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International Journal of Pharmaceutics 252 (2003) 111–122
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Stabilization of recombinant interferon-α by pegylation for encapsulation in PLGA microspheres

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Received 23 January 2002; received in revised form 11 November 2002; accepted 18 November 2002

Abstract

Interferon-α (IFN) was pegylated and encapsulated in biodegradable microspheres to achieve a long-acting formulation. IFN was pegylated with methoxy-polyethylene glycol (mPEG, MW 2000 or 5000). The conjugation procedures were optimized in terms of concentration of the reactants and the pH condition of the medium. The conjugates (IFN–$mPEG_{2000}$ and IFN–$mPEG_{5000}$) were characterized using SDS–PAGE, size-exclusion-HPLC (SE-HPLC) and matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectroscopy. The optimized IFN–mPEG conjugates consisted of mono- and multi-pegylated derivatives along with a small amount of native IFN (≤5%). In the simulation studies for microencapsulation, pegylated IFN showed better stability when exposed to dichloromethane as compared to native IFN. In vitro release profiles of IFN and IFN–mPEG from biodegradable poly(D,L-lactide-co-glycolide) microspheres were quite different. Native IFN was released only 16.3 ± 0.4% after 3 weeks, but IFN–$mPEG_{2000}$ and IFN–$mPEG_{5000}$ were released 72.5 ± 2.1 and 56.8 ± 2.5%, respectively after the same period. It was found that in contrast to native IFN, the pegylated IFN was able to largely retain its native aqueous solubility after being exposed to detrimental conditions of microencapsulation, resulting in a continuous release in vitro. These studies show the possibility of preparation of a long-acting dosage form for IFN using a combination of pegylation and microencapsulation techniques.

Keywords: Pegylation; Protein stability; Interferon; PEG; Microspheres

1. Introduction

Repeated administration of human interferon-α (IFN) is required in the treatments of hepatitis B, C, hairy cell leukemia, and Kaposi's sarcoma (Stuart-Harris et al., 1992; Spiegel, 1989; Baron et al., 1991; Itri, 1992). For instance, IFN is the prime drug for treatment of hepatitis C. Because IFN circulation half-life is only 5–7 h, the standard dosage regime is three times a week injection. Not surprisingly, the IFN treatment lasts for several years with a poor efficacy (Liang et al., 2000). Thus, a longer acting IFN formulation is highly desirable to enhance the efficacy as well as to improve the compliance of patients.

Conjugation of proteins with PEG or "pegylation" has been reported to enhance the circulation half-lives for many therapeutic proteins. They are adenosine

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PII: S0378-5173(02)00636-1

deaminase, asparaginase, TNFα-receptor, humanized antibody fragment, growth hormone receptor antagonists, G-CSF for chemotherapy of breast cancer (Hershfield et al., 1987; Jurgens et al., 1988; Edwards, 1999; Chapman et al., 1999; Leong et al., 2001; Olson et al., 1997). Although pegylated therapeutic proteins demonstrate lowered biological activities in vitro compared to unpegylated ones, their in vivo activities are substantially enhanced due to the long circulation effect (counter-acting effect). Conjugates of IFN and polyethylene glycol (IFN–PEG) have also been prepared and are currently being available in the market. The main objective of these attempts is to replace the current three-injection schedule of IFN with once-a-week injection using long-acting IFN–PEG conjugates (Hu et al., 1998). Early result for one such pegylated IFN-α 2a preparation (using PEG MW 5000) was not promising. There was only a limited improvement in the half-life of the drug, e.g. from 5 h for native IFN-α to 10 h for pegylated IFN-α, which was attributed to the poor result (Kozlowski and Harris, 2001). Methoxy-PEG (mPEG) derivatives of higher molecular weights have been used to increase the circulation half live of IFN-α up to 70 h in the subsequent trials (Glue et al., 2000; Bailon et al., 2001). We hypothesized that a sustained release formulation of pegylated IFN by encapsulating in biodegradable microspheres could possibly be another way to increase the blood circulation time of the drug. However, typical protein formulation steps involved in microspheres preparation, such as exposure of protein molecules to aqueous/organic interface and high shear stress of emulsification, adversely affect the physical and biological properties of the protein drugs (Diwan and Park, 2001; Li et al., 2000; Johansen et al., 1998; Xing et al., 1996).

In this paper, it was hypothesized that compared to native IFN, pegylated IFN could increase its physical stability and became more tolerant during the microencapsulation process, resulting in improved release kinetic patterns from biodegradable microspheres. IFN-α 2a (will be referred as IFN) was pegylated using two different molecular weights of mPEG derivatives (MW 2000 and 5000). The conjugates were characterized by various analytical methods and they were microencapsulated in biodegradable microspheres for sustained release.

2. Materials and methods

2.1. Materials

Interferon-α 2a was obtained from Cheiljedang Co. (Seoul, South Korea). Succinimidyl succinate (SSA) and succinimidyl propionate (SPA) derivatives of methoxy-poly(ethylene glycol) (MW 5000 and 2000, respectively) were purchased from Shearwater Polymers (Huntsville, AL). Biodegradable poly (D,L-lactide-co-glycolide) copolymer (RG502H, 50/50 lactide:glycolide molar ratio, MW 10,000 with uncapped carboxylic end group) was purchased from Boehringer Ingelheim (Ingelheim, Germany).

