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Multivesicular liposome formulations for the sustained delivery of interferon α-2b[1]

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Key words

interferon α-2b; liposomes; delayed-action preparations

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Abstract

Aim: To develop and optimize a sustained release multivesicular liposome (MVL) formulation of interferon (IFN) α-2b. **Methods:** IFN α-2b MVL were prepared using a typical double-emulsion procedure. The sustained release effects of IFN α-2b MVL were investigated by monitoring the blood IFN α-2b concentration using an enzyme-linked immunosorbent assay test after subcutaneous administration to healthy mice. **Results:** IFN α-2b was successfully encapsulated in MVL with high efficiency, and the integrity of encapsulated protein was maintained. After subcutaneous injection, the MVL slowly released IFN α-2b into systemic circulation in a sustained manner. The estimated serum half-life of IFN α-2b was approximately 30 h. In addition, varying the size of the MVL preparations could further modify the *in vivo* release profile. **Conclusion:** IFN α-2b MVL may be a useful sustained release formulation in the clinical treatment of viral diseases.

Introduction

Interferon (IFN) α-2b is an important cytokine and has been used widely as a therapeutic agent to treat patients with viral and oncological diseases. It is an essential component of the treatment of chronic hepatitis B infection[1]. However, in the recommended dosing regimen, the protein needs to be administered every other day for 3 months, which brings about much inconvenience to the patients. The $t_{1/2}$ of IFN α-2b, when administered subcutaneously, is only about 4 h[2].The protein was shown to be cleared quickly, therefore frequent repeated administrations are necessary.

Much effort has been devoted to the development of IFN α-2b-based products with persistent effects. One approach, covalent attachment of polyethylene glycol (PEG) to the protein surface (PEGylation), has been the most successful. Several PEGylated IFNα products are already on the market. The half-life of the PEGylated protein is 40 h, thus it only needs to be administered once a week for similar therapeutic effects[3]. However, the chemical conjugation process of PEGylation is rather complex and the PEGylated products are usually mixtures with different PEG conjugation sites. In addition, a few studies have suggested that the chemical modifications can sometimes affect the structure as well as the bioactivity of the protein[4].

An alternative approach is to develop sustained-release depot formulations of IFN α-2b. Liposome formulations of IFNγ have been developed and have been reported to have prolonged release profiles of up to 160 h. Even so, using conventional liposome formulations, the drug loading capacity and encapsulation efficiency are still rather low and variable[5].

Multivesicular liposomes (MVL), on the other hand, have a different structure and possess some distinctive properties. They usually contain a larger internal space, which would allow more drug to be loaded. Their larger size would also deter rapid clearance by tissue macrophages so that they may act as drug depots to enable sustained release of drugs[6]. The MVL formulation of the anticancer drug cytarabine (Depocyt™) has been developed successfully and is now being used widely for the treatment of leukemia[7].

We took a similar approach in the present study and evalu-

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ated the MVL formulation of IFN α-2b and its biopharmaceutical properties. Some of the parameters that affected the MVL *in vivo* pharmacokinetic behaviors were further investigated. Our data suggest that MVL formulation of IFN α-2b can be developed with satisfactory sustained release properties *in vivo*, which may have useful clinical applications.

Materials and methods

Materials 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol, triolein and 1,2-dipalmitoyl-sn-3-phosphoglycerol (DPPG) were all purchased from Sigma (St Louis, MO, USA). *L*-lysine was purchased from Sangon Biological Engineering Technology and Service Co (Shanghai, China). IFN α-2b (recombinant human interferon α-2b) was kindly provided by Pan Asia Bio (Shanghai, China), and all other reagents were of analytical grade and purchased from Shanghai Chemical Reagent Co (Shanghai, China).

