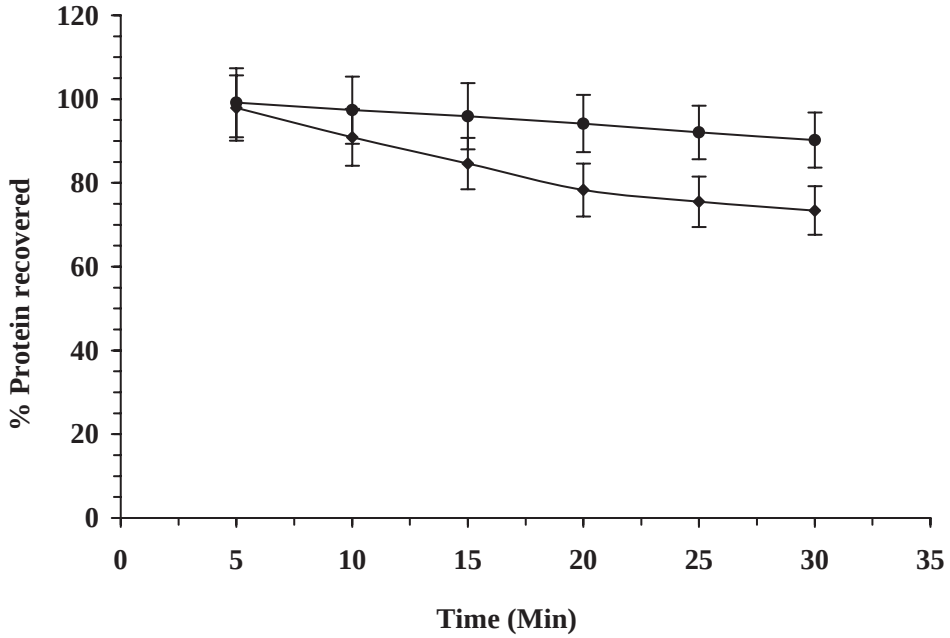

FIGURE 4 **Effect of Vortexing Time on Percent of Soluble Protein Recovered. Smooth Line (-●-) and Dashed Line (-✦-) Indicated the Pegylated IFN α and Native IFN α Respectively. Results Are Given as Means ± S.D. ($P < 0.05$).**

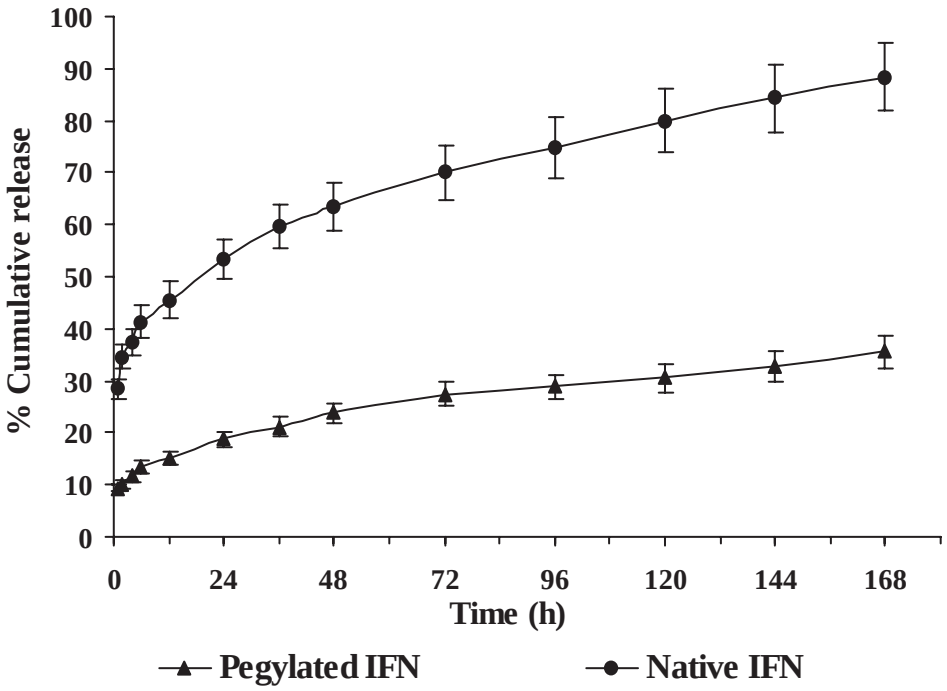

FIGURE 5 **In Vitro Release Profile of Interferon α from Optimized Native and Pegylated IFN α Entrapped MVLs. Results Are Given as Means ± S.D. ($P < 0.05$).**

TABLE 4 **Long-Term Storage (6-Month) Stability Parameters for Interferon α Entrapped Multivesicular Liposomes**

	% Residual drug		Vesicle size (μm)		Vesicle count (No. of vesicles/mm^3) × 1000	
MVLs formulations	4 ± 0.5°C	25 ± 0.5°C	4 ± 0.5°C	25 ± 0.5°C	4 ± 0.5°C	25 ± 0.5°C
IFN α MVLs	93.07 ± 5.14	90.2 ± 6.38	21.07 ± 1.41	18.8 ± 1.3	47.60 ± 2.83	44.57 ± 3.2
IFN α-mPEG$_{5000}$ MVLs	91.82 ± 4.92	88.31 ± 5.81	19.12 ± 1.17	17.28 ± 1.14	51.72 ± 3.06	48.6 ± 3.10

All the values are representative of mean ± S.D. for three independent determinations ($P < 0.05$).

additional possible reason for increased burst release. Based on in vitro release experiments using fluorescent-labeled lipids and aqueous-phase markers, several mechanisms of drug release from these MVLs in vivo have been postulated. These include (a) diffusion; (b) surface erosion of outer vesicles and presentation of internal vesicles to the tissue milieu; and (c) reorganization of the lipid membranes and redistribution of the lipids, leading to extrusion of the lipids from the particle surface (Langston et al., 2003). Data presented in this paper and the results presented in earlier reports support the hypothesis that pegylated protein encapsulated in MVLs composed of both phospholipid and triglyceride components impact on drug delivery, and can be used as a tool to optimize the in vivo delivery profiles.

The percent free drug in the MVLs suspensions of pegylated and nonpegylated IFN α was 3.1 ± 0.13 and 3.4 ± 0.16, respectively, in the final multivesicular liposomal preparations. There was no or very little leakage of any of the drugs from the MVL particles over a 6-month period when stored at 4°C, indicating that the MVLs were stable to storage at 4°C over the indicated time period (Table 4). This has been demonstrated previously that MVLs as depofoam particles were stable over 1 year at 4°C in a long-term storage stability study (Katre et al., 1998).

CONCLUSION

In conclusion, results demonstrated that pegylated protein encapsulation in MVLs could be a versatile vehicle for controlled and sustained delivery of proteins and their release could be controlled up to several weeks. Pegylation may modulate the stability and maintain the integrity of protein during the formulation

process. MVLs containing the higher encapsulation efficiency could be successfully prepared by double emulsification, as their industrial scale-up may be more easy and applicable. Taken together with both pegylation and encapsulation in multivesicular liposomes, this unique MVLs technology offers a safe and convenient sustained-release platform to deliver antiviral protein for further evaluation in animal model for pharmacokinetic and therapeutic efficacy.

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