2.2. Conjugation of IFN and mPEG

IFN was conjugated with methoxy-PEG of different molecular weights, i.e. 2000 and 5000 using mPEG–SPA and mPEG–SSA derivatives, respectively. The reaction conditions were optimized to obtain high conjugation yield in a reproducible manner. Briefly, IFN solution (3 mg/ml) in phosphate buffered saline (50 mM, pH 8.0) was filtered through a 0.45 μm pore size syringe filter. MPEG–SPA (15 M excess) dissolved in 100 μl of dimethylsulfoxide was added dropwise to the cold solution of IFN (1.5 ml). The conjugation was allowed to proceed overnight at 4 °C with an end-to-end rotation and quenched with the addition of glycine (15 mM final concentration). The reaction mixture was dialyzed against PBS (50 mM, pH 7.4) using a membrane with pore size of 5 kDa. The IFN–$mPEG_{2000}$ conjugate solution was stored at 4 °C. Similarly, IFN–mPEG conjugate (IFN–$mPEG_{5000}$) with PEG molecular weight of 5000 was prepared using mPEG–SSA (30 M excess).

2.3. Physico-chemical characterization of the conjugate

2.3.1. SDS–PAGE analysis

SDS–PAGE was performed using a Bio-Rad MiniProtean II gel apparatus (Hercules, CA). The samples were prepared under reducing conditions for application on a gel consisting of 5 and 15% stacking and resolving gel, respectively. Coomassie brilliant blue staining was employed to reveal the separated protein bands.

2.3.2. Size-exclusion-HPLC (SE-HPLC)

The composition of IFN–mPEG conjugates was analyzed on a HPLC system consisting of Waters 660E pump and UV detector equipped with an Autochro data module (Younglin, South Korea). 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 the eluate was monitored by UV detection at 280 nm.

2.3.3. Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectroscopy

The presence of different species of mono-, di-, or higher derivatives of PEG in a mixture of IFN–mPEG conjugates was confirmed using MALDI-TOF mass spectroscopy (PE Biosystems Voyager System 4095).

2.4. Simulation studies

The formulation steps involved in the preparation of microspheres were simulated to assess the stability of IFN and IFN–mPEG 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.

2.4.1. Exposure to organic solvent

Pegylated or native IFN aqueous solution (0.05% w/v, 50 μl) was added into a test tube containing dichloromethane (500 μl). After gentle mixing, the solvent was allowed to evaporate at room temperature. PBS (250 μl) was added to each tube and incubated at 37 °C. After 2 h the samples were centrifuged at 14,000 rpm for 10 min and supernatant was removed. Protein concentration in the supernatant was estimated by microBCA protein assay kit (Pierce, IL). Loss of protein as insoluble aggregates was estimated by subtracting the amount of protein recovered in the supernatant from the known amount initially taken.

2.4.2. Shear-induced stress of emulsification

Native IFN or IFN–mPEG aqueous solution (0.05% w/v, 75 μl) was emulsified with dichloromethane (750 μl) using a homogenizer (7 mm probe, PowerGen, Fischer Scientific, Germany) at a constant low speed. The duration of homogenization was varied from 30 to 600 s. 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.

2.5. Preparation of microspheres of IFN and IFN–mPEG

Microspheres loaded either with IFN or IFN–mPEG, were prepared using PLGA 50:50 copolymer (Diwan and Park, 2001). A primary emulsion (w/o type) of IFN solution (300 μl, 2% w/v) in PBS (0.05 M, pH 7.4) and polymer solution (20% w/v, 3 ml) in dichloromethane was prepared by homogenization (PowerGen 700, Fischer Scientific, Germany). The primary emulsion was re-emulsified in polyvinylalcohol solution (0.1% w/v) to obtain secondary emulsion (w/o/w type). The organic solvent was allowed to evaporate at room temperature by constant stirring of the reaction mixture. Microspheres obtained were washed three times with deionized water before freeze-drying. Pegylated IFN solution, when concentrated beyond 0.2% (w/v), resulted in fine precipitation of the protein. For encapsulation, a cloudy solution of pegylated IFN (0.5% w/v) was employed.

2.6. Characterization of prepared microspheres

2.6.1. SEM analysis and particle size

Surface morphology of microspheres loaded with protein was studied with a scanning electron microscope (535 M, Phillips). The average particle size was visually determined from SEM photomicrographs by counting 100 microspheres.

2.6.2. Protein entrapment

The amount of protein loaded in microspheres was estimated by a modified “two-step” extraction method (Diwan et al., 1998). Briefly, known amounts of microspheres (about 10 mg) were dissolved in 1 ml of acetonitrile. The samples were centrifuged at 14,000 rpm for 10 min. The supernatant comprising of polymer solution was discarded and the precipitated protein was then extracted in NaOH (0.1N, 1 ml). Protein content of the neutralized alkaline extraction was estimated using microBCA assay and expressed as amount of protein entrapped per milligram of dry microspheres.

2.7. In vitro release studies

Microspheres containing native or pegylated IFN (125–200 mg) were incubated in 4 ml release medium containing phosphate buffered saline (50 mM, pH 7.4), Tween 20 (0.2% w/v), and sodium azide (0.02% w/v). The temperature of air-bath incubator was maintained at 37 °C (±2 °C) with continuous agitation at 200 rpm. At each sampling time, the samples were centrifuged and clear supernatant of the release medium (3 ml) was withdrawn and replaced with fresh medium. The amount of protein released was estimated by microBCA protein assay.