Multivesicular liposome preparation The MVL formulations of IFN α-2b were prepared based on the typical double-emulsion procedure developed by Kim *et al*[8–12]. Briefly, 1 mL chloroform containing the lipids (molar ratio DOPC:cholesterol:DPPG: triolein, 7:11:1:1; two other molar ratios were also used: 7:11:1:4 and 7:11:1: 8) was emulsified at 10 000 r/min for 10 min with 1 mL aqueous solution containing IFN α-2b in phosphate-buffered solution (PBS) and various sucrose concentrations to produce a water-in-oil emulsion. This water-in-oil emulsion was subsequently emulsified with 4 mL of an aqueous solution containing 4% glucose (*w*/*v*) and 20 mmol/L lysine at 2500 r/min for 10 s, and then poured into another 4 mL of the same aqueous solution. Chloroform was removed by flushing nitrogen over the surface of the double emulsion at 37 °C for approximately 15 min. The resultant MVL were pelleted at 600 ×*g* and washed twice with PBS to remove unencapsulated IFN α-2b. The IFN α-2b concentration in MVL was determined by HPLC quantification and adjusted accordingly.

For preparing MVL samples with narrower size distributions, the procedures were further modified. For large-sized MVL, a smaller emulsification force (1000 r/min) was applied during the second emulsification and the chloroform was removed slowly (over 30 min). The large MVL were purified and harvested by centrifugation at 100×*g* and only the pellet was collected. For small-sized MVL, the second emulsification step was carried out at 10 000 r/min. Chloroform was removed over approximately 15 min. The resultant MVL were then centrifuged twice at 100×*g* for 10 min, and the precipitants were discarded. Small-sized MVL were then harvested in the pellet after centrifugation at 600×*g* for 10 min. The size distributions were quite reproducible because of the purification-by-centrifugation step. The IFN α-2b concentration was determined by HPLC quantification and adjusted accordingly.

Multivesicular liposome size measurements The MVL suspensions were diluted in saline. The particle size distribution was measured using a CIS100 particle size analyzer (Ankersmid, the Netherlands).

Encapsulation efficiency determination IFN α-2b encapsulation efficiency was determined by measuring the amount of encapsulated protein as compared to the total amount added[13]. Briefly, the MVL were pelleted by centrifugation at 600×*g* for 10 min. The pellet was then treated with extraction solution (0.2% Triton X-100, 28% ethanol, 71.2% water, *v*/*v*) and quantified using the HPLC assay described below.

IFN α-2b characterization IFN α-2b was characterized using reverse phase (RP)-HPLC, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked immunosorbent assays (ELISA). RP-HPLC was carried out on a Agilent 1100 liquid chromatography system at 45 °C using a linear gradient of 45%–70% solvent B [CH_3CN, 0.1% Trifluoroacetic Acid (TFA)] over 11 min, and then a sharp linear gradient of 70%–100% solvent B over 9 min at a flow rate of 1.0 mL/min. Solvent A was water (0.1% TFA). IFN α-2b was detected by UV absorbance at 280 nm. The standard curve showed a linear correlation within the range of 2.0 μg/mL–100 μg/mL. The intra-day and inter-day assay precisions were determined to be less than 3% and 2%, respectively. SDS-PAGE analyses of encapsulated IFN α-2b were carried out using 12% acrylamide gels under reducing conditions and stained with silver stain. ELISA were carried out using the human interferon α ELISA Kit (sandwich method) from PBL Biomedical Laboratories (Piscataway,NJ, USA). The protein control and the MVL samples were both treated with extraction solution (0.2% Triton X-100, 28% ethanol, 71.2% water) for 30 min and then applied to the ELISA plate. IFN α-2b concentrations were determined according to the standard curve supplied with the kit.

***In vitro* drug release study** Aliquots of IFN α-2b MVL (500 μL) were pipetted into a 50 mL beaker containing 25 mL of saline solution. The beaker was incubated at 37 °C under constant rotation at 12 r/min. Three samples were collected at each time point (0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h) and were centrifuged at 600×*g* for 10 min. The protein concentrations in the pellets were determined using the RP-HPLC assay[14].