3. Results and discussion

IFN–mPEG conjugates of different polymer chain lengths (MW 2000 and 5000) were prepared. SDS–PAGE analysis shows the formation of mono- and mutli-pegylated IFN species (Fig. 1). Reaction conditions were optimized in terms of the concentration of reactants and pH of conjugation buffer. It was observed that by increasing the molar ratio of protein and $mPEG_{2000}$–SPA from 1:5 to 1:40, the extent of pegylation was also increased (Fig. 1a). A similar trend was noticed for $mPEG_{5000}$–SSA when the proportion was increased from 1:8 to 1:40 (Fig. 1b). In both cases, the increase in the pegylation degree was accompanied by a rise in the formation of multi-pegylated over mono-pegylated derivatives of IFN. The pH of the conjugation medium also plays an important role. Increasing the pH from 7.4 to 8.0 enhanced the extent of mPEG substitution on IFN molecule. A further increase in pH to 9.0, resulted in lowering of the conjugation efficiency (data not shown).

Optimized IFN–mPEG conjugates were analyzed by size-exclusion HPLC. Fig. 2 shows chromatograms for IFN–$mPEG_{2000}$ and IFN–$mPEG_{5000}$ conjugates. For IFN–$mPEG_{2000}$, multi-pegylated IFN species were eluted as a single broad peak. This indicates that multi-pegylated IFN–$mPEG_{2000}$ species could not be resolved in the SE-HPLC. On the other hand, when using mPEG derivative of MW 5000, mono-, di-, and multi-pegylated IFN conjugates were separated due to the a larger MW difference between IFN–mPEG species. Some lower molecular weight peptide fragments (MW ~6500 Da) were also present in native IFN (2–3%). These were persistently observed as small peaks in the SE-chromatograms with a retention time longer than 14 min.

Fig. 3a and b gives MALDI-TOF mass spectra of IFN–$mPEG_{2000}$ and IFN–$mPEG_{5000}$, respectively. Pegylation of IFN using the low molecular weight PEG produced a wide distribution range of multi-pegylated IFN species as compared to the higher molecular weight PEG. Not surprisingly, for IFN–$mPEG_{2000}$ conjugates, unpegylated and mono-pegylated IFN were minimally present in the mixture. A broad range of multi-pegylated IFN conjugates was detected. Fig. 3b shows that the IFN–$mPEG_{5000}$ conjugates mainly consisted of mono-, di-, tri-pegylated IFN along with a small fraction of native IFN. The number of conjugated lysine residues for pegylation depends on the number and length of PEG chains. The effect of PEG chain length on the extent of pegylation was clearly caused by the molecular weight-dependent steric effect of mPEG derivatives. The accessibility of high molecular weight mPEG derivatives to the conjugation site in IFN would be more limited than that of low molecular weight mPEG derivatives. Thus IFN–$mPEG_{2000}$ had greater fraction of multi-pegylated IFN species than IFN–$mPEG_{5000}$.

Previous studies from our laboratory and others have shown that the harsh steps involved in the preparation of microspheres, adversely affect the stability of the encapsulated protein (Diwan and Park, 2001; Zu et al., 2000; Kishnamurthy et al., 2000). We have recently shown that after pegylation, the stability of lysozyme was significantly enhanced to mostly overcome the destabilization influences of microencapsulation process. For instance, when aqueous solution of lysozyme was emulsified with organic phase (dichloromethane) for 300 s, more then 60% of native lysozyme formed insoluble aggregates. In contrast, about 80% of the pegylated lysozyme retained its native aqueous solubility (Diwan and Park, 2001). For aqueous solution of IFN, the effects of exposure to organic solvent and shear-induced stress of homogenization were individually studied. After treatment, soluble protein fraction recovered in aqueous media was considered as a stable form, i.e. “soluble protein fraction”, while the insoluble fraction that comprises of aggregated species was regarded as an unstable form.

Dichloromethane is the most commonly used solvent for the preparation of microspheres. Table 1