***In vivo* pharmacokinetic studies** Free IFN α-2b and IFN α-2b MVL suspensions were injected subcutaneously

in a single dose in female SD rats. Three rats were included in each group. Blood samples (0.3 mL) were collected at specific time points (5 min, 30 min, 2 h, 8 h, 12 h, 24 h, 48 h, 72 h, 96 h, and 120 h after injection) and were placed aside for 30 min at room temperature. The supernatant (serum) was collected by centrifugation at 700×*g* for 10 min. IFN α-2b concentrations were determined using ELISA, which has a detection limit of 30 pg/mL. Any values lower than 30 pg/mL were considered undetectable.

Results

Interferon α-2b encapsulation in multivesicular liposomes Multivesicular liposomes containing IFN α-2b were prepared according to the standard double-emulsion method. The preparations were highly reproducible, usually yielding MVL with similar size distributions and encapsulation efficiencies. A representative light microscope image of the resultant MVL is shown in Figure 1A. The particle size distribution analysis is shown in Figure 1B. The MVL had a rather broad size distribution ranging from 2 μm to 50 μm in diameter. The median size was approximately 18 μm.

The encapsulated proteins were characterized using SDS-PAGE, ELISA, and HPLC. There was no chemical degradation in the peptide chain after the preparation. The structural integrity of the protein is considered crucial to its activity. We used an ELISA to partially characterize the 3-dimensional conformational change in the protein. Our data showed that the antibody binding affinity to the protein was only slightly reduced, indicating that there was a substantial amount of native structure remaining in the protein sample after preparation (Figure 2).

Interferon α-2b encapsulation efficiencies Several parameters were evaluated for their effects in optimizing IFN α-2b encapsulation efficiencies. Table 1 lists some of the representative scenarios. Using the standard lipid MVL formulation (48.3 mmol/L DOPC, 70.7 mmol/L cholesterol, 6.7 mmol/L DPPG and 6.7 mmol/L triolein), the encapsulation efficiency was approximately 30%. It can be further increased by adding more lipids. At a protein-to-lipid ratio of 0.031 (*w/w*), the encapsulation efficiency was more than 60%. In contrast to the reported development of MVL formulation of progenipoietin, we did not find any evident correlation between the sucrose concentration in the first aqueous phase and IFN α-2b encapsulation efficiency[15]. Furthermore, the encapsulation capacity only seemed to vary slightly with different triolein contents.

Interferon α-2b release from multivesicular liposomes *in vitro* The MVL were stable when stored in saline in small volumes at 4 °C, with less than 2% protein leaked after 3 months (data not shown). When the MVL were diluted

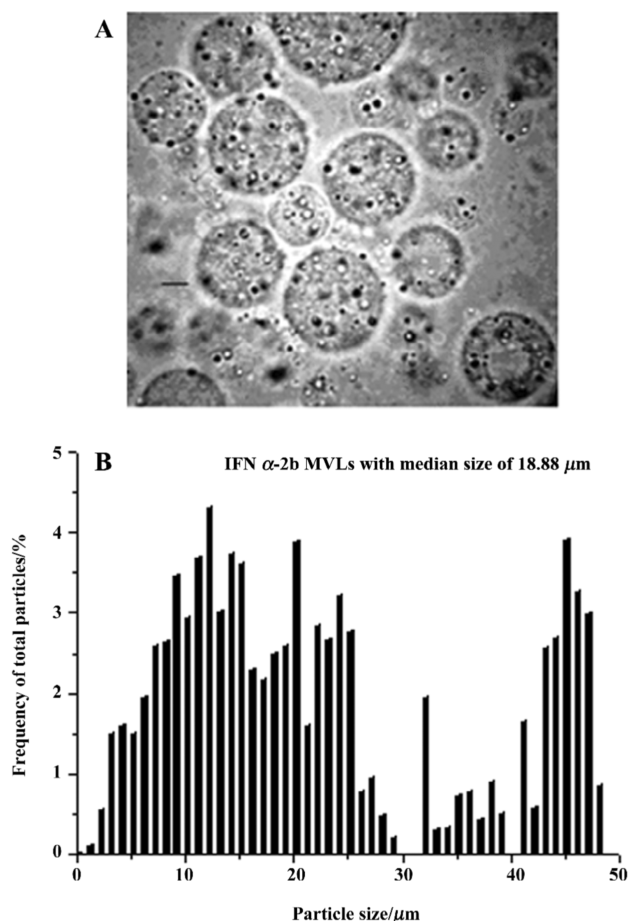

Figure 1. (A) Light micrograph of interferon (IFN) α-2b multivesicular liposomes (MVL) at 400×magnification. Scale bar=5 μm. (B) Particles size distribution of IFN α-2b MVL.

Table 1. Interferon α-2b encapsulation efficiencies in various multivesicular liposome formulations. *n*=3. Mean±SD.

Sucrose concentration (%, *w/v*)	Protein-to-lipid ratio (mg/mg)	Triolein-to-DOPC ratio (molar)	Encapsulation efficiency (%)
2.5	0.063	0.139	34.10±0.70
4.0	0.063	0.139	23.60±0.50
5.0	0.063	0.139	36.25±2.15
5.0	0.031	0.139	66.70±1.30
5.0	0.042	0.139	53.0±0.60
5.0	0.126	0.139	21.85±0.55
7.0	0.063	0.139	39.0±1.15
5.0	0.063	0.596	29.60±1.20
5.0	0.063	1.190	27.25±0.85

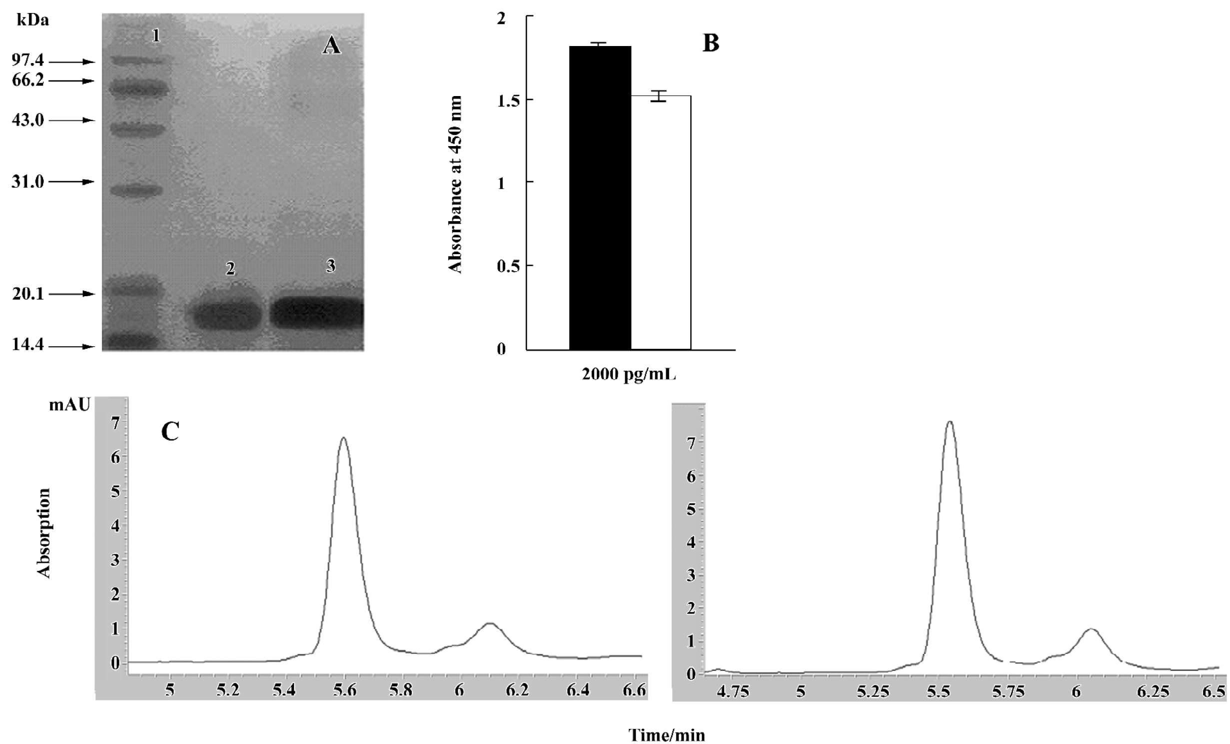

Figure 2. (A) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of interferon (IFN) α-2b before and after multivesicular liposome (MVL) encapsulation. Lane 1, low molecular weight standard; lane 2, IFN α-2b extracted from MVL; lane 3, native IFN α-2b. (B) Enzyme-linked immunosorbent assay binding activities of unencapsulated IFN α-2b and IFN α-2b extracted from MVL (2000 pg/mL). □: Unencapsulated IFN α-2b; ■: IFN α-2b from MVL. The data represent the mean±SD (n=3). (C) Comparison of the reverse phase high performance liquid chromatography profile of unencapsulated (free form) IFN α-2b to that of IFN α-2b extracted from DepoFoam particles. Above: unencapsulated IFN α-2b; below: IFN α-2b encapsulated in MVL.

into a large amount of saline (1:50 dilution) under well-mixed conditions, the encapsulated protein would gradually leak out (Figure 3). Approximately 90% of the content was shown to have been released after 7 d.