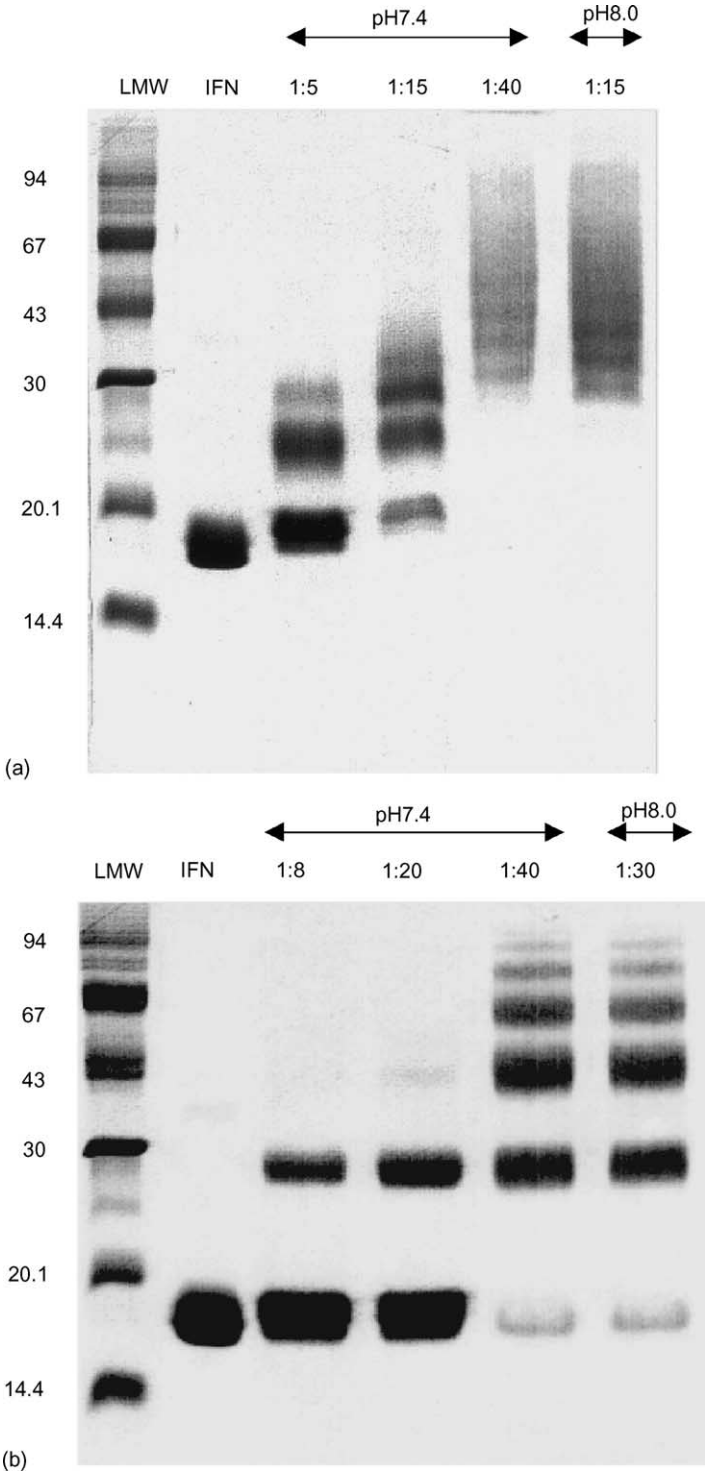

Fig. 1. SDS–PAGE analysis of IFN–mPEG conjugates and optimization of conjugation process. Individual lane indicates the molar ratio of IFN:mPEG derivative employed under different pH conditions. Protein marker lane is indicated as LMW. Pegylation of IFN was carried out using different molecular weight PEG derivatives (a) $mPEG_{2000}$–SPA and (b) $mPEG_{5000}$–SSA.