***In vivo* pharmacokinetic profiles** After subcutaneous injection of a dose of 2.5 mg/kg, free IFN α-2b proteins were cleared quickly within 1 d (the detection limit of the ELISA kit was at 30 pg/mL). The MVL sustained release formulations, however, would provide a continuous supply of IFN α-2b to the systemic circulation, which lasted more than 2 d. The detailed pharmacokinetic behavior was found to be related to the triolein content in the MVL formulation (Figure 4). Increases in triolein content resulted in longer release times.

Effect of multivesicular liposome size on *in vivo* protein release profiles To further optimize the sustained release profile of IFN α-2b MVL formulations, we specifically compared the *in vivo* release properties of MVL with different sizes. As the typical MVL preparation procedure yielded MVL with rather broad size distributions (Figure 1B), we modified some emulsification parameters and added a final fractionation step to obtain MVL samples in much narrower size distributions. The lipid formulation remained the same.

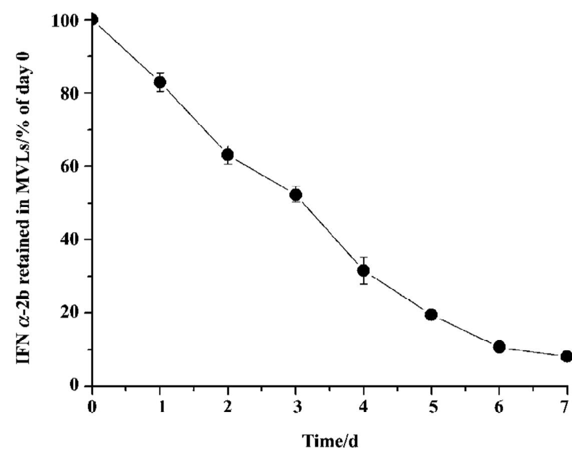

Figure 3. Interferon (IFN) α-2b *in vitro* release profile from multivesicular liposomes (MVL) in saline. The data represent the percentage of total IFN α-2b retained in MVL at various incubation time points. n=3. Mean±SD.