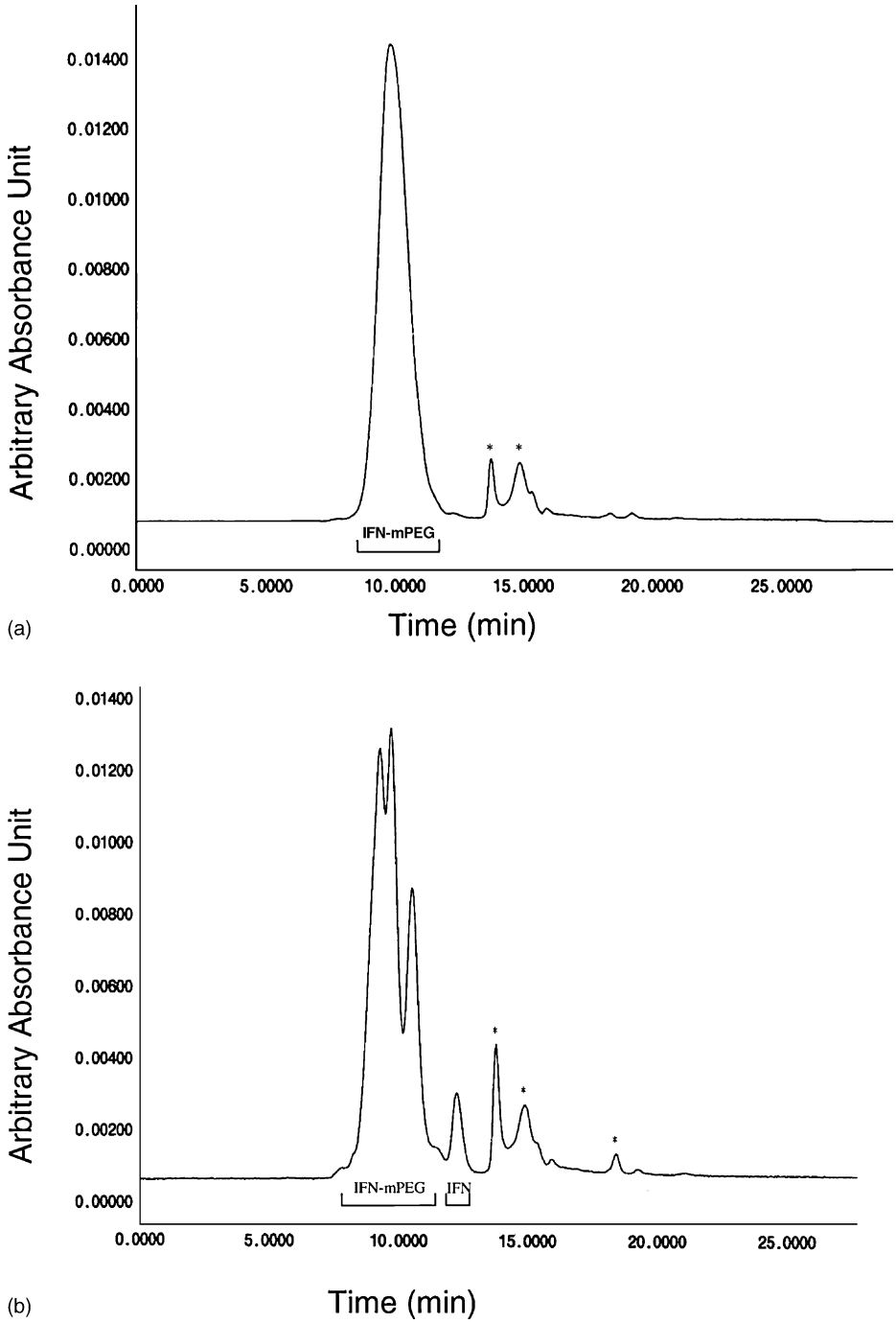

Fig. 2. Size-exclusion-HPLC chromatograms show conjugation products after pegylation of IFN with methoxy-PEG of molecular weights (a) 2000, and (b) 5000. Low molecular weight impurities (RT 13.7 min and higher) eluting after the main peak are marked with asterisk.

Table 1
Recovery of the soluble protein fraction after exposure to dichloromethane

Protein	Recovery ± S.D. (%)
IFN	75.9 ± 4.0
IFN–mPEG$_{2000}$	98.0 ± 3.5
IFN–mPEG$_{5000}$	86.9 ± 13.7

shows the effect of exposure of IFN to dichloromethane on its solubility in PBS. A substantial loss, 24.1 ± 4.0%, of soluble fraction for native IFN was measured. On the other hand, pegylated IFN, i.e. IFN–mPEG$_{2000}$ and IFN–mPEG$_{5000}$, showed considerably higher recovery of the soluble protein fraction. The IFN–mPEG$_{2000}$ had higher recovery yield than the IFN–mPEG$_{5000}$, possibly due to the effect of multi-pegylation as described above. The IFN–mPEG$_{2000}$, having a greater number of PEG chains on the protein surface than the IFN–mPEG$_{5000}$, is more likely to resist against non-specific adsorption on the interface between water and organic solvent phases. The protein molecule may undergo nonspecific adsorption on the water/dichloromethane interface leading to surface-induced unfolding and subsequent aggregation. This physical aggregation, being irreversible

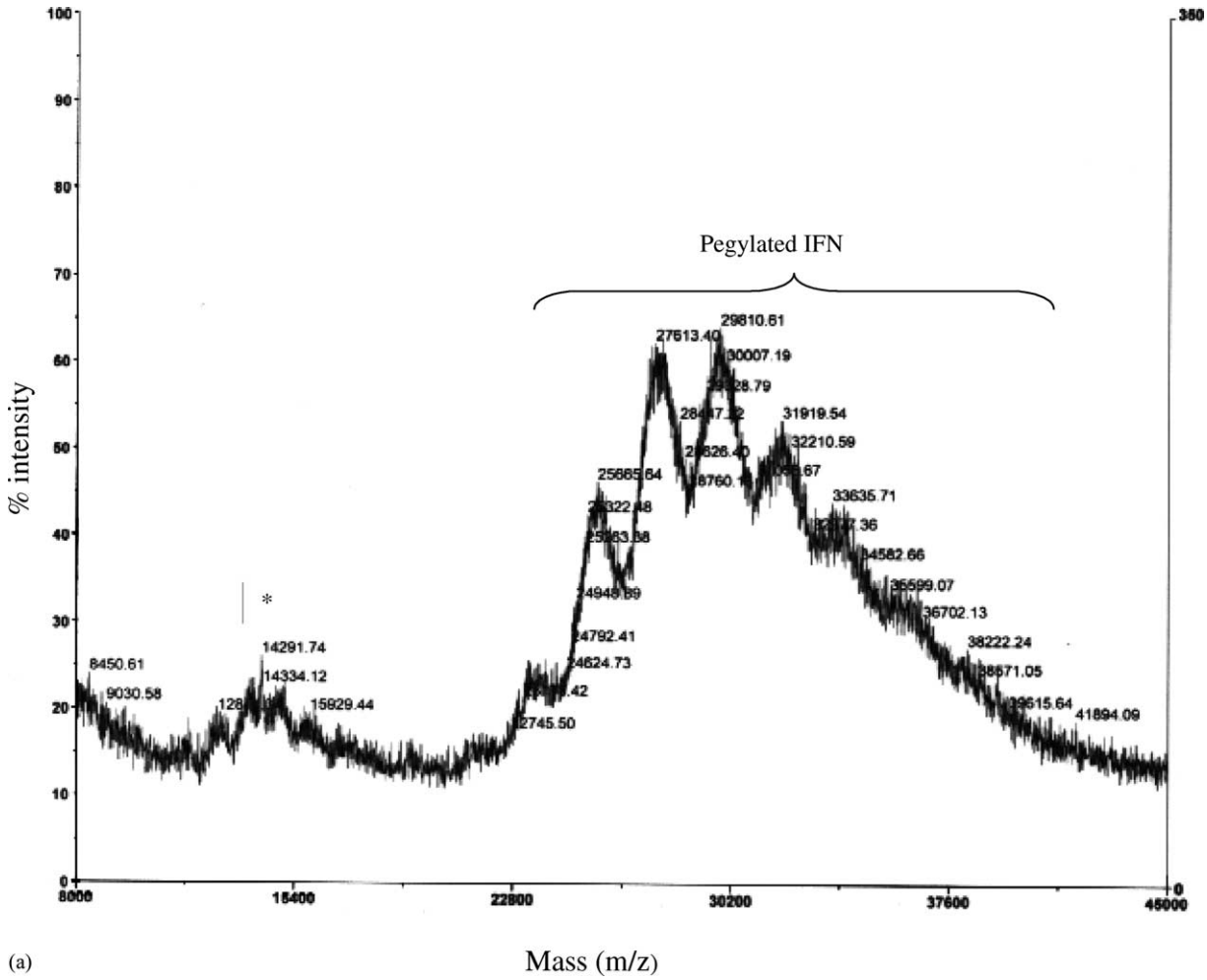

Fig. 3. Mono- or multi-pegylated IFN species can be seen in the MALDI-TOF mass spectrograms for (a) IFN–mPEG$_{2000}$, and (b) IFN–mPEG$_{5000}$. Pegylated IFN fragments are identified with asterisk.

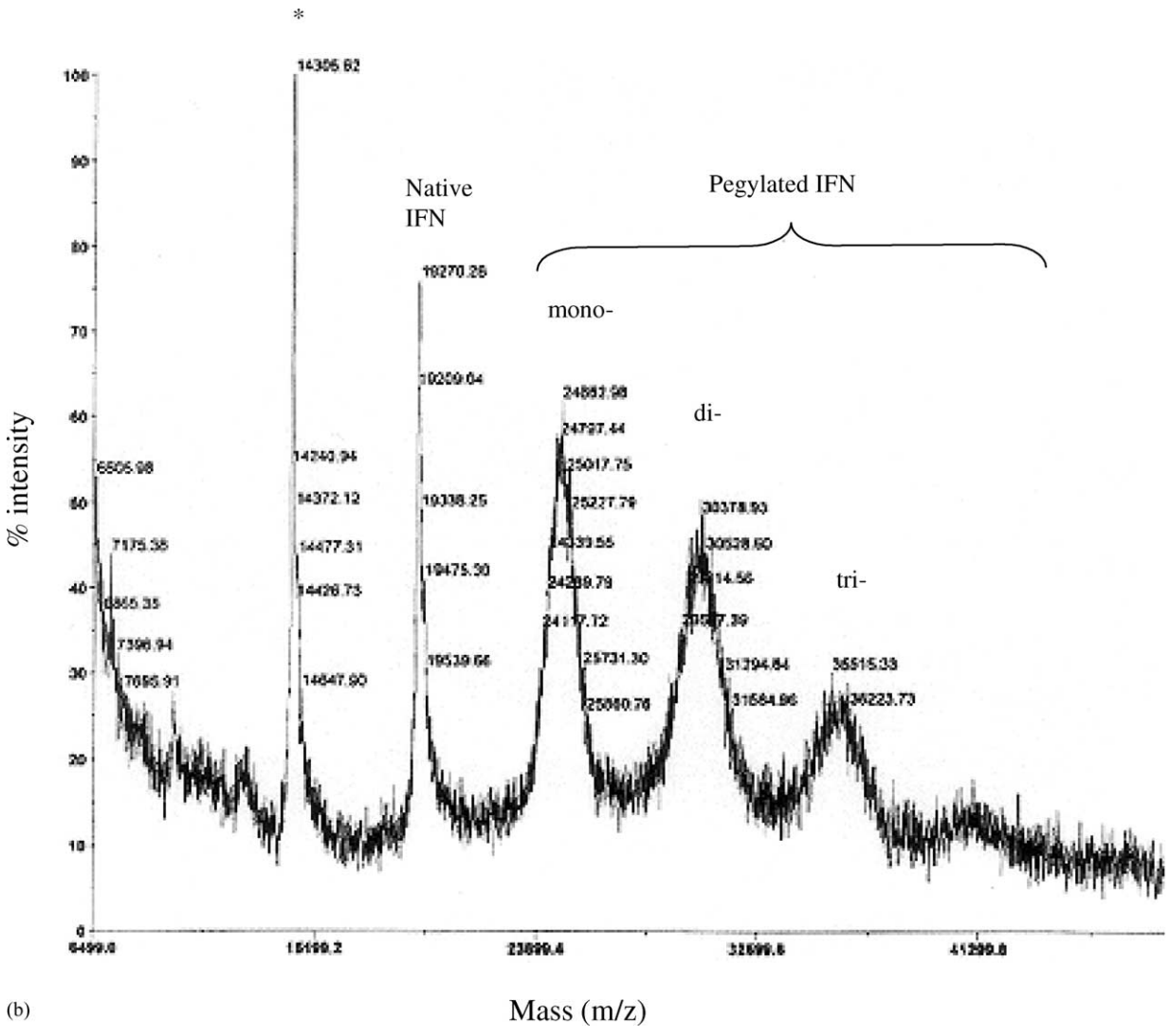

Fig. 3 (*Continued*).

in nature, is reflected as a loss in the soluble protein fraction. Pegylated IFN is likely to resist any nonspecific protein adsorption onto the interface due to steric repulsion of PEG chains (McPherson et al., 1995). Apparently, a greater protection was mediated by $mPEG_{2000}$ than $mPEG_{5000}$ moieties in the two conjugates as seen in Table 1. It may be due to either the presence of some unconjugated and hence unprotected protein in the IFN–$mPEG_{5000}$ samples, or greater PEG substitution in the IFN–$mPEG_{2000}$ conjugates.