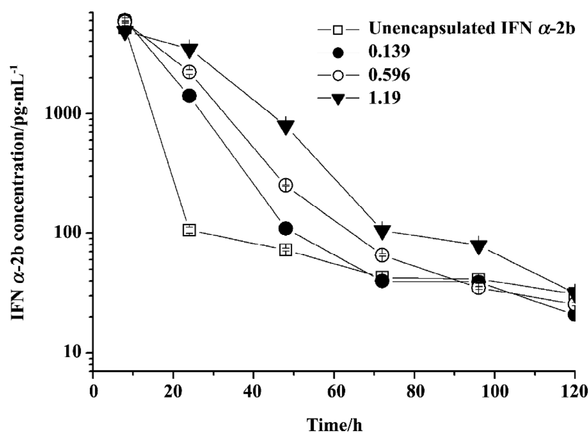

Figure 4. Serum interferon (IFN) α-2b concentrations after subcutaneous injection of IFN α-2b multivesicular liposomes (MVL) prepared using various molar ratios of triolein to DOPC (0.139, 0.596 or 1.19). The dosage is 1.6 mg/kg. □: Unencapsulated IFN α-2b; ●: triolein/DOPC=0.139; ○: triolein/DOPC=0.596; ▼: triolein/DOPC=1.19. Blood samples were collected at 8 h, 24 h, 48 h, 72 h, 96 h, 120 h after administration. n=3. Mean±SD.

Two different-sized populations were obtained and their size profiles are shown in Figure 5A. The large MVL had sizes of approximately 40 μm–60 μm diameter, and the small MVL were approximately 10 μm–25 μm in diameter. The samples were administered subcutaneously at a dose of 1.2 mg/kg and the IFN α-2b serum concentrations were determined and plotted in Figure 5B. It shows that small MVL released the encapsulated protein content over a longer time compared with large MVL.

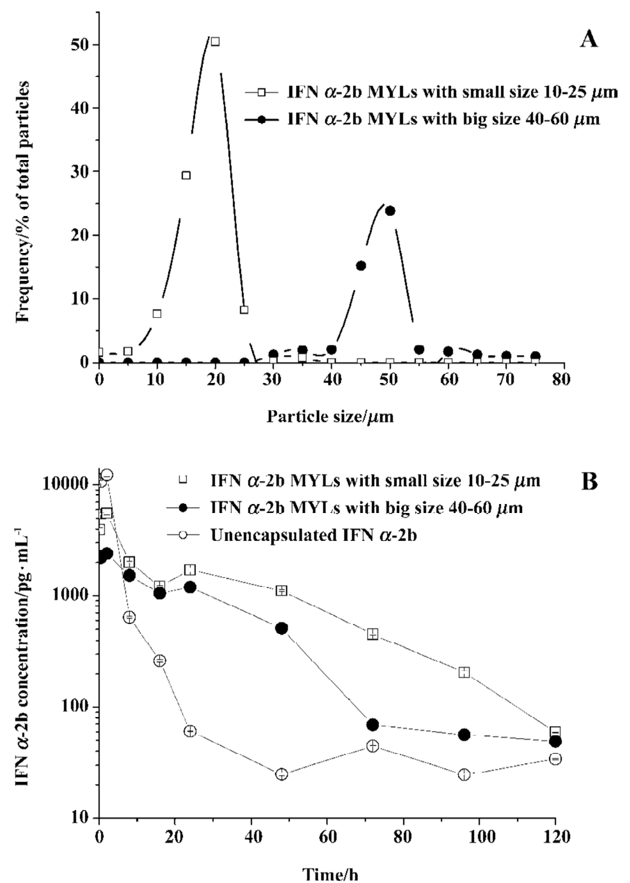

Figure 5. (A) Particle size distribution of 2 different preparations of interferon (IFN) α-2b multivesicular liposomes (MVL). (B) Serum IFN α-2b concentrations after subcutaneous injection of IFN α-2b MVL with different sizes. ○: Free IFN α-2b; ●: IFN α-2b MVL with larger sizes (40 μm–60 μm); □: IFN α-2b MVL with smaller sizes (10 μm–25 μm). The dosage was 800 μg/kg. Blood samples were collected at 5 min, 30 min, 2 h, 8 h, 16 h, 24 h, 48 h, 72 h, 96 h, and 120 h after administration. n=3. Mean±SD.

Discussion

Multivesicular liposome formulations have been developed successfully for the prolonged release of cytarabine, morphine and other drugs[16,17]. The long-lasting sustained release properties were most evident when the formulations were administered in a small confined space, such as the epidural. We showed here that MVL could also be used to achieve reasonable prolonged release properties after subcutaneous administration, and MVL IFN α-2b formulations may be developed for the treatment of viral infections requiring less frequent dosing. Our data indicate that MVL can maintain their structure in the subcutaneous interstitial space for a few days and slowly release the encapsulated proteins into the systemic circulation. There was considerable protein detected in the circulation for more than 5 d, and the serum half-life was estimated to be approximately 30 h.