Microencapsulation using single (w/o) or double emulsion (w/o/w) techniques requires 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. Aqueous solution of IFN when homogenized with DCM for only 60 s, resulted in a loss of $59.3 \pm 4.9\%$ of soluble protein fraction. Under similar conditions, only 12.1 ± 2.1 and $9.0 \pm 7.4\%$

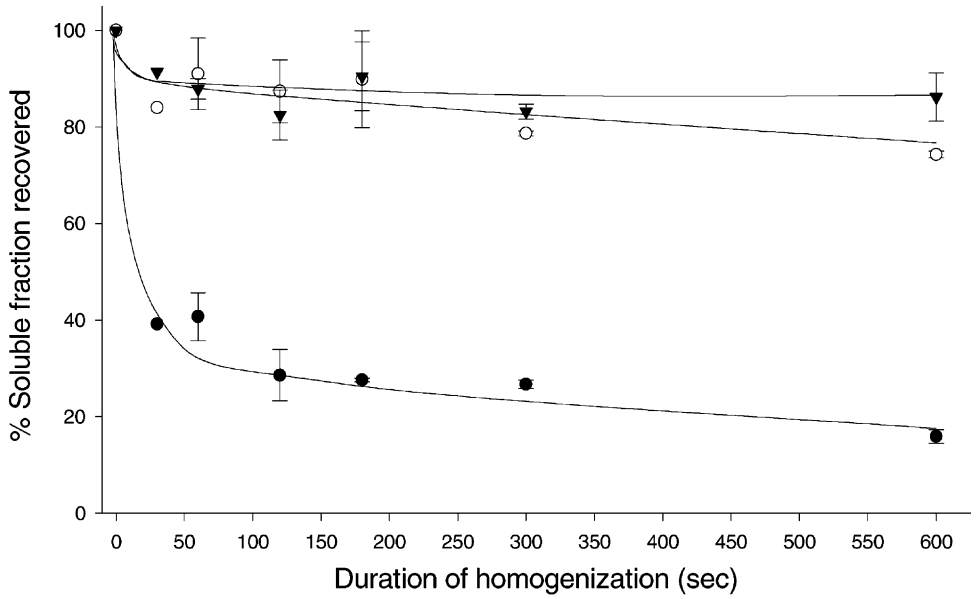

Fig. 4. Loss in the aqueous solubility of native or pegylated IFN as a function of shear-induced stress of homogenization in the presence of dichloromethane. Soluble protein was recovered in PBS after complete removal of organic solvent. Closed circle, inverted closed triangle, and open circle symbols depict the profiles for native IFN, IFN–$mPEG_{2000}$, and IFN–$mPEG_{5000}$, respectively.

reduction in the soluble protein fraction were observed for IFN–$mPEG_{2000}$ and IFN–$mPEG_{5000}$, respectively (Fig. 4). Further increase in the duration of homogenization for native IFN sample, caused greater aggregation of the protein and hence a decline in the percent soluble recovered fraction that reduced down to merely $15.9 \pm 1.4\%$ after 600 s. In contrast, the two types of pegylated IFN, IFN–$mPEG_{2000}$ and IFN–$mPEG_{5000}$, largely retained their soluble fraction, 86.2 ± 5.0 and $74.3 \pm 0.7\%$, respectively even after undergoing the homogenization stress for the same duration. It is evident that during encapsulation process, protein was prone to denaturation when exposed onto w/o interface under an added stress of homogenization. Its stabilization during these steps thus becomes critical for the preparation of "protein-releasing" microspheres (Diwan and Park, 2001; Li et al., 2000). The formation of insoluble aggregates of encapsulated protein may result in incomplete release of the protein from microspheres in vitro (Weert et al., 2000; Morlock et al., 1998; Kim and Park, 1999). Furthermore, surface adsorption of protein on microspheres and an acidic microclimate generated by polymer degradation may also cause instability of the protein during the release (Crotts et al., 1997; Marinina et al., 2000).

IFN and IFN–mPEG conjugates were encapsulated in biodegradable microspheres made of fast degrading PLGA 50:50 copolymer. The average size of the native IFN microspheres was 1.8 μm with protein loading of 1.1% w/w. For pegylated IFN, the size and protein loading values were 1.2 μm and 0.18% w/w for IFN–$mPEG_{2000}$, and 1.5 μm and 0.14% w/w for IFN–$mPEG_{5000}$. Fig. 5 shows in vitro release profiles of IFN and IFN–mPEG conjugates from PLGA microspheres. The IFN microspheres show an initial burst release followed by a nearly no release, a pattern similar to most protein release profiles from PLGA microspheres (Diwan and Park, 2001; Kishnamurthy et al., 2000; Weert et al., 2000; Morlock et al., 1998; Kim and Park, 1999; Lu and Park, 1995). In contrast, pegylated IFN shows continuous releases for the same period of observation. For native IFN, after a period of 21 days, only $16.3 \pm 0.4\%$ of the total encapsulated IFN was released out, of which 11.0% was released within the first hour of the study. On the other hand, pegylated IFNs, IFN–$mPEG_{2000}$ and IFN–$mPEG_{5000}$, exhibit net IFN release of 72.5 ± 2.1