The prolonged serum half-life that we achieved is actually comparable to what has been reported for the PEGylated IFN α-2b product currently in clinical use, even though their mechanisms for sustained serum concentration are quite different. PEGylated IFN α-2b requires chemical modification of the protein structure, which might affect its bioactivity. The PEGylated proteins are absorbed into the systemic circulation quickly after administration but remain there for a long time by avoiding various clearance mechanisms. In contrast, the proteins in MVL formulations are unmodified, which wait inside the subcutaneous MVL depot, slowly leak out, and enter the circulation. They should maintain their original structure, and most likely their full bioactivities. Their distribution and clearance mechanisms should also follow the same pathway as natural IFN α-2b. Therefore, compared to the PEGylated product, MVL formulations would have a more defined safety profile, established manufacture proce-

dures and drug efficacy, and side effects that are easier to evaluate. We therefore believe that MVL formulation may be an attractive candidate for the sustained delivery of IFN α-2b for the treatment of viral infections.

Another significant advantage of the MVL formulations is its high drug loading capacity. Compared with conventional liposomes, which often have limited encapsulation for hydrophilic proteins, the MVL offer a much larger internal space and therefore usually have higher encapsulation efficiencies. For IFN α-2b, the encapsulation efficiency was usually more than 30%. However, the double-emulsion preparation method has been shown to cause protein degradation and denaturation[18]. Also, there might exist protein-liposomal bilayer interactions that may affect protein conformation and activity[19,20]. We used three methods to test protein chemical and structural changes after encapsulation. Both the SDS-PAGE and HPLC analyses showed that the proteins were chemically intact. For protein conformational changes, some studies have used biophysical methods such as circular dichroism and fluorescence spectroscopy to detect the secondary structure or local amino acid environment changes[19]. We adopted a biochemical approach using an ELISA to probe possible 3-dimensional conformational changes. The ELISA may have its limitations because it can only detect changes of structure near binding sites. However, antibody binding has been shown to be very sensitive to protein denaturing effects, and ELISA are commonly used in protein formulation studies to assay protein structure integrity[21]. Our data showed that the antibody binding affinity for the protein after encapsulation was only slightly reduced, indicating that substantial native structure remained after preparation. Further studies are needed to confirm the detailed bioactivity of the encapsulated IFN α-2b.

We also tested several parameters that might affect the release profile of MVL. Triolein is used as a hydrophobic space filler at lipid membrane intersection points and can stabilize the junctions[11]. The amount of triolein in the MVL formulation was suggested to be important for MVL morphology and stability[11,22]. We showed that it had a significant impact on the *in vivo* release profiles of IFN α-2b (Figure 4). It is possible that when more triolein is present, the lipid walls are more stable and therefore the protein is released more slowly.

With a similar argument, we hypothesized that the size of the MVL would also be important for the drug release profile, because the inter-compartmental fusion and diffusion of the proteins in larger MVL would add another rate-limiting step and would eventually result in faster protein release into the environment. However, when we used the typical preparation procedure, the resultant MVL size distribution was rather broad, ranging from 2 μm to 50 μm (Figure 1). It is difficult to differentiate the release profile of different-sized MVL. Therefore, we developed a modified procedure to make MVL with much narrower size distributions (Figure 5). Based on our data, the protein release from MVL with smaller sizes (10 μm–25 μm) was indeed slower than that from larger MVL (40 μm–60 μm), which is an important observation. We therefore suggest that, in further development of control-released formulations, MVL sizes will need to be optimized and well controlled.

In summary, we have demonstrated that IFN α-2b MVL formulation can achieve high encapsulation efficiency, good stability and sustained release effects. The sustained release effect can be affected by the triolein content and particle sizes. Further optimization is needed in order to develop a clinically valuable sustained release formulation of IFN α-2b.

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