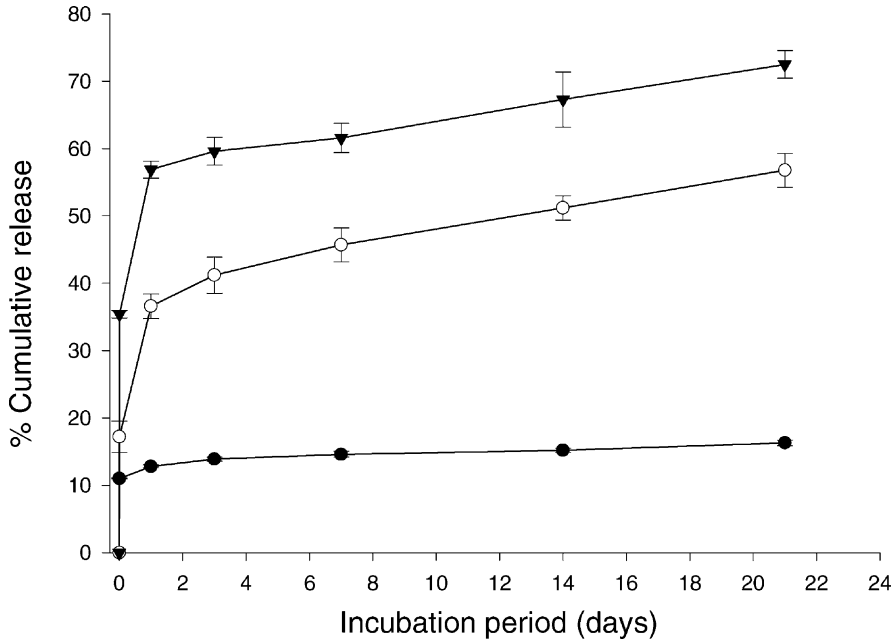

Fig. 5. In vitro release profile of protein from PLGA microspheres carrying native or pegylated IFN (IFN–mPEG$_{2000}$ and IFN–mPEG$_{5000}$) as shown with closed circle, inverted closed triangle, and open circle, respectively.

and 56.8 ± 2.5%, respectively in a 3-week incubation period. The difference in the relative release amounts of protein between the two types of encapsulated pegylated IFN may be due to the different PEG chain lengths. The longer PEG chain would offer more resistance of pegylated IFN to diffuse out from the micropores of microspheres than the shorter one, due to the increased hydrodynamic size. The size effect is more pronounced during the initial burst of protein release. After 1 h of incubation, 35.4 ± 0.6% of IFN–mPEG$_{2000}$ was detected in the release medium as compared to 17.2 ± 2.3% of IFN–mPEG$_{5000}$. Higher burst release was observed for IFN–mPEG$_{5000}$ as compared to that of lysozyme–mPEG$_{5000}$ (6.4 ± 0.7%) (Diwan and Park, 2001). This discrepancy was likely to be caused by physico-chemically different characters of the two pegylated proteins, IFN and lysozyme, although the underlying reasons are still obscure. It should be noted that freeze-dried pegylated IFN, when dissolved in a small amount of water for microencapsulation, was not readily dissolved, but became finely dispersed particles. This might result in an uneven distribution of pegylated IFN in the polymer matrix. The preferential location of surface-active pegylated IFN in the vicinity of the surface of microspheres could be an additional possible reason for the increased burst release.

As reported earlier (Diwan and Park, 2001), the protein fraction retaining its native soluble conformation is preferentially released out from microspheres whereas that of insoluble aggregated species fails to come out in the release medium until the polymer matrix is sufficiently disintegrated. Thus, it is reasonable to hypothesize that nearly all the stable fraction of IFN microspheres was released as a burst within 1 h of incubation. It should be recalled that a maximum of about 80% of native IFN formed insoluble aggregates under shear-induced stress of homogenization during simulation studies (Fig. 4). Further, pegylated IFN containing about 80% of protein in the stable state (based on simulation studies data) continued the release of that amount in a sustained manner up to 21 days. The hydrophilic polymer chain of PEG, when conjugated to IFN, might help the retention of its native conformation under detrimental conditions. A possible mechanism of its protective action could be that PEG acts as a barrier onto the interface between aqueous and polymer phases. This would prevent the

exposure and adsorption of protein to the interface, which has been described as the primary reason for unfolding of its three-dimensional structure and subsequently aggregation (McPherson et al., 1995). It was also reported that PEG could be used as an additive for physical stabilization of protein drugs encapsulated within PLGA microspheres (Péan et al., 1999). PEG is additionally known to help in the recovery (refolding) of proteins from denaturing reagents such as urea and guanidine hydrochloride (Kuboi et al., 2000; Cleland et al., 1992). Thus, it is possible that pegylated proteins within PLGA microspheres becomes more stable than unpegylated ones, resulting in better release profiles.

4. Conclusions

The pegylated IFN showed significantly greater resistance towards aggregation induced under the harsh conditions for microencapsulation in contrast to native IFN. Encapsulation of IFN–mPEG conjugates in fast degrading PLGA microspheres, although they exhibited relatively high burst releases, demonstrated sustained in vitro releases for at least 3 weeks. The PEG chain length also influenced the release rate of IFN from microspheres. This study suggested that protein pegylation approach would be a very useful strategy for a long-acting microspheres formulation of various therapeutic proteins.

Acknowledgements

This work was supported by the BK-21 grant from the Cheiljedang Co., South Korea.

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